FOLIA VETERINARIA

The scientific journal of the UNIVERSITY
OF VETERINARY MEDICINE IN KOŠICE
— The Slovak Republic

ISSN 0015-5748

3—4
LI • 2007

UNIVERSITY OF VETERINARY MEDICINE IN KOŠICE

EDITORIAL BOARD

FOLIA VETERINARIA is issued by the *University of Veterinary Medicine* in Košice (UVL); address: Komenského 73, 041 81 Košice, The Slovak Republic (tel.: +421 55 633 51 03, fax: +421 55 633 51 03, E-mail: vargova@uvm.sk).

The journal is published quarterly in English (numbers 1–4) and distributed worldwide.

Subscription rate for 1 year is 200 Sk, for foreigners 80 euros. Orders are accepted by *The Department of The Scientific Information – The Library of The University of Veterinary Medicine, Košice* (UVIK); the subscription is accepted by the National bank of Slovakia in Košice (at the account number mentioned below).

Bank contact: *National bank of Slovakia*, 040 01 Košice, Strojárenská 1, our account number: 19–1924–512/0720.

FOLIA VETERINARIA, vydáva *Univerzita veterinárskeho lekárstva* v Košiciach (UVL), Komenského 73, 041 81 Košice, Slovenská republika (tel.: 055/633 51 03, fax: 055/633 51 03, E-mail: vargova@uvm.sk).

Časopis vychádza kvartálne (č. 1–4) a je distribuovaný celosvetove.

Ročné predplatné 200 Sk (6,64 €), pre zahraničných odberateľov 80 eur. Objednávky prijíma *Ústav vedeckých informácií a knižnice Univerzity veterinárskeho lekárstva v Košiciach* (UVIK); predplatné Národná banka Slovenska v Košiciach (na nižšie uvedené číslo účtu).

Bankové spojenie: Národná banka Slovenska, Košice, Strojárenská 1, číslo príjmového účtu: 19–1924–512/0720.

Tlač: **EMILENA**, Čermeľská 3, 040 01 Košice
Sadzba: **Aprilla, s.r.o.**, Szakkayho 1, 040 01 Košice

EV 3485/09

For basic information about the journal see Internet home pages: www.uvm.sk

Indexed and abstracted in AGRIS, CAB Abstracts

FOLIA VETERINARIA, 51, 3–4, 2007

CONTENTS

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 113–117, 2007

SEROPREVALENCE OF ANTIBODIES TO *Toxoplasma gondii* IN SOME FOOD AND COMPANION ANIMALS IN THE SOUTHWESTERN NIGERIA

Okewole, E. A.

Department of Veterinary Medicine, University of Ibadan, Ibadan Nigeria

doktorokewole@yahoo.com

ABSTRACT

Serum samples from traditionally and institutionally raised food and companion animals from four strategically selected farms and veterinary clinics in the Southwestern Nigeria were tested for *Toxoplasma gondii* IgG antibodies by the Modified Agglutination Test (MAT) with whole formalized tachyzoites as antigen and pre-mercaptoethanol-treated sera. Seropositivities were cattle, 10.1% of 316 (12.0% of 200 white Fulani cattle and 6.9% of 116 N'dama cattle), sheep, 50.1% of 112, goat, 44.1% of 102, pigs, 41.5% of 164, rabbit, 5.8% of 120, horse, 4.3% of 23, dog, 3.5% of 820 and cat 47.2% of 265. Data were compared by host species and age groups. Lower seropositivity in cattle was suggested to be due to the genetic lower susceptibility of cattle to toxoplasmosis and their grazing on cat feace-free pasture while, higher seropositivity in sheep and goats was blamed on grazing very close to ground with high risk of picking contaminants. Similarly, high seropositivity in pigs was blamed on habitual rooting in contaminated soil and the delibrate consumption of dead animals when available and accessible. The relatively low seropositivity in the domestic dogs was blamed on the inhibition of hunting tendency as against the wild canids, while the high seropositivity in the cats was blamed on carnivorism, promoted by hunting and eating of preys, raw meat, etc. Control measures recommended for cat included keeping in-door, feeding of only cooked meat, burying of dead animal carcases; while for humans, eating of well-cooked meat and good personal hygiene.

Key words: cat; infection; seropositivity; *Toxoplasma*

INTRODUCTION

Toxoplasma gondii is an obligate intracellular coccidia parasite that infects virtually all species of warm-blooded animals including people. Domestic cats and other Felidae are the definitive hosts, while all infectable non-feline species are intermediate hosts (13). There are three infectious stages of toxoplasmosis; the oocyst that are excreted in faeces, the tachyzoites and bradyzoites that are found in tissues (13).

In nature numerous species of mammals and birds are commonly infected with *Toxoplasma*. Cats account for the infection of herbivores and indirectly for the infection of predators (18). Cattle, sheep, goat and other herbivores are infected by eating grass with roots contaminated by oocyst-infected soil (17) while birds, rats, house mice, rabbit, etc. get infected through close interaction between persisting oocysts in close human and animal co-habitation.

Serological evidences of toxoplasmosis in different food animal species in Africa have been published (14, 32, 31, 28) and the economic significances highlighted in some of these reports included abortions, stillbirths and high mortality in sheep and goat; and less commonly in cattle, as documented elsewhere (5). Perhaps, a more important consideration of toxoplasmosis is its zoonotic potential, which manifests from transplacental infection, and the consumption of raw or undercooked meat or other unhygienic handling of raw meat or meat products (25). Mental retardation, loss of vision (9), toxoplasma encephalitis (15), uveitis (34, 30) were amongst the clinical signs and lesions widely reported, particularly in the pre-natally infected children and the immunocompromised adults, particularly the HIV/AIDS patients in Africa.

Gill and Stone (19) estimated a 10.0 % incidence of toxoplasmosis among the AIDS patients in the USA and also attributed 30 % of the CNS complications in these patients to the same zoonosis. Giving the rising prevalence of the HIV/AIDS pandemic in Africa, a closer study of the epidemiology of one of its predisposing zoonosis in the food and companion animals would be a worth, while contribution to the national control effort.

This survey was done to determine the seroprevalence of antibodies to *T. gondii* in the common food and companion animals in the Southwestern Nigeria, with a view to formulate more appropriate control strategies that would complement the national and international efforts on the HIV/AIDS control.

MATERIALS AND METHODS

Animals

Food animals

The food animals used for this investigation consisted of the normadic white Fulani cattle that were kept under the traditional husbandry system of grazing and occasional feeding of concentrates and the N'dama cattle that were kept on sedentary institutional farms with regular but restricted grazing on cultivated paddocks and occasional feeding of concentrates. Sheep and goats were similarly managed on the same farms, but a higher proportion of the sample was from the institutional farms with more of supplementary feeding. Pigs were kept on table remnants, supplemented with occasional scavenging scrabs from the neighourhood; while rabbits were principally on pelleted ration supplemented with occasional herb feeding.

They included 316 cattle made from 200 normadic while Fulani cattle and 116 sedentary N'dama cattle. Others were 112 West African dwarf sheep, 102 West African dwarf goats, 164 large white breed of pigs and 120 adult New Zealand white rabbits.

Companion animals

The 820 dogs and 265 cats presented for routine vaccination and deworming at the Mokola Veterinary Clinic, Ibadan and the Metropolitan Veterinary Clinic, Lagos. Nigeria between May and September, 2006 were included. These dogs and cats were of different breeds, purposes and husbandry systems, particularly with respect to feed and feeding. Only 23 horses from the Ibadan Polo club, Ibadan. Nigeria were available for screening.

Serum samples

Blood samples were taken from all animals into plain bottles, allowed to clot at room temperature before separation by centrifugation and storage at –20 °C until use.

Serological testing

Sera were assayed for *T. gondii* antibodies by the modified agglutination test (MAT) with whole formalized tachyzoites as antigen on 0.2 mol mercapthoethanol pre-treated sera. Sera were diluted at 1 : 25, 1 : 50 and 1 : 500 for MAT testing as described (12). The MAT is a specific and sensitive test for *T. gondii* as the later does not cross-react with other related parasite (11, 10). A titre of 1:25 dilution was taken as positive.

RESULTS

Of the 200 white fulani cattle tested, 12.0 % were positive, while 6.9 % of the 116 N'dama cattle were positive. The combined total of 316 cattle had a seropositivity of 10.1 %. The mean ages were 2.7 years and 2.0 years for the respective cattle breeds. Sex appeared to have no effect as there were almost equal number of both sexes among the reactors. The prevalence of positive reactions seemed to increase with age from 1½ to 3½ years. Positive reactors were more (almost double) in the nomadic white Fulani cattle than in the sedentary N'dama type (Table 1).

Of the 112 sheep tested, 50 % were positive, while 44.1 % of the 102 goats were positive. Mean ages were similar at 2.2 and 2.5 years, respectively. Both sexes were almost equally represented and seropositivity tended to rise with age, like in cattle. Disproportionately higher number of positive goats were in the highest titre range (1 : 500) (Table 1).

Sixty-eight (41.5 %) of the 164 domestic pigs tested were positive, mean age was 1.9 years, with a higher number of boars than sow (2 : 1, respectively) especially at the upper titre range (1.500) (Table 1). Seven (5.8 %) of the 120 rabbits tested were positive, but all reactors were all within a lower titre range (1 : 25).

Only 1 (4.3 %) of the 23 horses tested positive. Age mean was 2.8 years. Of the 820 dogs tested, 35.0 % were positive, while 47.2 % of the 265 cats were positive. Age means were 2.2 years and 1.1 years for dogs and cats, respectively. It was only in the dog and cat that the reactive titre values were evenly spread across the three ranges with 47 (16.4 %) and 68 (54.4 %) of their respective reactors in the highest titre range (1 : 500) (Table 1).

DISCUSSION

The seropositivity to *T. gondii* was highest for dog and cat and lowest for rabbit and horse in this survey. This trend is consistent with the earlier reports from Canada, (36) and from the USA (4), but at variance with those of Nation and Allen (29) who reported a prevalence of 3.4 % for cats in Saskatchewon, in Canada and Gondim *et al.* (20) who reported a higher prevalence of 28.9 % for goat and 18.7 % for sheep in Brazil. These discrepancies might not be unrelated to the variation in the sensitivities and specificities of diagnostic assays used by the investigators.

Tizard *et al.* (36) and Dubey (4) used the Sabin-Feldman dye test (SFDT), while Nation and Allen (29) and Gondim *et al.* (20) used the latex

Table 1. Prevalence of *Toxoplasma gondii* antibodies in some food and companion animals in the Southwestern Nigeria

ANIMAL SPECIES	NO. EXAMINED	NO (%) POSITIVE	AGE RANGE IN YEARS (MEAN)	HUSBANDRY: HOUSING & FEEDING SYSTEMS	ANTIBODY TITRES		
					1:25	1:50	1:500
White Fulani cattle	200	24 (12)	1–3.5 (2.7)	Open range, grazing & occasional supplementary feeding	16	8	–
N'dama cattle	116	8 (6.9)	1–4 (2.0)	Night housing, grazing & regular supplementary feeding	6	2	–
Sheep (wad)	112	57 (50.1)	1–6 (2.2)	Night housing, grazing & regular supplementary feeding	8	37	12
Goat (wad)	102	45 (44.1)	1–4 (2.5)	Night housing, grazing & regular supplementary feeding	6	21	18
Domestic pig (lw)	164	68 (41.5)	1–4 (1.9)	Regular housing with occasional release for scavenging & rooting	17	37	14
Rabbit (nzw)	120	7 (5.8)	1–2 (1.2)	Regular housing with feeding of pelleted feed and occasional herbs	7	–	–
Stable horse	23	1 (4.3)	2–5 (2.8)	Regular housing with occasional grazing and supplementary concentrate	1	–	–
Domestic dog	820	287 (35.0)	2–4.5 (2.2)	Varied housing and feeding with little opportunity for scavenging & hunting for some	152	88	47
Domestic cat	265	125 (47.2)	1–3 (1.1)	Varied housing and feeding with little opportunity for scavenging & hunting for some	13	44	68
Total	**1.922**	**622 (32.4)**	**–**	**–**	**226**	**237**	**159**

agglutination test (LAT). While the SFDT is valuable as a diagnostic assay for toxoplasmosis, it is also fallible as its specificity is low, especially when the closely related coccidian; *Hammondia hammondi* (16) and sarcocystosis (1) are included. Similarly, LAT has a low sensitivity, especially on swine sera (2). However, MAT as applied in this survey (i. e. previous treatment of sera with 2.0 mol mercapethanol) is a specific and sensitive test for *T. gondii*, as the latter does not cross-react with other related or unrelated parasites (11, 10).

The seropositivity of 10.1 % for cattle (white Fulani plus N'dama) in this survey is relatively low when compared with sheep and goats, but is also consistent with earlier reports (4, 5, 21, 20). A genetic lower susceptibility of cattle to *T. gondii* (5, 11) and a management system that reduced contact of cattle with cat or cat wastes (20) might be responsible. These latter factors were also speculated to be responsible for the higher seropositivity of the nomadic white Fulani cattle breed than the sedentary N'dama, as also previously reported (24) from this place.

The higher seropositivity of 50.1 % and 44.1 % in sheep and goat respectively are consistent with the earlier reports (36, 11, 21, 20) and also confirmed the higher susceptibility of sheep, in particular to *T. gondii* with resultant abortion, stillbirths and death as reported from Canada and the USA (23). In one reported survey (7) from the USA, 65.0% of the ewes on 33 farms had *T. gondii* antibodies in their serum at a 1 : 64 dilution as determined by MAT and up to 93.0 % of ewes on certain farms were seropositive.

A possible contributory factor might be that sheep and goat graze very close to the ground and so are potentially capable of picking up oocysts from a ground contaminated with cat's faeces. Seropositivity in the domestic pig was relatively high and also comparable with those earlier reported (33, 22). This high seropositivity may be a reflection of the feeding habits of this species, which might involve accidental ingestion of oocysts while rooting and the delibrate consumption of dead animals when available. Seropositivity in the horse was relatively low, when compared with other herbivores, but the stable practice of feeding horses from an elevated manager which reduces the possibility of oocysts contamination of feed might be contributory. Rabbit's seropositivity was very low and this probably reflected their regular housing in cages and the feeding of pelleted feed with occasional herbs that might be contaminated with oocysts. The level

of infection in the domestic dog was comparatively low when compared with the reported values in the wild canids, e.g. coyotes (*Canis latrans*), which was reported as 65.0 % positive (35). The difference may be attributable to the domestication effect on the domestic dog that has reduced their hunting urge.

The prevalence of *T. gondii* in domestic cat, obtained here was high and compares well with values reported elsewhere (37, 3, 26). Carnivorism has been identified as the major source of infection for the cat, being an obligate definitive carnivorous host (8) and their seropositivity tends to increase with age as more and more infected rodents, mice, birds etc are consumed with age (3).

Seropositive cats pass oocysts in faeces to contaminate the ground and pastures for the ultimate infection of the food animals and humans. Infected sheep and goat can abort or died, while children infected in-utero have mental retardation, loss of vision, toxoplasma encephalitis or could even die; if immunocompromised by the raphaging HIV/AIDS (9). L u f t *et al.* (27) estimated that 2–20% of all AIDS patients die of toxoplasmosis.

The control of toxoplasmosis is therefore critical, given the rising prevalence of the HIV/AIDS pandemic and this again is pivotal on its three modes of transmission. Control in food animals is currently by vaccination. A live vaccine that uses a non-persistent strain of *T. gondii* is available in New Zealand, Australia, UK and other parts of Europe for use on sheep to prevent abortion. Also, a live vaccine that uses a mutant strain of *T. gondii* (T-263) is being developed in the USA to prevent oocyst shedding in infected cats (9). Scavenging on dead animals by pigs and cats should be prevented by burying or incinerating such animals (8).

Cats should be kept in door to prevent hunting and fed only dry, canned or cooked food and not uncooked meat, viscera or bones (8). Since no drug is currently available to kill *T. gondii* tissue cysts, meat for human consumption could be frozen at –20 °C, cooked to internal temperature of 67 °C or gamma irradiated (0.5 kGy) to kill tissue cysts in such meat, as recommended (9). Fortunately, freezing at normal household freezer temperature has been reported to kill cysts (6).

In conclusion, a good personal hygiene is needed, when handling raw meat, with the hands, tabletops and utensils thoroughly washed and rinsed by a non-pregnant person. Pregnant women, in particular should avoid close contact with cat and cat wastes and should also avoid contact with cat litters (8). Children's sandboxes should be covered when not in use and contaminated sand replaced. Files and cockroaches should also be biologically controlled as much as possible.

ACKNOWLEDGEMENTS

The technical assistance of Messrs. A. J. R o b e r t s and A. A. W e i r both of the General Diagnostic Laboratory, Obafemi Awolowo University, Ile-ife, Nigeria is gratefully acknowledged. So also is the cooperation of the management and staff of the Fashola and Iwo Road farm centres, Ibadan, Nigeria.

REFERENCES

1. Awad, F. I., Lainson, R., 1954: A note on the serology of sarcosporidiosis and toxoplasmosis. *J. Clin. Path.*, 7, 152–156.

2. Dubey, J. P., Thulliez, P., Weigel, R. M., Andrews, C. D., Lind, P., Powell, E. C., 1995: Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sows. *Am. J. Vet. Res.*, 56, 1030–1036.

3. Dubey, J. P., 1973: Feline Toxoplasmosis and Coccidiosis: a survey of domiciled and stray cats. *J. Am. Vet. Med. Assoc.*, 162, 873–877.

4. Dubey, J. P., 1985: Serologic prevalence of toxoplasmosis in cattle, sheep, goats, pigs, bisom and olk in Montana. *J. Am. Vet. Med. Assoc.*, 186, 969–970.

5. Dubey, J. P., 1986: A review of toxoplasmosis in cattle. *Vet. Parasitol.*, 122, 177–202.

6. Dubey, J. P., 1988: Long-term persistence of *Toxoplasma gondii* in tissue of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. *Am. J. Vet. Res.*, 49, 910–913.

7. Dubey, J. P., 1990: Status of toxoplasmosis in sheep and goats in the United States. *J. Am. Vet. Med. Assoc.*, 196, 259–262.

8. Dubey, J. P., 1994: Toxoplasmosis. *J. Am. Vet. Med. Assoc.*, 205, 1593–1598.

9. Dubey, J. P., 1996: Strategies to reduce transmission of *Toxoplasmosis gondii* to animals and humans. *Vet. Parasitol.*, 64, 65–70.

10. Dubey, J. P., 1997: Validation of the specificity of the modified agglutination test for toxoplasmosis in pigs. *Vet. Parasitol.*, 71, 307–310.

11. Dubey, J. P., Beattie, P., 1988: *Toxoplasmosis of Man and Animals*. CRC press, Boca Raton, Florida, pp. 22–24.

12. Dubey, J. P., Desmonts, G., 1987: Serologic responses of equids fed with *Toxoplasma gondii* oocysts. *Eq. Vet. J.*, 19, 337–339.

13. Dubey, J. P., Lappin, M. R., 1998: Toxoplasmosis and neosporosis. In **Greene, C. E.** (ed.): *Infectious Diseases of the Dog and Cat*. Philadelphia, W.B. Saunders., pp. 493–509.

14. Falade, S., 1978: Toxoplasmosis antibodies in Nigerian goats. *Trop. Anim. Hlth. Prod.*, 10, 175–177.

15. Francis, P., Patel, V. B., Bill, P. L. A., Bhigjee, A. I., 2004: Oral trimethoprim-sulfadiazine famethoxazole in the treatment of cerebral toxoplasmosis in AIDS patients: a prospective study. *S. Afr. Med. J.*, 94, 51–53.

16. Frenkel, J. K., Dubey, J. P., 1976: *Hammondia hammondi* gen. nov. sp. nov. from domestic cats, a new coccidian related to *Toxoplasma* and *Sarcocystis. Z. Parasitkde*, 46, 3–12.

17. Frenkel, J. K., 1990: Transmission of toxoplasmosis and the role of immunity in limiting transmission of illness. *J. Am. Vet. Med. Assoc.*, 196, 233–240.

18. Frenkel, J. K., Dubey, J. P., 1972b: Toxoplasmosis and its prevention in cats and man. *J. Infect. Dis.*, 26, 664–673.

19. Gill, D. M., Stone, D. M., 1992: The veterinarian's role in the AIDS crisis. *J. Am. Vet. Med. Assoc.*, 201, 1683–1684.

20. Gondim, L. F. P. *et al.*, 1999: Serological survey of antibodies to *Toxoplasma gondii* in goats, sheep, cattle and water buffaloes in Bahia State, Brazil. *Vet. Parasitol.*, 82, 273–276.

21. Hashemi-Fesharki, R., 1996: Seroprevalence of *Toxoplasma gondii* in cattle, sheep and goats in Iran. *Vet. Parasitol.*, 61, 1–3.

22. Hove, T., Dubey, J. P., 1999: Prevalence of *Toxoplasma gondii* antibodies in sera of domestic pigs and some wild game species from Zimbabwe. *J. Parasitol.*, 82, 372–373.

23. Hulland, T. J., Tobe, S. B., 1961: Toxoplasmosis as a cause of abortion in Ontario sheep. *Can. Vet. J.*, 2, 45–51.

24. Joshua, R. A., Akinwumi, K. A., 2003: Prevalence of antibodies to *Toxoplasma gondii* in four breeds of cattle at Ibadan. *Nigeria Trop. Vet.*, 21, 134–137.

25. Lappin, M. R., 1996: Feline toxoplasmosis: interpretation of diagnostic test results. *Semin. Vet. Med. Surg.*, 11, 154–160.

26. Lappin, M. R., Powell, C. C., 1991: Comparison of latex agglutination, indirect hemagglutination and ELIZA techniques for the detection of *Toxoplasma gondii* specific-antibodies in the serum of cats. *J. Vet. Intern. Med.*, 5, 299–301.

27. Luft, B. J., Hafner, M. D., Korzun A. H. *et al.*, 1993: *Toxoplasma encephalitis* in patients with AIDS. *N. Engl. J. Med.*, 329, 995–1000.

28. Makinde, A. A., Ezeh, A. O., 1981: Serological survey of *Toxoplasma gondii* in Nigerian cattle: a preliminary report. *Brit. Vet. J.*, 137, 485–488.

29. Nation, P. N., Allen, J. R., 1976: Antibodies to *Toxoplasma gondii* in Saskatchewan cats, sheep and cattle. *Can. Vet. J.*, 17, 308–310.

30. Ogun, S. A., Ogini, F., Okubadejo, N., Danesi, M., Kolapo, K., Osalusi, B., Boyle, B., 2005: Pattern and outcome of neurological manifestations of HIV/AIDS: a review of 154 cases in a Nigerian University Teaching Hospital: a preliminary report. *Afr. J. Neurol. Sc.*, 24, 29–36.

31. Ogunba, E. O., Thomas. V., 1979: Antibodies to *Toxoplasma gondii* in Ibadan. *Nig. J. Med. Sc.*, 2, 77–80.

32. Ogunrinade, A. F., 1978: Studies on Toxoplasmosis of domestic animals in Nigeria: *Toxoplasma* antibodies in pet dogs in Ibadan area. Nig. Med. J., 8, 446-448.

33. Pandey, V. S., Knapen, F., Van: 1992: The seroprevalence of toxoplasmosis in sheep, goats and pigs in Zimbabwe. *Annals. Trop. Med. Parasitol.*, 86, 313–315.

34. Ronday, M. J., Stilma, J. S., Barbe, R. F., McElroy, W., Luyendik, L., 1996: Aethiology of *uveitis* in Sierra Leone, West Africa. *Br. J. Ophthalmol.*, 80, 956–961.

35. Tizard, I. R., Billett, J. B., Ramsden, R. O., 1976: The prevalence of antibodies against *Toxoplasma gondii* in some Ontario mammals. *J. Wild. Dis.*, 12, 322–325.

36. Tizard, I. R., Harmeson, J., Lai, C. H., 1978: The prevalence of serum antibodies to *Toxoplasma gondii* in Ontario Mammals. *Can. J. Comp. Med.*, 42, 177–183.

37. Work, K, 1969: The Incidence of *Toxoplasma* antibodies among dogs and cats in Denmark. *Acta. Path. Microbiol. Scand.*, 75, 447–456.

Received August 9, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 118–121, 2007

IMMUNOHISTOCHEMICAL LOCALIZATION OF NERVE FIBERS IN THE SHEEP AND GOAT MANDIBULAR SALIVARY GLAND

Maretta, M., Marettová, M., Legáth, J.

University of Veterinary Medicine, Komenského 73, 041 81 Košice
The Slovak Republic

maretta@uvm.sk

ABSTRACT

An immunohistochemical method and acetylated α-tubulin antibodies were used to demonstrate the nerve fibres in the mandibular salivary glands in the sheep and goat. Positivity to both antibodies was localised in prevalence in the stroma alongside the blood vessels and in close contact to interlobulary excretory ducts. In the glandular parenchyma immunoreactivity to acetylated α-tubulin was observed in the nerve fibers localized next to intralobulary excretory ducts and intercalated ducts. In a periacinar space single fine nerve fibres positive to acetylated α-tubulin terminated in the area around the secretory acini. Close contact of these nerve fibers to secretory cells were located only at the basal side with close contact with myoepithelial cells. Nerve fibers were dominantly seen in the area of the mucous cells. No positive reaction was observed inside the acini and on the lateral side of the acinar cells. Acetylated α-tubulin revealed strong reaction and high density of the nerve fibers in connection with the intralobular duct system.

Key words: acetylated α-tubulin; nerve fibres; salivary glands

INTRODUCTION

Salivary secretion is nerve mediated. Each major salivary glands are inervated by both sympathetic and parasympathetic divisions of the autonomic nerve system (13). The parasympathetic system is influencing the movement of ions and water, while the sympathetic system influences secretion of organic material (10). It has been suggested that the sympathetic stimulation may affect the semilune cells, whereas the parasympathetic stimulation may act on the acinar cells (8).

The presence of the nerve fibres in the parotid and sublingual salivary glands were studied earlier histochemically in the rat (4, 5, 17) in the cat (9, 14) in the rabbit (10), in the sheep (1), and in the human salivary glands (12, 23, 25, 26), electron microscopically in the submandibular and parotid glands of cats, rabbit, and monkey (11, 19, 21) and later immunohistochemically in various animal species and in human salivary glands (3, 7, 16). In histochemical methods, acetylcholinesterase activity was used as a marker for the cholinergic junction between nerve and effector cells in the rat mandibular gland (4, 5, 8). A quantitative study of nerve fiber density in the submandibular gland of rats was made by T s u k o i *et al.* (27).

Acetylated α-tubulin as a modified form of tubulin has been found to be present in different cellular structures but has also been found to be preferentially expressed in nerve fibres, both *in vivo* and *in vitro* (2, 3). For this property it was used as one of the antibodies for demonstration of the nervous tissue. Despite numerous histochemical studies performed in human and in laboratory animals the detailed morphology of innervation of salivary glands in domestic animals is absent.

The aim of the present study was to localize acetylated tubulin-positive nerve fibers in ovine and goat mandibular salivary gland. This paper is a part of research in which the effect of toxic substances on the digestive functions is studied.

MATERIAL AND METHODS

Five adult sheeps and five goats were used in this study. Mandibular salivary glands were dissected out at the local slaughterhouse immediately upon death. Samples of the tis-

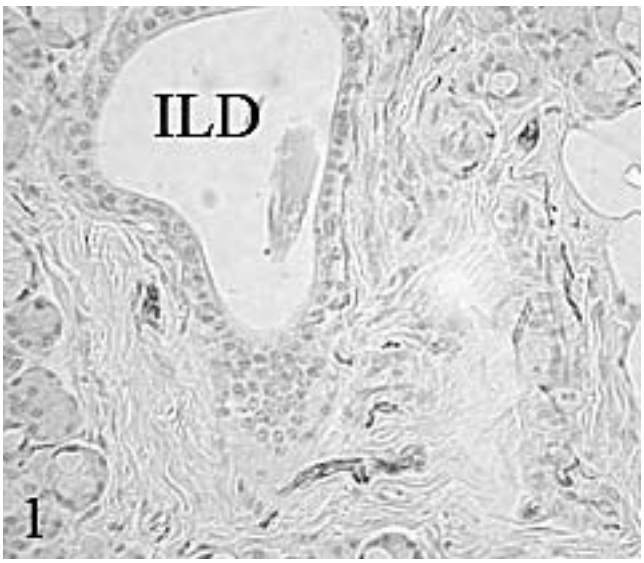


Fig. 1. Mandibular gland of the sheep. AT-positive nerve fibers located in the supporting tissue next to interlobular ducts (ILD) – 100 ×

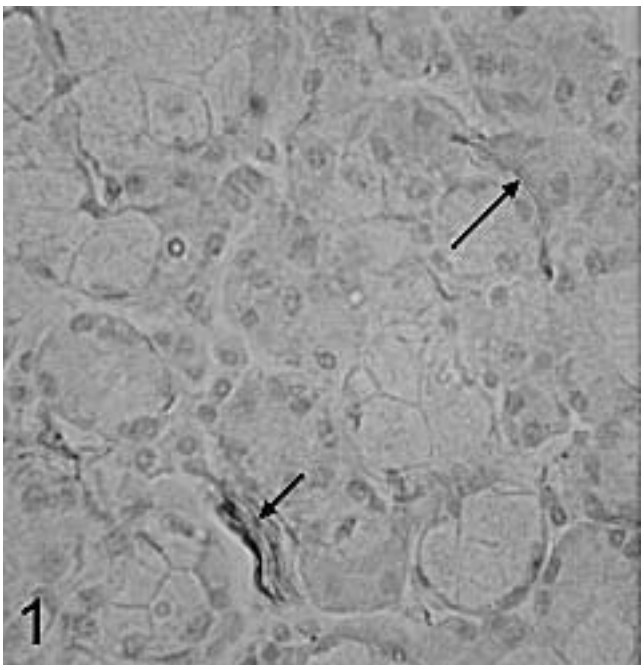


Fig. 2. Mandibular gland of the sheep. AT-immunoreactive nerve fibres were seen on the basal sides of secretory acini (arrow) – 200 ×

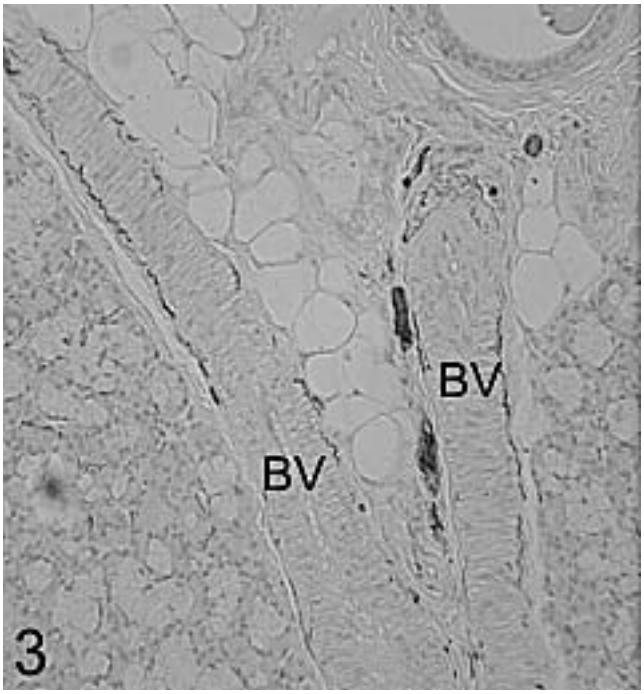


Fig. 3. Mandibular gland of the goat. AT-immunoreactive nerve fibers are in adventicia of the blood vessels (BV) – 400 ×

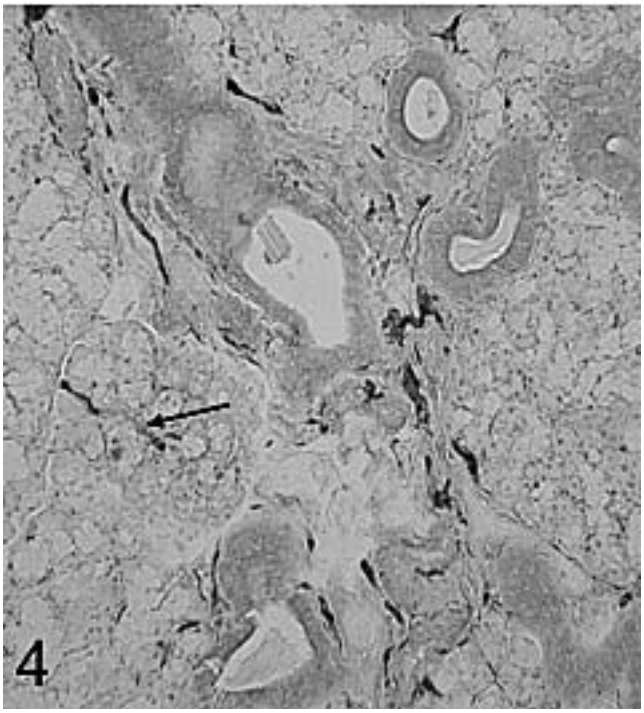


Fig. 4. Mandibular gland of the goat. S-100 protein immunoreactive fibers are in the space of interlobulary ducts, next to blood vessels and among the acini (arrow) – 10 ×

sue were fixed in 10 % neutral buffered formalin for 24 h and routinely embedded in paraffin. Sections at 5 μm thickness were cut and mounted on 3-aminopropyltriethoxysilane (APES)-coated slides. Consecutive sections were used for histological, control and immunohistochemical procedures.

Immunohistochemistry

For immunostaining procedure Avidin-biotin complex method was used (18). Histological section were deparafinized and rehydrated, pretreated with 3 % H_2O_2 in methanol to block endogenous peroxidase activity and preincubated with 2 % goat serum to mask unspecific binding sites. Washed section were incubated overnight with monoclonal anti-acetylated α-tubulin antibody (mouse IgG2b, clon 611 B-1, Sigma). The sections were washed in phosphate-balanced salt solution (PBS), and incubated with biotinylated secondary antibody for 30 min.

Washed sections in PBS, were incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame, USA). After washing with PBS, peroxidase activity was visualized with diaminobenzidine (DAB) and H2O2 in TRIS buffer within 5 min at room temperature. Sections were counterstained with Mayer's haematoxylin. For negative controls, the first antibody was substituted by PBS.

RESULTS

In the mandibular salivary gland acetylated α-tubulin immunoreactivity was restricted to the nervous tissue. Positive nerve fibers as a single fibers, thick fiber bundles or forming small nerves were observed mainly inside the interlobular connective tissue of the mandibular salivary gland along the wall of the excretory ducts (Fig. 1). In this area the nerve fibers positive for acetylated tubulin were in close contact to major blood vessels located in the adventitia. Single nerve fibers make continuous layer on the surface of the blood vessel (Fig. 3). Thicker nerve bundles course through the interlobular connective tissue.

Inside the glandular lobules nerve fibers positive to acetylated α-tubulin follow small blood vessels and intralobular ducts located in the fine supporting tissue. As for intercalary salivary ducts only fine bundles and single nerve fibres were seen on their surface. Here, the density of the nerve fibres decreased with their entrance among the glandular acini (Figs. 2, 4). The immunoreactive single nerve fibres were oberved at the base of secretory cells in close connection to the myoepithelial cells. There were no nerve fibers observed to penetrate the acinar basal lamina and and to acquire an intraepithelial position.

DISCUSSION

The salivary glands are supplied by parasympathetic and sympathetic efferent nerves which travel to the glands by separate routes. Once in the glands the axons from each type of nerve intermingle and travel together in association with Schwann cells, forming Schwann-axon bundles (13). This association permit to identify nerve fibers with both antibodies we used.

The observations of Bogart (5) in the sublingual gland correspond with our observations made in the mandibular glands that activity of the nerve fibers was observed in the nerve trunks in relationship to the ducts and blood vessels. On the other hand, the electron microscopic studies reveal that direct contacts exist between nerve terminals and parenchymal elements in both sublingual and parotis glands of the rat, while much direct contacts are absent in the submandibular gland (24).

Dardig *et al.* (7) and the present study also demonstrated the presence of nerve fibres in close contact with the secretory acini of the salivary gland. Ultrastructural observations on the cat submandibular gland indicate that terminations of both sympathetic and parasympathetic fibers are associated with the surface of one acinar cells. Hand (17) localized activity at the axon-Schwann cell interface in association with the axolemma and autonomic nerve fibers penetrated the acinar basal lamina and acquire an intraepithelial position. These axons were seen to be ensheated by Schwann cells and reaction for acetycholinesterase activity was found at the axolemma-Schwann cell surface (5).

According to Bogart (5) some axons give not reaction, even though they are enwrapped by a Schwann cell. The author observed axons with their investing Schwann cell in relationship to the basal surface of the acinus separated from these acinar cells by them interceding basement laminae. The axonemal enlargements were seen to have contact with acinar cells, being found either abutt in the base of a single acinar cell or lying in the lateral intercellular spaces and abbuting two acinar cells.

In our study nerve fibers were seen only at the basal side of the secretory cells. Two types of neuro-effector relationships exist with salivary parenchymal and myoepithelial cells: epilemmal (outside the parenchymal basement membrane) and hypolemmal (within the parenchymal basement membrane). Their relative frequencies with either type of nerve differ greatly between glands and species.

The presence of hypolemnal axons between striated duct cells in submandibular glands of cats has been established electron microscopically (22). Axons were found between light and dark cells and between light and basal cells. Hypolemnal axons were observed most frequently in the junctional region between striated and intercalary ducts. In this area we observed nerve fibers also both in the sheep and goat which fibers may correspond to hypolemnal axons.

The close contact of the nerve fibres and the myoepithelial cells lead to some misinterpretations of these structures. Nerve fibres which Dardick *et al.* (7) identified beneath acinar epithelium in the normal salivary gland the other authors (16, 20) have clasified as a myoepithelial cells. Our findings correspond with observations of mentioned authors and the presence of nerve fibres was demonstrated in close contact with the secretory acini of the mandibular salivary gland. The presence of the nerve fibers was also confirmed by Ceccarelli *et al.* (6) who studied innervation of the bovine naso-labial glands and established the occurrence of cholinergic and peptidergic nerve fibres. The authors localized parasympathetic nerve fibres around the secretory units and intralobular excretory ducts while adrenergic fibres were only seen in the interlobular connective tissue around blood vessels of medium size.

Similar distribution of the nerve fibers was observed also in other type of salivary glands though some differencies were observed. Bogart (5) in the rat sublingual gland did not find as rich an innervation as in the parotid or submandibular glands. Axonal bundles consisting of axons ensheated by Schwann cells were observed infrequently in the stroma. These observation we observed also in the sheep and goat mandibular gland.

Variability in axonal terminatioin inside the glandular tissue were also described. The nerve fibers were found to penetrate the

basement lamina and loose their Schwann cell investment. Many of the axons were observed adjacent to acinar cells or between acinar cells and myoepithelial cells. G a r g i u l o *et al.* (15) found small bundles of myelinated and unmyelinated fibres in the connective tissue among the adenomeres of the horse Ebner´s salivary gland. Electron-microscopically they identified bulb-like nerve terminals between secretory cells as well as between the basal lamina and basal pole of the glandular cells.

B o g a r t (5) observed axonal terminal nerve endings that make contact on a parenchymal cell denote nerve-effector-cell junction. Such situation have been demonstrated to exist in the basal and lateral intercellular spaces of the acinus of the parotid gland, in the intercellular spaces between acinar cells, and between acinar and myoepithelial cells in the sublingual gland. Many of these axons are ensheated by a Schwann cell that also invest axons that do possess activity. Similarly, nerve terminals devoid of acitivty may contact an acinus that also contains nerve terminals that have enzyme activity.

In recent years afferent nerves have started to be identified and are found in greatest numbers around the main salivary ducts, where they may form a hypolemmal association with the epithelial cells. Morphological assessments of changes in the parenchymal cells after nerve stimulations or denervations add greatly to our understanding of the nerve functions. According to G a r r e t t and K i d (13) at least four types of influence can be exerted on salivary parenchymal cells by the nerves: hydrokinetic (water mobilizing), proteokinetic (protein secreting), synthetic (inducing synthesis), and trophic (maintaining normal functional size and state). In respect to each role, wide glandular and species differences exist between the relative contributions made by each type of nerve.

ACKNOWLEGEMENT

This work was supported by Referential Laboratory for Pesticides, UVM, Košice.

REFERENCES

1. Arciszewski, M. B., Arciszewski, A., Zacharko, R., Lalak., 2004: Co-expression of tyrosine hydroxylase, dopamine beta-hydroxylase and neuropeptide Y in the sympathetic neurons projecting to the submandibular gland in the sheep. *Anat. Embr.*, 208, 161–167.

2. Bass, P. W., Black. M. M., l990: Individual microtubules in the axon consist of domains that differ in both composition and stability. *J. Cell Biol.*, 111, 495–509.

3. Black, M. M., Baas, P. W., Humpries, S., l989: Dynamic of α-tubulin deacetylation in intact neurons. *J. Neurosci.*, 9, 358–368.

4. Bogart, B. I., 1970: Fine structural localization of cholinesterase activity in rat submandibular gland. *J. Histochem. Cytochem.*, 18, 730–739.

5. Bogart, B. I., 1971: The fine structural localization of cholinesterase activity in the rat parotid and sublingual glands. *Am. J. Anat.*, 132, 259–266.

6. Ceccarelli, P., Garguilo, A. M., Pedini, V.,. l986: Innervation of the bovine naso labial glands. *Bas. Appl. Histochem.*, 30, 447–452.

7. Dardick, I., Stratis, M., Parks, W. R., DeNardi, F. G., Kahn, H. J., 1991: S-100 protein antibodies do not label normal salivary gland myoepithelium. *Am. J. Pathol.*, 138, 619–629.

8. Dellmann, H. D., Brown, E. M., 1987: *Textbook of Veterinary Histology*. *Digestive system*. Ed. Lea and Febiger, Philadelphia, pp. 209–263.

9. Emmelin, N., 1967: Nervous control of the salivary glands. In *Handbook of Physiology*. Alimentary Canal, Vol., *II. Secretion*. Am. Physiool. Soc., Washington D. C., pp. 595–632.

10. Freitag, P., Engel, M. B., 1970: Autonomic innervation in rabbit salivary gland. *Anat. Rec.*, 167, 87–106.

11. Garrett, J. R., 1966: The innervationm of salivary glands. II. The ultrastructure of nerves in normal glands of the cat. *J. Roy. Micr. Soc.*, 85, 149–162.

12. Garrett, J. R., 1967: The innervation of normal human submandibular and parotid salivary glands. *Arch. Oral Biol.*, 12, 1417–1436.

18. Hsu, S. M., Raine, L., Fanger, H., 1981: The use of avidin-biotin peroxidase complex in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.*, 29, 577–580.

19. Kagayama, M., 1971: The fine structure of the monkey submandibular gland with a special reference to intra-acinar nerve endings. *Am. J. Anat.*, 131, 185–196.

20. Kahn, H. J., Baumal, R., Marks, A., Dardick, I., Van Nostrand, P., 1985: Myoepithelial cells in salivary gland tumors. *Arch. Pathol. Lab. Med.*, 109, 190–195.

21. Kope, G. H., 1977: Ultrastructure of the innervation of the ducts within the rabbit parotid gland. *Archs. Oral Biol.*, 22, 715–719.

22. Kidd, A., Garret, J. R. 1979: New observations on the innervation of striated ducts in submandibular glands of cats, including possible peptidergic nerves. *Cell Tissue Res.*, 200, 205–214.

23. Kronberger, C., Albeger, C., Saria, A., Hacker, G. W., 1992: Neuropeptides in human salivary (submandibular and parotid) glands. *Acta Otolaryngol.*, 112, 343–348.

24. Kurtz, S. M., 1964: The salivary gland. In **Kurtz, S. M.:** *Electron Microscopic Anatomy*. ed. Academic Press, New York, pp. 97–122.

25. Kusakabe, T., Matsuda, H., Kawakami, T., Syoui, N., Kurihara, K., Tsukuda, M., Takenaka, T., 1997: Distribution of neuropeptide-containing nerve fibers in the human submandibular gland, with special reference to the difference between serous and mucous acini. *Cell Tiss. Res.*, 288, 25–31.

26. Soinila, J., Nuorva, K. Soinila, S., 2006: Nitric oxide synthase in human salivary glands. *Histochem. Cell Biol.*, 125, 717–723.

27. Tsukoi, T., Honda, T., Hishida, S., Shigetomi, T., Ueda, M., Sugita, Y., 2004: A quantitative study of nerve fiber density in the submandibular gland of rats. *Nagoa J. Med. Sci.*, 67, 25–34.

Received Octobet 17, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 122–125, 2007

IMMUNOHISTOCHEMICAL DETECTION OF SMOOTH MUSCLE ACTIN IN THE SHEEP MANDIBULAR AND PAROTID SALIVARY GLAND

Marettová, M., Maretta, M., Legáth, J.,

University of Veterinary Medicine, Komenského 73, 041 81 Košice
The Slovak Republic

marettova@uvm.sk

ABSTRACT

The smooth muscle actin in the sheep mandibular and parotid salivary glands was studied immunohistochemically using alpha-smooth muscle actin and S-100 protein antibodies. After using alpha-smooth muscle actin antibody positive reaction has been recognized in the myoepithelial cells located on the periphery of secretory acines and around the small ducts. The presence of the smooth muscle actin was absent on the periphery of intralobular and interlobular ducts. Positive myoepithelial cells with cytoplasmic processes envelop partially or completely secretory acini and intercalated ducts. The presence of contractile protein filaments in the cytoplasm and the localization of the myoepithelial cells confirm their contractile function in the passage of secretion from the acini into and along the ducts. Besides, the reactivity for smooth muscle actin has been observed in the wall of small and larger blood vessels where smooth muscle cells were located in the *tunica media*. S-100 protein did not give positive reaction for the myoepithelial cell of both salivary glands studied.

Key words: immunohistochemistry; salivary glands; sheep

INTRODUCTION

Smooth muscle actin is considered the marker for smooth muscle cells as well as for myoepithelial cell (6). It has been observed in myoepithelial cells in normal human submandibular gland (3, 14), in developing bovine parotid gland (13) and in various exocrine glands of guinea pigs (18). Z a i d a and F a r i n a (23) who studied different cellular types in bovine nasolabial glands with particular emphasis on the cytoskeletal protein expression found anti-smooth muscle actin in basket cells and basal cells of intercalated ducts.

Myoepithelial cells positive for S-100 protein were described in apocrine sweat glands (4) but rat myoepithelial cell (10) as well as cells in bovine glands (11) were found to be negative for S-100. Neither myoepithelial cells in human submandibular gland (15) give a positive reaction for S-l00 protein. On the other hand H a s h i m o t o *et al.* (8) in the rat salivary gland and D a r d i c *et al.* (3) in human salivary gland tumor described positivity for S-l00 protein in myoepithelial cells. Also F e r r e r *et al.* (4) in the appocrine sweat gland of the dog showed anti protein S-100 in proliferating myoepithelial cells of sweat gland carcinomas.

The aim of the study was immunohistochemically to localize anti-alpha smooth actin and S-100 protein in relation with myoepithelial cells in two large salivary gland. The results will be applied in the study of the effect of toxic substances both in the secretory cells and in the myoepithelial cells in the process of digestion of food before it reaches the stomac.

MATERIAL AND METHODS

Seven adult sheeps were used in this study mandibular and parotid salivary glands were dissected out at the local slaughterhouse immediately upon death. Samples of the tissue were fixed in 10 % formalin in 0.2 mol phosphate buffer for 24 h and routinely embedded in paraffin. Sections at 5 µm thickness were cut and mounted on 3-aminopropyltriethoxysilane (APES)-coated

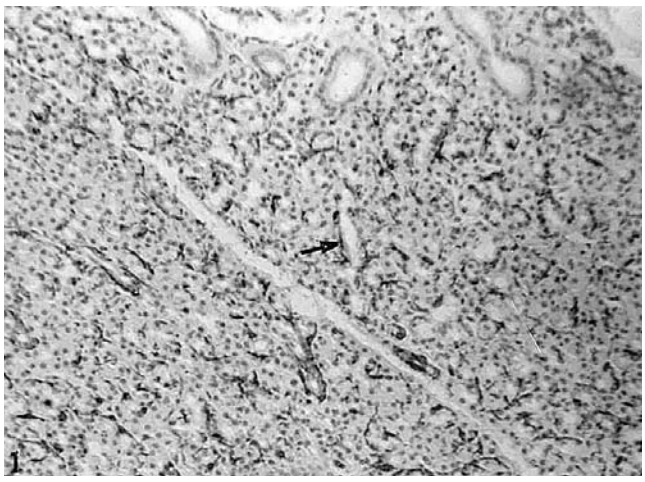


Fig. 1. Parotid gland showing immunoreactivity for smooth muscle actin and counterstain with haematoxylin. Reactive myoepithelial cells are seen on the periphery of intercalated ducts (arrow) — 120 ×

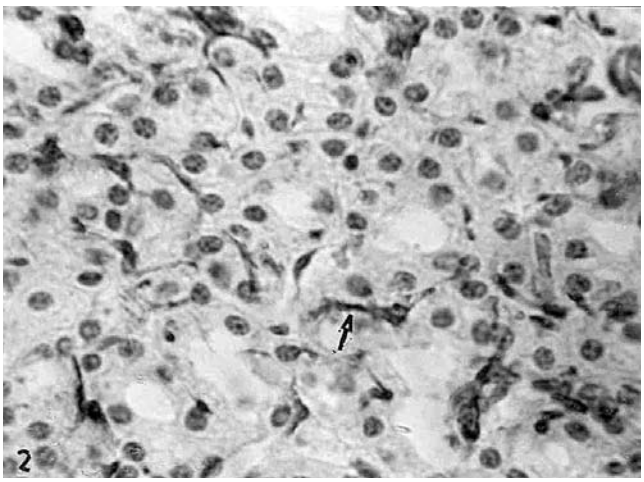


Fig. 2. Parotid gland showing immunoreactivity for smooth muscle actin and counterstain with haematoxilin. Reactive myoepithelial cells are seen between acinar cells (arrow) — 460 ×

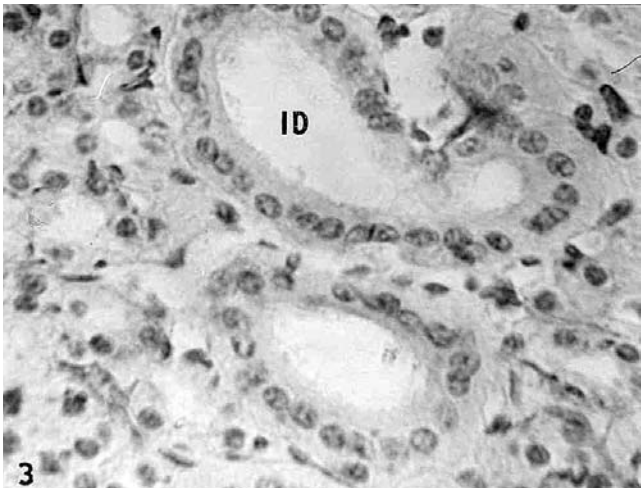


Fig. 3. Parotid gland showing immunoreactivity for smooth muscle actin. No reactive myoepithelial cells are seen arround the intralobular duct (ID) — 460 ×

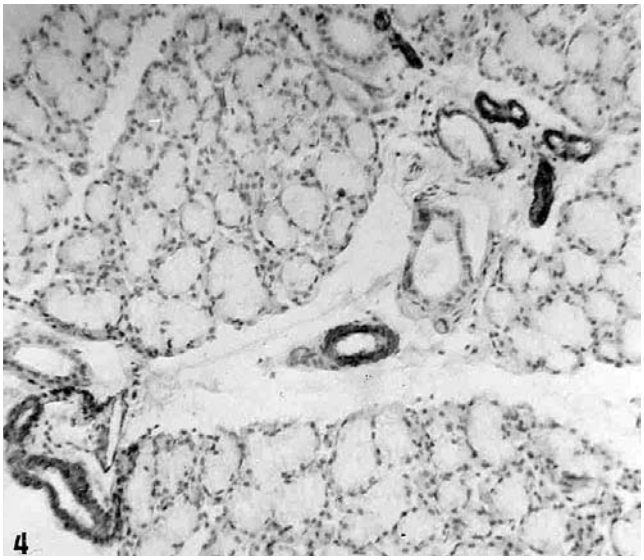


Fig. 4. Submandibular gland showing immunoreactivity for smooth muscle actin. Strong positive reaction is in the wall of the small blood vessels — 120 ×

slides. Consecutive sections were used for histological, control and immunohistochemical procedures.

Immunohistochemistry

For immunostaining procedure Avidin-biotin complex method (9) was used. Histological section were deparafinized and rehydrated, pretreated with 3 % H_2O_2 in methanol to block endogenous peroxidase activity and preincubated with 2% goat serum to mask unspecific binding sites. Washed section were incubated overnight with monoclonal anti-α-smooth muscle actin antibody (mouse IgE1, clon 1A4, Sigma) and polyclonal S-100 protein (rabbit, IgG, Sigma). The sections were washed in phosphate-balanced salt solution (PBS), and incubated with biotinylated secondary antibody for 30 min. Washed sections in PBS, were incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame, USA). After washing with PBS, peroxidase activity was visualized with diaminobenzidine (DAB) and H_2O_2 in TRIS buffer within 5 min at room temperature. Sections were counterstined with Mayer's haematoxylin. For negative controls, the first antibody was substituted by PBS.

RESULTS

The myoepithelial cell in the ovine salivary gland has a form of flat cell with long cytoplasmic processes located on the periphery of secretory acini and small ducts. In routine histologic sections and haematoxilin-eosin staining it was hardly to identify precise localiza-

tion myoepithelial cell with their cytoplasmic processes. After using alpha-smooth muscle actin antibody strong positive reaction has been observed in the myoepithelial cells located on the periphery of secretory acini and small intercalated ducts between secretory cells and the basement lamina.

The cytoplasmic processes of myoepithelial cells envelop partially or completely secretory acini and intercalated ducts (Figs. l, 2). The cytoplasmic processes surrounding the secretory aciny often display differences in stainability. Positive reaction for smooth muscle actin was not observed on the periphery of larger salivary ducts – intralobular and interlobular ducts (Fig. 3). Smooth muscle actin was observed in the wall of small and large blood vessels where smooth muscle positive cells were located in the *tunica media* (Fig. 4). S-100 protein used in this study did not give positive reaction for the myoepithelial cell of both the mandibular and parotis salivary glands. The ductal cells, on the other hand, were weakly to mildly positive for S-100 protein.

DISCUSSION

The distribution of the smooth muscle actin in the ovine large salivary glands manifested similar position to those described in other animal species and it was considered the marker for smooth muscle cells as well as for myoepithelial cell (6).

Myoepithelial cells have structural features of both epithelium and smooth muscle cells (17). They are characterized by their flat shape with long processes situated on the periphery of the secretory acines and small ducts localized between glandular cells and basement membrane (13). Myoepithelial cells were observed also in human parotid salivary gland (12) and in bovine small salivary gland in the same position as in the large, i.e. at the basal surface (5). As in the bovine parotid gland also in the ovine the processes of myoepithelial cells were in contact with several underlying secretory acinar cells or along the edges of intercalated duct cell.

In contrast, anti-smooth muscle actin reactivity has been never found along other duct segments, i.e., intralobular or interlobular ductal cells. The absence of myoepithelial cells in this kind of excretory ducts was described also in the small salivary gland – G a r g i u l o *et al.* (5). The absence of these cells we observed also in large ovine salivary glands. M i u r a *et al.* (13) in bovine parotid gland found that cell processes were often in intercellular regions, i.e. beneath the lateral folds of epithellial cells. This localization of myoepithelial cells we could not identify in the ovine salivary glands.

In other studies the myoepithelial cells were positively stained with antibody to S-l00 protein (1, 7). M a k i n o *et al.* (12) consider the S-l00 protein to be a marker for myoepithelial cells. On the other hand, D a r d i c k *et al.* (3) after using double immunoflorescent staining with antibodies to either S-l00 protein or neuron-specific enolase combined with muscle specific actin do not reveal colocalization of these antigens in myoepithelial cells. O k u r a *et al.* (l5) who described that no of S-l00 protein occurred in the smooth muscle actin containing myoepithelial cells in a human submandibular gland and none of the bovine exocrine gland studied give a positive reaction for S-l00 protein (11).

Distribution of myoepithelial cells seems to be stable in a salivary glands of various animal species. M i u r a *et al.* (13) observed that in bovine foetuses were essencially the same as those reported in rats – T a g a and S e s s o (20) and R e d m a n and B a l l (l6). Stable position of myoepithelial cells in relation to the secretory acines was not seen in glandular ducts. S h a c k l e f o r d and W i k l b o r n (l9) studied localization of myoepithelial cells in the bovine parotid gland and noted their limited occurrence in relation to the junctional area betwen acini and intercalated ducts.

Z a i d a and F a r i n a (23) who studied different cellular types in bovine nasolabial glands with particular emphasis on the cytoskeletal protein expression found basket cells and basal cells of intercalated ducts to be reactive to anti-smooth muscle actin. The authors suggested that the basal duct cells are contractile, myoepithelial cells. After using other monoclonal antibodies to cytoskeletal proteins they consider intermediate filaments of the duct epithelium to be more complex and heterogenous in comparison with those present in the acinar cells.

M i u r a *et al.* (13) in developing bovine parotid gland observed cellular bodies of the myoepithelium to surround entire acini and intercalated ducts with some cellular processes distributed similarly as in the sheep. Also S a t o h *et al.* (18) in the parotid and submandibular glands of guinea pig observed cellular bodies of the myoepithelium to surround entire acini. In this species the glandular acini are very small and each acinus had one or two myoepithelial cells attached. In ovine mandibular salivary glands only part of the acini and length intercalated ducts was covered by one myoepithelial cells. This discrepancy may be caused by differences in size of the acini and length of intercalated ducts among animal species. According to T a n d l e r *et al.* (2l) they tend to be the longest in the parotid gland, intermediate long in the submandibular gland, and short to nonexistent in the sublingual gland but there are exceptions between animal species. Localization of contractile filaments in the cytoplasm and the localization of the myoepithelial cells ralated with secretory cells confirm their contractile function – to facilitate the passage of secretion from the acini into and along the ducts.

REFERENCES

1. Batzakis, J. G., Ordoez, N. G. H., Ro, J., Meis, J. M., Bruner, J. M., 1986: S-100 protein and myoepithelial neoplasms. *J. Laryngol. Otol.*, 100, 687–698.

2. Cullar, L. S., Chaudry, A. P., l973: Differentiation of myoepithelial cells of the rat submandibular gland *in vivo* and *in vitro*. An ultrastructural study. *J. Morphol.*, 140, 343–353.

3. Dardick, I., Stratis, M., Parks, W. R., DeNardi, F. G., Kahn, H. J., 1991: S-100 protein antibodies do not label normal salivary gland myoepithelium. *Am. J. Pathol.*, 138, 61–629.

4. Ferrer, L., Rabanal, R. M., Fondevilla, D., Prats, N., 1990: Immunocytochemical demonstration of intermediate filament proteins, S-100 protein and CEA in apocrine sweat glands and apocrine gland derived lesions of the dog. *J. Vet. Med. Ser. A.*, 37, 569–576.

5. Gargiulo, A. M., Ceccarelli, P., Dall'Aglio, C., Pedini, V., l995: Ultrastucture of bovine von Ebner's salivary glands. *Ann. Anat.*, 177: 33–37.

6. Gugliotta, P., Sapino, A., Macri, L., Skalli, O., Gabbiani, G., Bussolati, G., 1988: Specific demonstration of myoepithelial cells by anti-alpha smooth muscle actin antibody. *J. Histochem. Cytochem.*, 36, 659–663.

7. Hara, K., Ito, M., Iijima, D., Endo, T., Hidaka, H., 1983: Distribution of S-100b protein in normal salivary glands and salivary glands tumors. *Virchows Arch. A.*, 401, 273–249.

8. Hashimoto, J., Yamada, K, Ogata, K., Takay, Y., Mori, M., 1992: Immunoreaction of keratin, actin, S-100 protein and rat-EGF in duct-ligated rat salivary glands. *J. Oral. Pathol. Med.*, 21, 214–220.

9. Hsu, S. M., Raine, L., Fanger, H., 1981: The use of avidin-biotin-peroxidase complex in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.*, 29, 577–580.

10. Jayasinghe, N., Hashimoto, J., Ogada, K., Shresta, P. Takai, Y., Mori, M., 1993: Changes of calmodulin, S-100 protein and keratin in duct-ligated salivary glands of rats. *Acta Histoch. Cytochem.*, 26, 555–562.

11. Lauboeck, S., Egerbacher, M., 1997: Distribution of S-100 protein and its subunits in bovine exocrine glands. *Histochem. Cell Biol.*, 108, 83–91.

12. Makino, I., Murakami, Y., Murakami, M., Saito, Y., Wada, Y., Nishiyama, Y., 1992: An immunohistological study of normal parotid gland. *Nippon Jibbinkoka Gakkaikaiho*, 95, 1923–1930.

13. Miura, K., Sasaki, K., Amasaki, H., Kano, Y., Asari, M., 1996: Expression and distribution of myoepithelial cells in developing bovine parotid gland. *Okajimas Folia Anat., Jpn.*, 73, 205–210.

14. Okura, M., Shirasuna, K., Hiranuma, T., Yoshioka, H., Nakahara, H., Aikawa, T., Matsuya, T., 1993: Characterization of growth and differentiation of normal human submandibular gland epithelial cells in a serum-free medium. *Differentiation*, 54, 143–153.

15. Okura, M., Hiranuma, T., Tominaga, G., Yoshioka, H., Aikawa, T., Shirasuna, K., Matsuya, T., 1996: Expression of S-100 protein and glial fibrillary acidic protein in cultured submandibular gland epithelial cells and salivary gland tissues: Histogenetic implication for salivary gland tumors. *Amer. J. Path.*, 148, 1709–1716.

16. Redman, R. S., Ball, W. D., l979: Differentiation of myoepithelial cells in the developing rat sublingual gland. *Am. J. Anat.*, 156, 543–566.

17. Redman, R. S., 2005: Myoepithelium of salivary glands. *Micr. Res. Techn.*, 27, 25–45.

18. Satoh, Y., Oomori, Y., Ishikawa, K., Ono, K., 1994: Configuration of myoepithelial cells in various exocrine glands of guinea pigs. *Anat. Embryol.*, 189, 227–236.

19. Shackleford, J. M., Wiklborn, W. H., 1969: Ultrastructure of bovine parotid glands. *J. Morphol.*, 127, 453–474.

20. Taga, R., Sesso, A., l979: Ultrastructural studies on developing parotid gland of the rat at early periods. *Arch. Histol. Jpn.*, 427–444.

21. Tandler, B., Nagato, T., Toyshima, K., Phillips, C. J., 1998: Comparative ultrastructure of intercalated ducts in major salivary glands. *A review. Anat Rec.*, 252, 64–91.

22. Van Lennep, E. W., Kennerson, A. R., Compton, J. S., l977: The ultrastructure of the sheep parotid gland. *Cell Tiss. Res.*, 179, 377–392.

23. Zaida, M., Farina, V., l998: Immunocytochemical identification of different cell types in bovine nasolabial glands with particular emphasis on cytoskeletal protein expression. *Anat. Histol. Embryol.*, 27, 37–44.

ACKNOWLEDGEMENT

This study was supported by National laboratory for pesticides, UVM Košice.

Received September 17, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 126–129, 2007

THE SPERMIOGRAM OF MALE WISTAR RATS TREATED WITH AQUEOUS LEAF EXTRACT OF *Vernonia amygdalina*

Oyeyemi, M. O., Oluwatoyin, O. Ajala
Leigh, O. O., Adesiji T., Fisayo

Department of Veterinary Surgery and Reproduction
Faculty of Veterinary Medicine, University of Ibadan
Nigeria

mo.oyeyemi@mail.ui.edu.

ABSTRACT

The reproductive implications of the aqueous leaf extract of *Vernonia amygdalina* were studied using twelve male albino rats (Wistar strain). Two experimental groups (A and B) were treated with 500 mg.kg^{-1} body weight and 250 mg.kg^{-1} body weight respectively for 15 days and the control group C were not treated with the extract. The spermatozoa from the caudal epididymides were then studied in these three groups. It was observed that the mobility of spermatozoa from groups A and B with percentage values of 34.00 ± 2.28 and 60.00 ± 0.00 respectively were significantly lower (P > 0.05) than for group C which had 92.50 ± 0.00. The percentage livability of the sperm cells were also lower in groups A and B with 92.00 ± 0.54 and 95.20 ± 0.75 respectively than for the control of group with value of C 98.00 concentration of the experimental groups 53.20 ± 1.49 for group B) were also significantly lower (P > 0.05) than for the control group (C 88.50 ± 0.00). The histopathology of the testes revealed hypoplasia of the seminiferous tubules in groups A and B as compared to the normal somniferous tubules in the control group (C). It can be concluded, therefore, that the uncontrolled use of *Vernonia amygdalina* have an adverse effect on the spermiogram and spermatozoa/morphology of the intact male Wistar rats.

Key words: spermiogram; *Vernonia amygdalina*; Wistar rats

Corresponding author:
Dr. M. O. Oyeyemi
Department of Veterinary Surgery and Reproduction
Faculty of Veterinary Medicine
University of Ibadan
Nigeria

INTRODUCTION

Vernonia amygdalina is a shrub of 2–5 m with petiolate leaf of about 6 mm in diameter and elliptic shape (C o m p o s i t a e). The leaves are green with a characteristic odour and a bitter taste. It is propagated by cutting. There are about 200 species of *Vernonia* and it grows under a range of ecological zones in Africa (11).

The use of medicinal herbs have increased over the past few years and research interest has focused on various herbs that possess anti-tumour or immune stimulating properties that may be useful adjuncts in helping to reduce the risk of cancer (3). This was why N w u d e (8) said that s research into the efficacy of herbs used in traditional veterinary practice would be useful in establishing standard dosages for herbal preparations and to investigate their toxicity. One of such herbs is *Vernonia amygdalina*.

Vernonia amygdalina is commonly known as bitter leaf. It is known as ewuro in Yoruba and onugbu in Ebonyi State, and chusar doki in Hausa land. Traditionally the leaves of this herb are consumed in many parts of Nigeria as vegetable. The leaves and stems are also chewed to cure stomach aches, stimulate the digestive system. It can also be used as insecticide. The aqueous extract is used as tonic drink for prevention of certain illnesses for example as anti-helminthiasis, as laxative anti-malaria and trypanocide (1, 14). A cold infusion of the root bark is used to treat biharzias (4). The vegetable when incorporated in the diet of women may prevent or delay the onset of breast cancer.

The phytochemisry of this herb have been studies to some extent. According to P h i g a s h i *et al.* (11) and J i s a k a

et al. (7) reported that it is made up of several stigmastanetype saponins, such as vernonioside A, B1, A2, A3, B2, D3, A4, and C. In 2004, Akindahunsi and Salawu reported that the sundried leaves of this herb contain saponins, tannins and minerals, like iron, zinc, magnesium, sodium, potassium, calcium and phosphorus, Igile *et al.* (5) using coupled oxidation of B-carotene linoleic and demonstrated the anti-oxidant activities of luteolin-o-β-glucoside flavonoid compounds present in he leaves of *Vernonia amygdalina*. It was also discovered that the leaves contain vernodaline and vernolide which wee demonstrated by Jisaka *et al.* (7) to elicit antitumoural activity in leukaemia cell P-388 and C 1210 with IC 50 values of 0.11 and 0.17 $\mu g.ml^{-1}$ for vernodaline and 0.13 and 0.11 $\mu g.ml^{-1}$ for vernolide. Extracts from the herb have cell growth inhibitory effects in prostate cancer cell 1 ms (PC-3) and no effect on human peripheral blood mononuclear cells (PBMC) and some peptides from the aqueous extracts of the leaves are potent inhibitors of mitogen-activated proteinkinase (MAPKS) which are crucial for breast tumour growth and also inhibit DNA synthesis in a breast cancer cell line (6). The haemolytic effect of saponin extract of *V. amygdalina* was reported by Oboh (9).

It was discovered that there was no report on the effect of *Vernonia amygdalina* extract in any form either as aqueons or saponin on the reproductive functions of the male knowing that this herb is consumed by both male and female, hence this study was carried out to evaluate the sperm characteristics and morphology of the male Wistar rats that have undergone treatment with varying doses of the aqueous *V. amygdalina*. Since fertility in the male animal depend on the viability and concentration of the sperm cells produced by the testes, the outcome of this study will constitute a source of baseline data and basis for advising the ethnomedical practitioner and the general public on the usage of this herb.

MATERIALS AND METHODS

Fifteen sexually male albino rats (Wistar strains) were used for this study. The rats were housed in the Experimental Animal Unit (EAU) of the Faculty of Veterinary Medicine, University of Ibadan, Nigeria. The rats were fed *ad libitum* with commercially prepared rat feeds made up of 21 % protein, 3.5 % fat, 6 % fiber, 0.8 % phosphorus (Ladokun Feeds Limited, Ibadan, Nigeria) and given fresh water. They were kept in cages which have wood shaven as bedding and cleaned once a week. The feeds and water were provided *ad libitum* in earthen troughs. Three cages were used and each measured about 18 by 12 inches. The rats were in groups of 5, 5 and 5 in each cage marked A, B and C respectively under the same environmental and management conditions.

The aqueous extract was prepared using 400 ml of distilled water to 200 g of fresh *Vernonia amygdalina* leaves. The concentration of the extract was determined using this formula.

$$\text{Concentration} = \frac{\text{Weight of leaves g}}{\text{Quantity of water ml}}$$

And the actual dose to be administered was obtained using this formula:

$$\text{Dose (ml)} = \frac{\text{dosage mg.kg}^{-1} \times \text{body weight (kg)}}{\text{concentration mg.ml}^{-1}}$$

After 2 weeks of stabilization the rats in groups A and B were given aqueous extract of *Vernonia amygdalina* leaf at 500 $mg.kg^{-1}$ body weight and 250 $mg.kg^{-1}$ body weight respectively *per os* for a 5 days using needle and canular to introduce the extract directly into the stomach through the oesophagus. Group C rats were the control with no administration of *Vernonia amygdalina* extract.

The rats were then anaesthetized by placing them into a glass chamber containing cotton wool soaked in chloroform till they lost consciousness. The testicles were then removed through a lower abdominal incision. The testes were then separated from the epididymis with the scalped blade. Sperm cells were sucked into a pre-warmed (37 °C) Pasteur pipette from the caudal epididymis. It was flushed with 2–3 drops of 2.9 % sodium citrate self kept at 37 °C. Smears were prepared from these samples and strained with wells and Awa stain for morphological studies and eosin and nigrosin stain for live / dead ratio (16). Half of the spermatozoal samples collected were mixed with 0.5 ml of 2.9 % sodium citrate solution (7 °C) for head forward unidirectional progressive motility. These were studied at × 40 magnifications of the microscope (16).

The mean percentages and standard error of mean were calculated for motility, live/dead ratio, Spermatozoal concentration and morphological studies. ANOVA (Analysis of variance) was used to establish any significant difference in all these parameters (15).

RESULTS

It was observed that the groups of rats that had the highest dose of the aqueous extract (500 $mg.kg^{-1}$ body weight and those of 250 $mg.kg^{-1}$ body weight had their percentage motility 34.00 ± 2.8 and 60.00 ± 0.00 % respectively) significantly lower ($P < 0.05$) than that of the control group (92.5 ± 0.00 %). The higher the dosage the higher the gravity of the effect of the extract on the motility of the sperm cells (Table 1).

The percentage livability (live-dead ratio) of the sperm cells was 92.0 ± 0.5, 95.2 ± 0.75, and 98.0 ± 0.00 % for the doses of 500 $mg.kg^{-1}$, 250 $mg.kg^{-1}$ and the control group respectively. There was a reduction on the number of the live spermatozoa with 98.00 ± 0.05 % in the control group compared to the groups A & B with 92.00 ± 0.5 and 95.20 ± 0.75 % respectively.

The sperm cell concentration 53.20×10^6 $cells.ml^{-1}$ at group A and the count of 67.40×10^6 $cells.ml^{-1}$ were lower than the count 88.50×10^6 $cells.ml^{-1}$ in the control group and this difference is significant ($P < 0.05$) and this is confirmed by the histopathologic slide of the testes (Figs. 1, 2) where there was hypoplasia of the seminiferous tubules of the testes of rats in group A.

The morphological characteristic of the sperm cells in all the three groups of rats are as shown on Table 2, the following abnormalities were noticed both in the control group and the treated groups A & B; head less tail, rudimentary tail, curved mid-piece, curved tail, coiled tail, looped tail, bent mid piece tailless head and bent tail, but the percentage of abnormal sperm bells were highest (11.05 %) in the group A treated rats, followed by those (8.5 %) treated in group B. The control group was the lowest (6.45 %).

Table 1. Sperm characteristics of Wistar rats in groups A + B and C (± SEM)

Identification	Motility (%)	Live/dead ratio Livability (%)	Sperm count $\times 10^6$ cells.ml^{-1} (concentration)
A 500 mg.kg^{-1} bw	34.00 ± 2.8	92.00 ± 0.54	53.20 ± 0.53
B 250 mg.kg-1 bw	60.00 ± 0.00	95.20 ± 0.75	67.40 ± 1.49
C No treatment	92.50 ± 0.00	98.00 ± 0.00	88.50 ± 0.00

DISCUSSION AND CONLUSION

The study showed that the percentage motility of the control experiment (group C) rats 92.5 % was higher than 76.00 ± 2.40 % reported by O y e y e m i *et al.* (12) for same species of rats. These values are significantly higher ($P < 0.05$) than motility values of 34.00 % for group A rates (treated with 500 mg.kg^{-1} body weight) and 60.00 % for group B rates (250 mg.kg^{-1} body weight). Although the percentage livability of sperm for group A (92.00 %) was lower than group B (95.20 %) and also lower than group C value (98.00 %). However, livability percentages in the three groups are higher than what was recorded (88.00 ± 3.39 %) by O y e y e m i *et al.* (12) for male Wistar rats without any treatment.

The sperm count (concentration)of group C 88.50×10^6 cells.ml^{-1} was significantly higher ($P < 0.05$) than group A value (67.40×10^6 cells.ml^{-1}) and this difference was significant ($P < 0.05$) as evidenced by the hypoplasia of the seminiferous tubules in the histopathological slides of the testes of rats from group A indicated that very high dozes (250–500 mg.kg^{-1} body weight of aqueous leaf extract of *V. amygdalina* for a long time like 15 days will have adverse effect on the sperm concentration. The morphological abnormalities also increased as the concentration of the extract increased as observed in this study.

It can be therefore concluded that dosages between 250–500 mg.kg^{-1} body weight of the aqueous leaf extract of *Vernonia amygdalina* for as long as 15 days will have a deleterious effects on the reproductive functions of the male Wistar rat and so it is advised the continuous usage of aqueous leaf extracts of *V. amygdalina* should be with caution.

REFERENCES

1. Abosi, A. O., Raserok, B. H., 2003: *In vivo* anti-malaria activity of *V. amygdalina*. *Bri. J. Biomed. Sci.*, 60, 89–91.

2. Akindahunsi, A. A., Salawu, S. O., 2004: Phytochemical screening and nutrient – anti-nutrient composition of selected tropical leafy vegetables. *Africa J. Biotech.*, 14, 497–501.

3. Craigh, W. T., 1999: Health promoting properties of common herbs. *Am. J. Clin. Nutrit.*, 70, 491–499.

4. Fichtl, R., 2005: A/Bees for development tree vernonia. www.bees for dvlpt.org/infor/info/flora/tree vern. Amy Shtml.

5. Igile, G. O., Oleszek, W., Jurzyata, M., Burda, S., Fafunso, M., Fasonmade, A. A., 1994: Flavoids from *Vernonia amygdalina* and their antioxidant activities. *J. Agric. Food Chem.* (USA), 42, 2445–2448.

6. Izevbigie, E. B., 2003: Discovery of water-soluble anticancer agent (Etidotes) from a vegetable found in Benin City, Nigeria. *Exp. Biol. Med.*, 228, 293–248.

7. Jisaka, M., Ohigashi, H., Takagawa, K., Nozaki, H., Hirota, M., Irie, R., Huffman, M. A., Koshimizu, K., 1993: Steroid glucoside from *Vernonia amygdalina*. A possible chimpanzee medicinal plant. *Phytodiemistry*, 34, 409–413.

8. Nwude, I. N., 1976: T*oxicological investigations of Lassiosiphone kraussianus (Heisn)*. Abstract PhD Thesis, A.B.U., Zaria, Nigeria, 4 pp. Abst. 6731. Vet. Bull.

9. Oboh, G., 2001: Haemolytic effect of saponin extract from *Vernonia amygdalina* on humans erythrocyte. *ICTP Reprint* No. 1C 200115. www.ICTPtruste.IT/CTP/reprints/2001list html.

Table 2. Sperm morphology of Wistar Rats in Groups A + B and C (± SEM)

Identification	Headless tail (%)	Rudimentary tail (%)	Curved midpiece (%)	Curve tail (%)	Looped tail (%)	Bent mid-piece (%)	Tailless head (%)	Bent tail (%)	Total abnormal (%)	Total normal (%)	Total cells counted (%)
A	26 (1.37)	27 (1.42)	27 (1.42)	4 (0.021)	4 (0.021)	27 (1.42)	22 (1.16)	38 (2.01)	209 (11.05)	1681 (88.94)	1890 (100)
B	20 (1.01)	24 (1.21)	25 (1.26)	0	2 (0.10)	20 (1.01)	16 (0.80)	32 (1.61)	169 (8.53)	1811 (91.46)	1980 (1980)
C	6 (0.74)	5 (0.62)	10 (1.24)	0	0	6 (0.74)	5 (0.62)	11 (1.36)	52 (6.45)	753 (93.54)	805 (100)

10. Ohigashi, H., Jisaka, M., Huffman, M. A., Koshimizu, K., 1991: Butter principle and a related steroid glucoside from *Vernonia amygdalina* – a possible medicinal plant for wild chimpanzee. *Agric. Biol. Chem.*, 55, 1201–1203.

11. Ogundipe Oluwatoyin, 2005: Health bitter leaf extract may prevent delay breast cancer and diabetes. Friday October 21, 2005. http://www.utexas.edu/conferences/Africa/ads/ 1261. html.

12. Oyeyemi, M. O., Towobola Bisoye, A., Ola Davies Olufunke, E., 2006: The reproductive implications of Clomiphene citrate on sperm cells during the epididymal transit of spermatozoa in male Wistar Rats. *Folia veterinaria*, 50, 131–133.

13. Phillipson, J. E. Wright, C. W., Kirby, G. C., Warhust, D. C., 1993: Tropical plants as sources of antiprotozoal agents, Recent advances. *Phytochemistry*, 27, 1–40.

14. Snedecor, G. W., 1946: *Statistical Methods* (4th edn.). IOWA State College Press, Arnes, IOWA, pp. 3–56.

15. Zemjanis, R., 1970: *Diagnostic and Therapeutic Techniques in Animal Reproduction* (2nd edn.), pp. 139–154.

Received September 1, 2007

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 130–136, 2007

Clinical Case Report

INDIRECT CONTACT: A POSSIBLE DISSEMINATION ROUTE OF CAPRINE ARTHRITIS-ENCEPHALITIS VIRUS AMONG GOAT KIDS

Asefa Asmare, A.[a,b], Washburn, K. E.[c], Saliki, J. T.[d] Goetsch, A.L.[a], Dawson, L.J.[ec], Merkel, R. C.[a], Sahlu, T.[a]

[a]E (Kika) de la Garza American Institute for Goat Research, Langston University, P. O. Box 730, Langston, OK 73050 USA

[b]Department of Animal Sciences, Alemaya University, P. O. Box 138-212, Dire Dawa Ethiopia

[c]Department of Large Animal Clinical Sciences, Texas A&M University, 4475 TAMU, College Station, TX 77843 USA

[d]Oklahoma Animal Disease and Diagnostic Laboratory, Stillwater, OK 74078 USA

[e]College of Veterinary Medicine, Oklahoma State University, Farm Rd, Stillwater, OK 74078 USA

ABSTRACT

Twenty Alpine goat kids were randomly assigned to four groups each consisting of five animals. Kids were removed from their dams at birth and penned individually. Kids in all groups were fed colostrum during the first 48 h after birth. Group 1 was fed Caprine arthritis-encephalitis virus (CAEV) free colostrum, Group 2 received CAEV positive colostrum, Group 3 consumed CAEV positive colostrum subjected to conventional heat treatment, and Group 4 was given CAEV positive colostrum treated with methylene blue and fluorescent light. Thereafter, all kids were fed pasteurized goat milk until weaning. No CAEV specific antibodies were detected in serum of any kids collected prior to colostrum consumption. Despite efforts to avoid vertical transmission of the virus among kids, three goat kids of Group 1 showed seroconversion at the age of 48 h and the remaining two kids displayed seroconversion at 2 months of age. All animals in Groups 2, 3, and 4 had seroconvertedion at the age of 48 h. Presence of CAEV was confirmed in 17 of the kids (85 %) by polymerase chain reaction at the ages of 1.5 months. The early appearance of CAEV specific antibodies was probably caused by consumption of antibody containing colostrum and maintenance of maternal antibodies. Results of this study suggest that factors other than direct contact of kids with their dams i. e. ingestion of infected colostrum and milk could be means of CAEV transmission. Therefore, the risk of indirect contact in the dissemination of CAEV should be taken into account in control and eradication programs.

Key words: caprine arthritis-encephalitis virus; colostrum; dissemination; goats

INTRODUCTION

Caprine arthritis-encephalitis virus (CAEV) is an enveloped, single stranded RNA virus of the family Retroviridae and the sub-family Lentivirinae. The infection has been detected worldwide, but it is most prevalent in countries where goats are intensively dairied (11, 16). In the USA, Canada, and Europe, 30–80 % of dairy goats are infected compared with 0–10 % in Africa and South America (28, 11, 12, 33, 27). CAEV represents an economically important disease problem for the dairy goat industry (45). It leads to reproductive failure, low milk production, reduced lactation length, premature culling of high yielding goats, ban on importation of antibody positive goats, and incidence of intercurrent diseases (18).

CAEV is characterized by presence of an RNA-dependent DNA polymerase and is functional only when the viral RNA is transformed into viral DNA and integrated into the host cell DNA. Thus, the viral genome becomes a part of the cellular DNA and replicates efficiently in non-dividing, terminally differentiated cells (34). CAEV has a tropism for cells of the monocyte/macrophage lineage (35, 51, 17, 43, 41, 16). It is found in monocytes in latent form, and multiplies when the monocytes mature into macrophages in the different body tissues, thereby causing chronic inflammatory and degenerative diseases arthritis, mastitis, and interstitial pneumonia in adult goats and leukoencephalomyelitis in young kids (7, 9, 8, 6, 10, 37, 42).

CAEV-infected goats do not develop disease uniformly. Clinical signs of infection develop after a prolonged incubation period of months to years (22). The clinical syndrome is seen only in about 30–40 % of infected animals (8). Once an animal is infected with CAEV, the infection persists throughout life despite a high antibody titer 3). The depressed natural killer cell activity observed in infected goats may contribute to establishment of a persistent infection (30). Therefore, it is probably true that a large percentage of antibody positive goats carry persistent infections (42). It is also important to note that the maternal antibodies passed in the colostrum are not protective for kids ingesting the colostrum (1).

Transmission of CAEV is not completely understood (15). The mammary gland is an important target organ for infection with CAEV. It has recently been demonstrated that epithelial cells in goat milk are susceptible to CAEV infection both *in vitro* and *in vivo* (32). Infected mammary epithelial cells (21, 19, 46, 4) and infected macrophages (23, 29) in colostrum and milk are suspected to be the main source of natural infection from infected does to their kids (1, 12, 39). Viral transcripts have also been detected in epithelial cells from several organs of infected goats including the small intestine and kidneys (50) and granulosa cells derived from goat ovarian follicles (25), as well as blood mononuclear cells, seminal fluid, and non-spermatic cells of experimentally infected bucks (47).

Recent *in vitro* research has also demonstrated that caprine oviduct epithelial cells are susceptible to infection by CAEV (26, 24). Furthermore, Fieni *et al.* (15) detected CAEV-infected cells in the goat genital tract. The presence of CAEV-infected cells in the uterus and oviducts suggests a risk of foetal infection. According to Fieni *et al.* (15), all body secretions and excretions could also be a source of infection. Most researchers have found that the disease is primarily transferred through direct contact with infected goats and(or) their products, whereas the possible role of indirect contact was unclear. The purpose of this report is, therefore, to draw attention to the possible role of indirect contact in CAEV dissemination.

MATERIALS AND METHODS

An experiment was conducted at the American Institute for Goat Research of Langston University. The objectives of the experiment were to:

1) Compare the CAEV status of goat kids serologically and by PCR up to 6 months of age that were fed CAEV-containing colostrum at birth subjected to conventional heat treatment with the CAEV status of kids fed CAEV-containing colostrum at birth treated with methylene blue and fluorescent light,

2) Determine the effects of feeding colostrum treated with methylene blue on the general health of goat kids.

Twenty goat kids were randomly assigned to four groups each consisting of five animals and identified with ear tags. Dams of the kids were serologically tested for CAEV before mating and at the 4th month of pregnancy using competitive ELISA (cELISA) kits (VMRD, Inc, Pullman, WA) and were found to be serologically negative. However, by the serological examination conducted with the serum collected at delivery, 5 of the 19 dams were found to be serologically positive. After kids were removed from their dams at birth, avoiding contact between dams and kids, they were individually weighed. Then, each animal was fed colostrum at the rate of 10 % of BW for the first 48 h. Thereafter, kids were fed pasteurized milk until the experiment was terminated. The experiment ended before the initially planned time because of unexpected serology results. The colostrum treatments are described below:

1) Group 1 (negative control): CAEV free colostrum

2) Group 2 (positive control): CAEV positive colostrum, untreated

3) Group 3 (treatment group): CAEV positive colostrum, heat-treated

4) Group 4 (treatment group): CAEV positive colostrum, treated with methylene blue and fluorescent light.

Kids were housed individually and isolated from all other animals. They were handled as isolated individuals to prevent kids that seroconvert to CAEV during the course of the study from contacting other animals in the same or other groups. Separate bottles and nipples were allocated to each kid and were thoroughly cleaned before each feeding of the kids. However, the same farm workers cared for kids and does.

The CAEV negative or positive status of colostrum was determined by cELISA kit (VMRD, Inc, Pullman, WA) and the results were confirmed by a polymerase chain reaction (PCR) (1). CAEV-positive colostrum fed to Group 3 animals was heat-treated at 56 °C for 60 min (13). CAEV positive colostrum fed to Group 4 animals was treated with 0.1 mol methylene blue solution and 60 min of fluorescent illumination (photodynamic inactivation). Previously, 60 min of fluorescent

Fig. 1. Clinically infected kid at the early stage of encephalomyelitis, it had a tilted head usually supportedby the floor of the cage

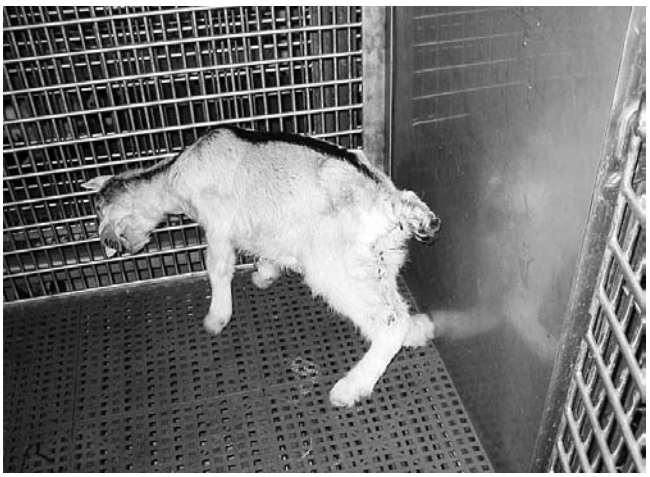


Fig. 2. The kid had swaying movement and was unable to stand for few minutes

Fig. 3. The kid was paralyzed and unable to walk

light treatment significantly reduced bovine viral diarrhoea virus (a RNA virus used as a model for CAEV) levels in colostrum samples containing 0.01 mol methylene blue as compared with dark treatment (49).

Blood was collected from each animal *via* jugular venipuncture at 0 h (immediately after birth prior to colostrum administration), 48 h, 1 week, 1 month, and 2 months of age. Thereafter, serum was harvested by centrifugation at 3,000 × g for 15 min and stored at –20 °C until analyses. Serum was examined for CAEV antibodies titers using a cELISA kit (VMRD, Inc, Pullman, WA). In addition, serum collected prior to colostrum consumption and at 48 h of age (after colostrum consumption) was analyzed by radial immunodiffusion test using the method of Fahey and McKelvey (14) for quantifying IgG in goat kids. Additional blood was collected from each animal at the age of 1.5 months for CAEV detection by PCR (1) in order to confirm results obtained by the cELISA test.

RESULTS AND DISCUSSION

No CAEV specific antibodies were detected in serum collected at birth prior to colostrum consumption in any kids (Table 1). In contrast to mice that are not immune competent at birth, the immune system of kids develops early in foetal life and both humoral and cellular responses against antigens can be observed more than 3 months before birth given an intrauterine CAEV challenge occurs (44). This suggests that even though some kids were born from dams that had seroconverted to CAEV, *in utero* transmission was a minimal contributor to the widespread seroconversion and infection of kids in this study to CAEV. Further evidence of a lack of *in utero* exposure of kids to CAEV is the fact that less than 19.2 to 28.6 mg.dl^{-1} of IgG were recorded in serum samples collected at birth prior to ingestion of colostrum. All kids in the study demonstrated adequate passive transfer of IgG 48 hours after birth indicating all kids had adequate absorption of colostrum. In Group 1, (negative control), three kids showed seroconversion to CAEV at the age of 48 h, and the remaining two had seroconverted by the age of 2 months. The presence of CAEV was confirmed in four of these kids by PCR analysis at 1.5 months of age (Table 1).

All animals in groups 2, 3, and 4 (Tables 2, 3, and 4, respectively) had seroconverted to CAEV at 48 h of age. According to Dawson (11), the mean time for seroconversion is 3–12 weeks after oral, intravenous, or intrarticular exposure to CAEV. The early appearance of CAEV specific antibodies in this study is probably due

Table 1. Caprine arthritis-encephalitis virus (CAEV) blood serum titer serology, IgG, and PCR results of goat kids fed CAEV-free colostrum that was CAEV free per PCR testing (negative control, Group 1)

			IgG (mg.dl^{-1})		CAEV titer serology					CAEV PCR
No.	Does CAEV status	Kid No.	0 h*	48 h**	0 hr	48 h	1 week	1 month	2 months	1–5 months
		395	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)	(-)
919	(-)	394	19.198	>170.162	(-)	(-)	(-)	(-)	(+)	(+)
201	(+)	411	19.198	>170.162	(-)	(+)	(+)	(+)	(+)	(+)
275	(-)	412	28.552	>170.162	(-)	(+)	(+)	(-)	(+)	(+)
171	(-)	406	<19.198	>170.162	(-)	(-)	(-)	(-)	(+)	(+)

* – before colostrum consumption; ** – after colostrum consumption

Table 2. Caprine arthritis-encephalitis virus (CAEV) blood serum titer serology, IgG, and PCR results of goat kids fed CAEV-positive colostrum containing CAEV per PCR testing (positive control, Group 2)

			IgG (mg.dl^{-1})		CAEV titer serology				CAEV PCR
No.	Does CAEV status	Kid No.	0 h*	48 h**	0 h	48 h	1 week	1 month	1.5 months
158	(-)	413	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
158	(-)	414	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
164	(-)	415	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
164	(-)	416	24.668	>170.162	(-)	(+)	(+)	(+)	(+)
205	(+)	400	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)

* – before colostrum consumption; ** – after colostrum consumption

Table 3. Caprine arthritis-encephalitis virus (CAEV) blood serum titer serology, IgG, and PCR results of goat kids fed heat-treated colostrum containing CAEV per PCR testing that was heat treated to eliminate the virus (Group 3)

			IgG (mg.dl^{-1})		CAEV titer serology				CAEV PCR
No.	Does CAEV Status	Kid No.	0 h*	48 h**	0 h	48 h	1 week	1 month	1.5 months
205	(+)	402	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
255	(-)	403	<19.198	>170.162	(-)	(+)	(+)	(+)	(-)
255	(-)	425	<19.198	>170.162	(-)	(+)	(-)	(+)	(+)
927	(+)	427	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
291	(+)	426	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)

* – before colostrum consumption; **– after colostrum consumption

Table 4. Caprine arthritis-encephalitis virus (CAEV) serum titer serology, IgG, and PCR results of positive goat kids fed methylene blue plus light-treated colostrum containing CAEV as per PCR testing that was subjected to methylene blue and light treatment to eliminate the virus (Group 4)

			IgG (mg.dl^{-1})		CAEV titer serology				CAEV PCR
No.	Does CAEV Status	Kid No.	0 h*	48 h**	0 h	48 h	1 week	1 month	1.5 months
285	(-)	407	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
285	(-)	404	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
822	(-)	405	<19.198	>170.162	(-)	(+)	(+)	(+)	(-)
822	(-)	428	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)
171	(-)	429	<19.198	>170.162	(-)	(+)	(+)	(+)	(+)

* – before colostrum consumption; ** – after colostrum consumption

to consumption and maintenance of colostral and maternal antibodies even though in some kids the colostrum was free of virus per PCR. The presence of CAEV was confirmed by PCR in all animals of group 2 and in four animals of groups 3 and 4 each at 1.5 months of age.

Goat kid number 400 of group 2 (positive control) developed clinical signs of encephalomyelitis at the age of 22 days (Figures 1–3). In the first day of clinical examination, the kid had poor body condition, was weak and depressed, and had a tilted head usually supported by the floor of the cage, swaying movements, and moderate diarrhoea. The stool was mildly foetid and bloodstained. Blood for serology and faeces for coccidial oocyte examinations were collected. The kid was previously given *Escherichia coli* antibody (Bovine Ecolizer, Grand Laboratories Novartis Animal Vaccines, Inc., Larchwood, IA). On the second day after clinical signs appeared, the goat kid was paralyzed and unable to walk (Figure 3). The faeces was found to be coccidian-negative, whereas CAEV specific antibodies were detected in the serum (Table 2).

Adams *et al.* (1), Peretz *et al.* (38) and Hanson *et al.* (20) noted that prevention of CAEV transmission is based on removal of kids from their mothers at birth and feeding heat-treated goat colostrum followed by pasteurized milk. Rowe *et al.* (40) and Barlough *et al.* (2), however, observed up to 10 % incidence of unexplained seroconversion after these measures. Despite measures taken to avoid vertical transmission of CAEV in this experiment, a high incidence of seroconversion and infection was demonstrated. The fact that all but one of the kids in the group fed conventionally heat treated colostrum (group 3) became infected with CAEV by 1.5 months of age suggests that in a herd with a high prevalence of CAEV, even standard control measures may not adequately prevent the spread of disease.

CONCLUSION

The unexpectedly high incidence of CAEV infection and transmission in our experiment designed to minimize or eliminate such occurrence suggests that factors other than direct contact of kids with their dams, *in utero* transmission and ingestion of infected colostrum and milk can be potential means of CAEV transmission. Therefore, the risk of indirect contact in the dissemination of CAEV should be taken into account during control and eradication programs.

ACKNOWLEDGEMENTS

This project was supported by the International Partnership in Higher Education for International Development Program of the Association Liaison Office for University Cooperation in Development/United States Agency for International Development.

REFERENCES

1. Adams, D. S., Klevjer-Anderson, P., Carlson, J. L., McGuire, T. C., Gorham, J. R., 1983: Transmission and control of caprine arthritis-encephalitis virus. *Am. J. Vet. Res.*, 44, 1670–1675.

2. Barlough, J., East, N., Rowe, J. D., Vanhoosear, K., Derock, E., Bigornia, L., 1994.: Double-nested polymerase chain reaction for detection of caprine arthritis-encephalitis virus proviral DNA in blood, milk, and tissues of infected goats. *J. Virol. Meth.*, 50, 101–113.

3. Bertoni, G., Hertig, C., Zahno, M. L., Vogt, H. R., Dufour, S., Cordano, P., Peterhans, E., Cheevers, W. P., Sonigo, P., Pancino, G., 2000: B-cell epithets of the envelope glycoprotein of caprine arthritis-encephalitis virus and antibody response in infected goats. *J. Gen. Virol.*, 81, 2929–2940.

4. Castro, R. S., Leite, R. C., Resende, M., Gouveia, A. M. G., 1999: A labeled avidin-biotin ELISA to detect antibodies to caprine arthritis-encephalitis virus in goats' sera. *Vet. Res. Commun.*, 23, 515–522.

5. Cheevers, W. P., Beyer, J. C., Knoweles, D. P., 1997: Type 1 and type 2 cytokine expression by viral gp135 surface protein-activated T- lymphocytes in caprine arthritis-encephalitis lentivirus infection. *J. Virol.*, 71, 6259–6263.

6. Cheevers, W. P., Knowles, D. P., McGuire, T. C., Cunningham, D. R., Adams, D. S., Gorham, J. R., 1988: Chronic disease in goats orally infected with two isolates of the caprine arthritis-encephalitis lentivirus. *Lab. Invest.*, 58, 510–517.

7. Cork, L. C., Hadlow, W. J., Crawford, T. B., Gorham, J. R., Piper, C., 1974: Infectious leucoencephalomyelitis of young goats. *J. Infect. Dis.*, 129, 134–141.

8. Crawford, T. B., Adams, D. S., 1981: Caprine arthritis-encephalitis virus: clinical features and presence of antibody in selected goat population. *J. Am. Vet. Med. Assoc.*, 178, 713–719.

9. Crawford, T. B., Adams, D. S., Cheevers, W. B., Cork, L. C., 1980: Chronic arthritis in goat caused by a retrovirus. *Science*, 207, 997–999.

10. Cutlip, R. C., Lehmukuhl, H., Sacks, J., Weaver, A., 1992: Prevalence of antibody to caprine arthritis-encephalitis virus in goats in the United States. *J. Am. Vet. Med. Assoc.*, 200, 802–805.

11. Dawson, M., 1988: Lentivirus diseases of domesticated animals. *J. Com. Pathol.*, 99, 401–419.

12. East, N. E., Rowe, J. D., Dahlberg, J. E., Theilen, G. H., Pedersen, N. C., 1993: Modes of transmission of caprine arthritis-encephalitis virus infection. *Small Rumin. Res.*, 10, 251–262.

13. Everman, J. F., 1990: Comparative features of retroviral infections of livestock. *Comp. Immunol. Microbiol. Infect. Dis.*, 13, 127–136.

14. Fahey, J. L., McKelvey, E. M., 1965: Quantitative determination of serum immunoglobulins in antibody agar plates. *J. Immunol.*, 94, 84.

15. Fieni, F., Rowe, J., Van Hoosear, K., Burucoa, C., Oppenheim, S., Anderson, G., Murra, J., Bondurant, R., 2003: Presence of caprine arthritis-encephalitis virus (CAEV) proviral DNA in general tract tissues of superovulated dairy goat does. *Theriogenol.*, 59, 1515–1523.

16. Fieni, F., Rowe, J., Van Hooser, K., Burucoa, C., Oppenheim, S., Anderson, G., Murray, J., Bondurant, R., 2002: Presence of caprine arthritis-encephalitis virus (CAEV) infected cells in flushing media following oviductal-stage embryo collection. *Theriogenol.*, 57, 931–940.

17. Gorrell, M. D., Brandon, M. R., Sheffer, D., Adams, R. J., Narayan, O., 1992: Ovine lentivirus is macrophagetropic and does not replicate productively in T- lymphocytes. *J. Virol.*, 66, 2679–2688.

18. Greenwood, P. L., 1995: Effects of caprine arthritis-encephalitis virus on productivity and health of dairy goats in New-South-Wales., Australiaralia. *Prevent. Vet. Med.*, 22, 71–87.

19. Greenwood, P. L., North, R. N., Kirkl, P. D., 1995: Prevalence, spread and control of caprine arthritis-encephalitis virus in dairy goat herds in New South Wales. *Aust. Vet. J.*, 72, 341–345.

20. Hanson, J., Hdbring, E., Ollson, K., 1996: A long term study of goats naturally infected with caprine arthritis-encephalitis virus. *Acta Vet. Scand.*, 37, 31–39.

21. Houwers, D. J., 1985: Experimental maedi-visna control in the Netherlands. In **Sharp, J. M., Hoff-Jorgensen, R.** (eds.): *Slow Virus in Sheep, Goats and Cattle*. Commission of the European Communities, Luxembourg, pp. 115–121.

22. Jolly, P. E., Gangopadhay, A., Chen, S., Reddy, P. G., Weiss, H. L., Sapp, W. J., 1997: Changes in the leukocyte phenotype profile of goats infected with the caprine arthritis-encephalitis virus. *Vet. Immunol. Immunopathol.*, 56, 97–106.

23. Kennedy-Stoskopf, S., Narayan, O., Strandberg, J. D., 1985: The mammary gland as a target organ for infection with caprine arthritis-encephalitis virus. *J. Comp. Pathol.*, 95, 609–617.

24. Lamara, A., Fieni, F., Mselli-lakhal, L., Chatagnon, G., Bruyas, J. F., Tainturier, D., Battut, I., Fornazero, C., Chebloune, Y., 2002: Early embryonic cells from *in vivo*-produced goat embryos transmit the caprine arthritis-encephalitis virus (CAEV). *Theriogenol.*, 58, 1153–1163.

25. Lamara, A., Fieni, F., Mselli-Lakhal, L., Tainturier, D., Chebloune, Y., 2001: Efficient replication of caprine arthritis-encephalitis virus in goat granulose cells. *Virol. Res.*, 79, 165–172.

26. Lamara, A., Fieni, F., Chebloume, Y., Bruyas, J. F., Tainturier, D., 1999: Susceptiblity of caprine arthritis-encephalitis virus (CAEV) preliminary results. In *Proceedings of the World Veterinary Congress*. Lyon, France, 23–26 September.

27. Lerondelle, C., Greenland, T., Jane, M., Mornex, J. F., 1995: Infection of lactating goats by mammary instillation of cell-borne caprine arthritis-encephalitis virus. *J. Dairy Sci.*, 78, 850–855.

28. Mackenzie, R. W., Olivier, R. E., Rooney, J. P., 1987: A successful attempt to raise goat kids free of infection with caprine arthritis-encephalitis in an endemically infected goat herd. *NZ Vet. J.*, 35, 184–186.

29. McGuire, T. C., 1987: The immune response to viral antigens as a determinant of arthritis in caprine arthritis-encephalitis virus infection. *Vet. Immunol. Immunopathol.*, 17, 465–470.

30. Mdurvwa, E. G., Ogunbiyi, P. O., Gakou, H. S., Reddy, P. G., 1994.: Pathogenic mechanisms of caprine arthritis-encephalitis virus. *Vet. Res. Commun.*, 18, 483–490.

31. Mselli-Lakhal, L., Favier, C., Leung, K., Guiguen, F., Grezel, D., Moissec, P., Mornex, J. F., Narayan, O., Quert, G., Chebloune, Y., 2000: Lack of functional receptors is the only barrier that prevents caprine arthritis-encephalitis virus from infecting human cells. *J. Virol.*, 74, 8343–8348.

32. Mselli-Lakhal, L., Guigen, F., Fornazero, C., Du, J., Favier, C., Durand, J., Grezel, D., Balleydier, S., Mornex, J. F., Chebloune, Y., 1999: Goat milk epithelial cells are highly permissive to CAEV infection *in vitro*. *Virol.*, 259, 67–73.

33. Narayan, O., Zink, M., Gorell, S., Crame, D., Huso, P., Jolly, P., 1993: The lentiviruses of sheep and goats. In **Levy, J. A.** (ed.): *The Retroviridae*. New York, NY.

34. Narayan, O., Kennedy-Stoskopf, S., Sheffer, D., Griffin, D. E., Clements, J. E., 1983: Activation of caprine arthritis-encephalitis virus expression during maturation of monocytes to macrophages. *Inf. Immun.*, 41, 67–73.

35. Narayan, O., Wolinsky, J. S., Clements, J. E., Strandberg, J. D., Griffin, D. E., Cork, L. C., 1982: Slow virus replication: the role macrophages in the persistence and expression of Visna viruses of sheep and goats. *J. Gen. Virol.*, 59, 345–356.

36. Narayan, O., Clements, J. E., Strandberg, J. D., Cork, L. C., Griffin, D. E., 1980: Biological characterization of the virus causing leucoencephalitis and arthritis in goats. *J. Gen. Virol.*, 50, 69–79.

37. Peretz, G., 1992: Caprine arthritis-encephalitis control in France. *Vet. Rec.*, 131, 495–496.

38. Peretz, G., Bugnard, F., Calavas, D., 1994: Study of a prevention program for caprine arthritis-encephalitis. *Vet. Res.*, 25, 322–326.

39. Rowe, J. D., East, N. E., 1997: Risk factors for transmission and methods for control of caprine arthritis-encephalitis virus infection. *Vet. Clin. North Am. Food Anim. Prac.*, 13, 35–53.

40. Rowe, J. D., East, N. E., Franti, C. E., Thurmond, M. C., Pedersen, N. C., Theilen, G. H., 1992: Risk factors associated with the incidence of seroconversion to caprine arthritis-encephalitis virus in goats on California dairies. *Am. J. Vet. Res.*, 53, 2396–2403.

41. Ryan, S., Tiley, L., McConnel, I., Blacklaws, B., 2000: Infection of dendritic cells by the maedi-visna lentivirus. *J. Virol.*, 74, 10096–10103.

42. Sherman, D. M., 1992: CAE: Caprine arthritis-encephalitis. In *Goat Handbook*, pp. 1–5.

43. Smith, M. C., Sherman, D. M., 1994: *Goat Medicine*. Lea & Febiger, Philadelphia, pp. 135–138.

44. Tizard, I. R., 2000: *Veterinary Immunology: An Introduction* (6th edn.). W. B. Saunders Co., Philadelphia, PA, pp. 73–79.

45. Toma, B., Eloit, M., Savey, M., 1990: Animal diseases caused by retroviruses: enzootic bovine leucosis, equine infectious anaemia, and caprine arthritis-encephalitis. *Rev. Sci. Technol.*, 9, 1039–1076.

46. Toniolo, A., Serra, C., Conaldi, P. G., Basolo, F., Falcone, V., Dolei, A., 1995: Productive HIV-1 infection of normal human mammary epithelial cells. *AIDS*, 9, 859–866.

47. Travassos, C., Benotic, C., Valas, S., da Silva, A., Perin, G., 1998: Detection of caprine arthritis-enmcephalitis

virus in sperm of experimentally infected bucks. *Vet. Res.*, 29, 579–584.

48. Turjillo, J. D., Hotzel, K. J., Snekvik, K. R., Cheevers, W. P., 2004: Antibody response to the surface envelope of caprine arthritis-encephalitis lentivirus: disease status is predicted by SU antibody isotype. *Virol.*, 325, 129–136.

49. Washburn, K. E., Streter, R. N., Saliki, J. T., Lehenbauer, T. W., Pardo, M. E., 2001: Photodynamic inactivation of an RNA enveloped virus in goat colostrums. *Small Rumin. Res.*, 42, 31–37.

50. Zink, M. C., Yager, J. A., Myers, J. D., 1990: Pathogenesis of caprine arthritis-encphalitis virus. Cellular localization of viral transcripts in tissues of infected goats. *Am. J. Pathol.*, 136, 843–854.

51. Zink, M. C., Narayan, O., Kennedy, P. G., Celements, J. E., 1987: Pathogenesis of visna/maedi and caprine arthritis-encephalitis: new leads on the mechanism of restricted virus replication and persistent inflammation. *Vet. Immunol. Immunopathol.*, 15, 167–180.

Received July 17, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 137–141, 2007

EFFECTS OF SHORT-TIME FED DEPRIVATION ON SOME BLOOD ELECTROLYTES IN HORSES

Piccione, G., Casella, S., Giannetto, C., Fazio, F., Caola, G.

Dipartimento di Morfologia, Biochimica, Fisiologia e Produzioni Animali
Università degli Studi di Messina
Italy

giuseppe.piccione@unime.it

ABSTRACT

The aim of the present study was to investigate the effect of short-time fed deprivation on serum concentrations of calcium, sodium, chloride, potassium, phosphorus and magnesium in horse. Ten horses were divided into two groups, each consisting of five subjects, housed in two different shelters: Group A was regularly fed twice a day (at 08 : 00 h and 14 : 00 h), and Group B was kept without food for 27 hours. Through blood collection by means of jugular venipuncture, concentrations of electrolytes were assessed for each subject on the following experimental conditions: one hour before meals and one hour, two hours and three hours after meals. The Bonferroni's test was applied to determine statistical significances within each group. Statistically significant differences were not observed for all the studied parameters. The results obtained during all experimental period indicate the influence of feeding on calcium only ($p \leq 0.04$). These results underline that feeding induce a different response in the organism; more specifically, obtained results suggest that calcium is influenced from feeding and circadian rhythm also.

Key words: fast; feeding; horse; serum electrolytes

INTRODUCTION

By nature the horse is grazing animal, but in practice horses are often fed high amounts of concentrates and low roughage in limited meals reflecting pasture availability, labour-time and owner preference (28). A larger volume, when fed is offered once a day, can be associated with an increased passage rate (15) resulting in decreased time for digestion (29). Dividing the total amount of feed into several small amounts throughout the day will not only be closer to the horse's natural grazing behaviour, but would also decrease passage rate (15) stimulate digestibility and reduce the risk of colic (18). For these reasons, increasing feeding frequency is often advised in clinical practise for horses with digestion problems.

A study of the available literature reveals no significant differences in digestibility between one and six (9) or twelve meals within a 24 h period (4, 6) and consequently it was not reveal significant difference in absorption of amino acid, electrolytes and other parameters.

Electrolytes are a critical element in cellular metabolism, muscle contraction, nerve transmission and enzyme reactions. Imbalances or deficits lead to impaired athletic performance at best, and life-threatening metabolic disruption or death at worst. It is important to realize that the body has no mechanism for storing "extra reserves" of electrolytes. Therefore, while electrolytes are closely regulated by the body, much is lost in the sweat, urine and faeces during exercise and hence are an important parameter in monitoring a horse's ongoing status.

Many studies have been published on electrolytes (2, 19), but only few about these are available on post-prandial changes of electrolytes in horses (8). J a n s s o n and D a h a l b o r n (10) investigated the effects of feeding frequency on voluntary salt intake on fluid and electrolyte regulation in athletic horses, discovering that the effect of voluntary sodium intake, but not feeding frequency, has significant effects on fluid and electrolyte regulation in athletic horses, it induced significant alterations in the plasma aldosterone concentration, water in-

take and faecal excretion of sodium and potassium. Schott and Hinchcliff (21) studied treatments affecting fluid and electrolyte status during exercise, too. Hambleton *et al.* (7) measured serum electrolytes and serum enzymes in muscle and observed a significant decrease in exercise-induced fluctuations of enzymes and electrolytes. Buchanan *et al.* (2) studied the effects of a 24-hour infusion of an isotonic electrolyte replacement fluid (IERF) on weight, serum and urine electrolyte concentrations, and other clinicopathologic variable in healthy neonatal foals. Results suggest that administration of an IERF containing a physiologic concentration of sodium may be not appropriate for use in neonatal foals that require maintenance fluid therapy.

Holbrook *et al.* (8) asserted that electrolyte supplementation is common in horses during endurance competitions, but the effect on the gastric mucosa is unknown. Findings suggest that one schedule of electrolyte supplementation used commonly in endurance horses may be harmful to the gastric mucosa. Toribio *et al.* (27) studied the effect of endotoxin administration to healthy horses on ionized calcium (Ca^{2+}) and total calcium (tCa), ionized magnesium (Mg^{2+}) and total magnesium (tMg), phosphate (Pi), potassium (K^{+}), sodium (Na^{+}), chloride (Cl^{-}) but no changes were found in serum Na+ and Cl^{-} concentrations.

So, endotoxemia in horses resulted in electrolyte abnormalities. On the basis of these knowledges we have studied the influence of feeding and of a short period of fast on some blood electrolytes in horse.

MATERIAL AND METHODS

Ten clinically healthy female Thoroughbred horses aged of 7 ± 2 years, with a body weight of 473 ± 43 kg were used. The horses were kept in individual box (4 × 4 meters) with a layer of wood shaving as bedding. All horse were divided in two groups: five horses were regularly wormed and fed twice a day (at 8 : 00 h – feed 1 and 14 : 00 h – feed 2) (Group A). The daily rations were 4.5 kg of hay and about 3 kg of concentrates (as-fed basis). The horses received half the amount of concentrate and half the amount of hay per meal. The hay was supplied after the concentrate was consumed. The horses were fed according to the energy and protein requirements of the feed evaluation system for horses and for trace elements and vitamins according to the NRC requirements for horses (NRC, 1989). Five horses were gone without food for all experimental period (Group B). The water was available *ad libitum*.

Blood samples were taken from the jugular vein by vacutainer tubes (Terumo Corporation, Japan) with no additive, one hour before meals (at 7 : 00 h and 13 : 00 h) and one hour (at 9 : 00 h and 15 : 00 h), two hours (at 10 : 00 h and 16 : 00 h), and three hours (at 11 : 00 h and 17 : 00 h) after meals, in Group A; in Group B blood samples were taken in the same time of Group A. Blood samples were centrifuged at 3000 r.p.m. for ten minutes. On the obtained serum, stored at –20 °C pending analysis calcium, chloride, phosphorus and magnesium were assessed by means of UV spectrophotometry; sodium and potassium were assessed by means of semiautomatic flame photometry (FP 20, SEAC, Italy).

All results were expressed as mean ± standard deviation of the means (SD). Two-way repeated measures analysis of variance (ANOVA) was used to determine significant differences. $P < 0.05$ were considered statistically significant. The Bonferroni's test was applied for post hoc comparison. Date were analyzed using software STATISTICA 5.5.

RESULTS

Table 1 shows the mean values of the parameters considered, together with their standard deviation of the means (SD), together with the statistical significance obtained on the different experimental conditions in the horses of group A and B.

Two-way ANOVA shows a statistical significant effect of experimental condition on calcium ($F(7.56) = 2.34$, $p \leq 0.04$). Calcium shows a significant increase ($p < 0.05$) at 09 : 00 respect to 07 : 00 in both groups. Also calcium shows a significant decrease ($p < 0.001$) at 17 : 00 respect to 07 : 00 in group A (Figure 1).

Two-way ANOVA no shows statistical significant effect of experimental condition on other electrolytes studied.

DISCUSSION

In our study calcium, sodium, chloride, potassium, phosphorus and magnesium values were within the physiological range for the goasts (11).

Argenzio and Stevens (1) demonstrated that animals fed the conventional diet shows cyclic variations in the ionic composition and osmolality of digesta with time after feeding; so, the ionic composition and water content of large intestinal digesta appeared largely dependent on the rate microbial digestion. But our results no show statistical significant modification of electrolyte's concentrations except for calcium. Calcium is present in the plasma in three form: protein-bound calcium, complexed calcium and ionized calcium. About 50 % of calcium is bound to protein. Serum calcium is reported as total calcium. Diet, albumin and endogenous hormone concentrations influence calcium concentration. Normally, adequate serum levels of ionized calcium will be maintained by mobilizing reserve stores in bone.

Our results showed a statistical significant increase of calcium at 09 : 00 in both groups. This peak is in the diurnal acrophases for this parameter as previously observed by Piccione *et al.* (16). In fact, it was shown mineral metabolism of bone has the daily rhythmicity, however the aetiology is not completely understood (13). The timing and magnitude of these rhythms appears to be constrained by the feeding schedule, LD cycle and endocrine relationships (3, 12, 17, 24, 25). This could explain the statistical significant increase of calcium in both groups at 09 : 00.

Table 1. Average values of electrolytes, expressed in their conventional units of measurement together with the related standard deviations in the different experimental conditions in the horses of Group A and B

Experimental conditions								
Parameters ($mmol.l^{-1}$)	**Group A**							
	07 : 00	**09 : 00**	**10 : 00**	**11 : 00**	**13 : 00**	**15 : 00**	**16 : 00**	**17 : 00**
Calcium	2.41 ± 0.12	2.85±0.19 *	2.49±0.24	2.49±0.24	2.49±0.24	2.48±0.27	2.63±0.29	1.88±0.24 ■
Sodium	141.10 ± 1.78	142.13±2.22	143.00±2.71	142.63±1.63	142.80±3.35	142.92±1.56	142.35±2.13	142.67±1.00
Chloride	100.13 ± 3.71	99.55±2.57	99.96±2.66	101.67±4.60	100.66±2.00	100.34±2.16	101.28±2.37	99.97±1.24
Potassium	4,89 ± 0.25	4.97±0.75	4.86±0.45	5.01±0.47	4.95±0.21	4.99±0.20	4.95±0.29	5.01±0.15
Phosphorus	1.71 ± 0.27	1.29±0.28	1.40±0.33	1.76±0.31	1.22±0.25	1.40±0.29	1.32±0.24	1.47±0.36
Magnesium	0.86 ± 0.05	0.93±0.08	0.98±0.13	0.87±0.07	0.85±0.09	0.97±0.14	0.96±0.13	0.88±0.09
Parameters ($mmol.l^{-1}$)	**Group B**							
	07 : 00	**09 : 00**	**10 : 00**	**11 : 00**	**13 : 00**	**15 : 00**	**16 : 00**	**17 : 00**
Calcium	2.40 ± 0.12	2.70±0.15 *	2.36±0.27	2.34±0.07	2.20±0.04	2.38±0.23	2.30±0.17	2.43±0.17
Sodium	143.96 ± 1.14	142.40±1.27	141.62±2.77	141.18±3.19	140.54±0.53	141.18±2.01	142.18±1.95	142.14±1.87
Chloride	101.95 ± 1.31	100.38±3.35	103.20±2.29	100.51±2.58	101.98±0.80	99.94±3.61	100.58±3.43	101.58±3.68
Potassium	4.98 ± 0.61	4.91±0.35	4.94±0.30	4.67±0.67	4.79±0.32	4.88±0.35	4.91±0.33	4.82±0.37
Phosphorus	1.39 ± 0.33	1.28±0.43	1.23±0.36	1.08±0.33	1.32±0.09	1.10±0.17	1.44±0.40	1.35±0.24
Magnesium	0.87 ± 0.04	0.88±0.06	0.73±0.39	0.87±0.04	0.87±0.01	0.88±0.02	0.84±0.03	0.92±0.11

Significance: * – *vs* 07 : 00 $p < 0.05$; ■ – *vs* 07 : 00 $p < 0.001$

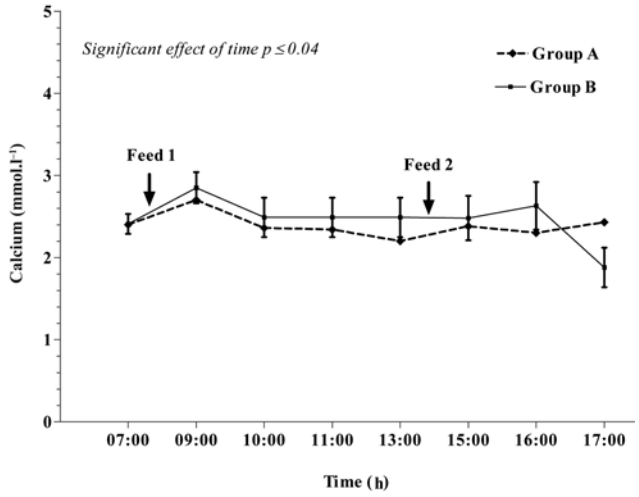


Fig. 1. Calcium ($mmol.l^{-1}$) mean value trend, with SD and statistical significance obtained on the different experimental conditions in horses of Group A and B

Little is know about the effects of lighting schedules on biochemical parameters of bone and mineral metabolism. Most of the data support the conception that the feeding schedule (meal timing) is a powerful "Zeitgeber" that can override the influence of the LD cycle (20, 23, 26). This could explain the statistical significant decrease of calcium observed only at 17 : 00 in group A.

Intestinal absorption, renal excretion and calcium removal from bone responded to, the dietary level of calcium to maintain calcium homeostasis. The rates of intestinal absorption and the removal of calcium from the skeleton increased while renal excretion decreased in response to low intake (22).

Sodium is a primary ion in the body involved in virtually every metabolic process from glucose transport to neural transmission. Chloride concentration increases and decreases may parallel changes in sodium concentration. The body does not store reserves of sodium and chloride (such as calcium), therefore losses which are not replaced through supplementation or other dietary intake will result in a progressive depletion, but we can explain that a fast of 27 hours no define statistical significant loss of these electrolytes respect to subject fed normally.

Potassium no showed statistical significant modification in both groups probably because it is present in relatively high concentration in most animal feeds but it is absorbed in the small intestine and colon with subsequent renal excretion accounting for more than 90% of the daily potassium intake. The lack of statistical significant modification of phosphorus concentrations is probably due to the very low content of this mineral in hays and straws, also serum Magnesium concentrations are diet depend (5).

In conclusion it is possible to establish that in horses feeding twice a day determines statistical significant modifications of calcium only, even though these modifications could be attribute circadian rhythm of calcium, too.

The lack of statistical significant modifications of other electrolytes allow us to establish that feeding has

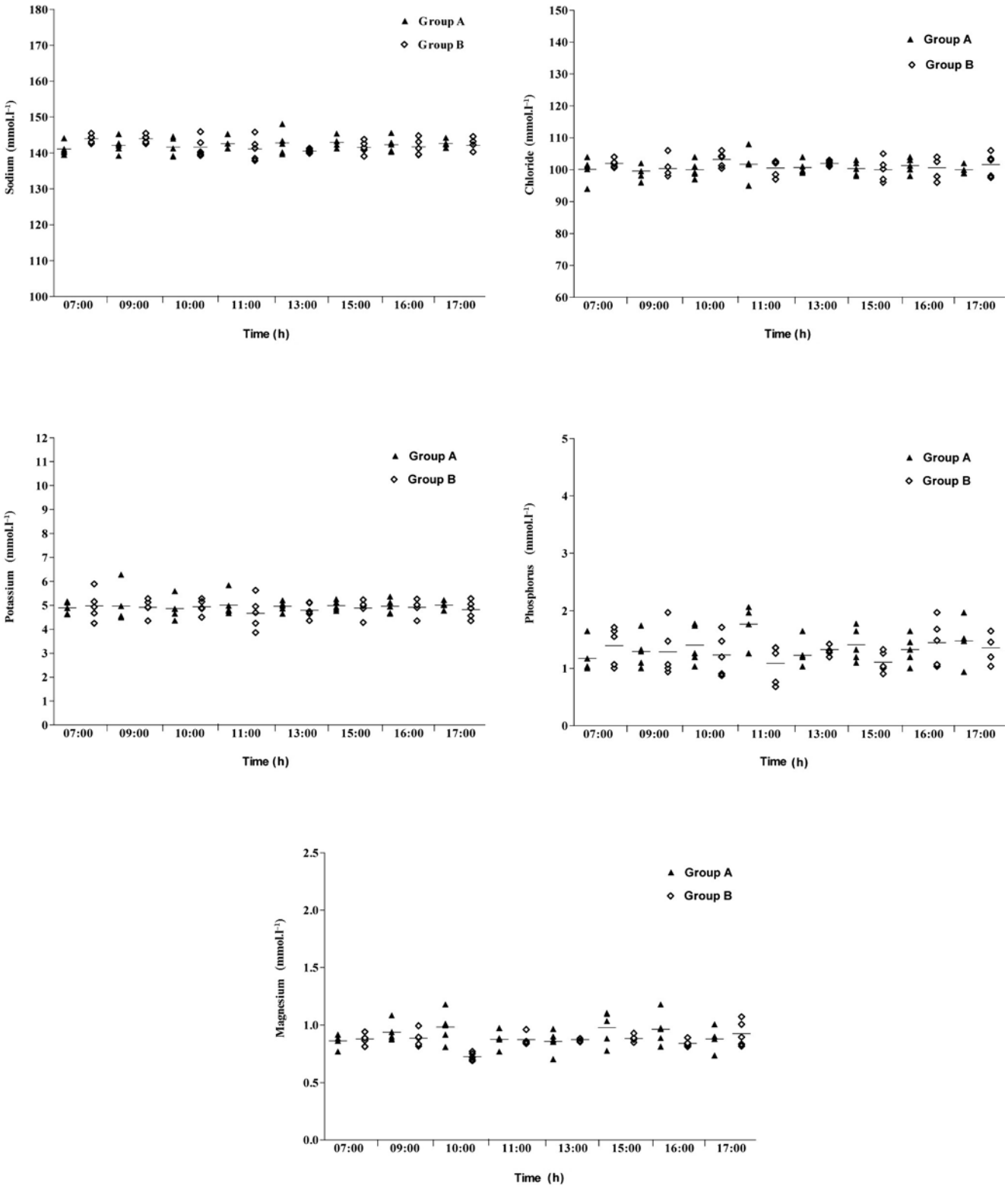


Fig. 2. Scatter graph of sodium, chloride, potasium, phosphous and magnesium meanvalue obtained on the different experimental conditions in horses of Group A and B

not effect on sodium, chloride, potassium, phosphorus and magnesium; therefore further investigation with different food rations and with long periods of fasting will be done in order to better understand the influence of these variables on the serum concentration of the studied electrolytes.

REFERENCES

1. Argenzio, R. A., Stevens, C. E., 1975: Cyclic changes in ionic composition of digesta in the equine intestinal tract. *J. nim. Physiol.,* 228, 1224–1230.

2. Buchanan, B. R., Sommardahl, C. S., Rohrbach, B. W., Andrews F. M., 2005: Effect of 24-hour infusion of an isotonic electrolyte replacement fluid on the renal clearance of electrolytes in healthy neonatal foals. *J. Vet. Intern. Med. Assoc.*, 227, 1123–1129.

3. Calvo, M. S., Eastell, R., Offort, K. P., Bergstralh, E. J., Burritt, M. S., 1991: Circadian variations in ionized calcium and intact parathyroid hormone: evidence for sex difference in calcium homeostasis. *J. Clinic. Endocrinol. Metab.*, 72, 69–76.

4. Clarke, L. L., Argenzio, R. A., Roberts, M. C., 1990: Effect of feeding on plasma volume and urinary electrolyte clearance in ponies. *Am. J. Vet. Res.*, 51, 571–576.

5. Eades, S. C., Bounous, D. I., 1997: Significance of laboratory tests. In *Laboratory Profiles of Equine Diseases*. Ed. Mosby – Year Book, St. Louis, 9–11.

6. Gill, A. M., Parker, A., Leak, S., Lawrence, L. M., 1998: Effect of meal of feeding frequency and roughage on behaviours of stabled horses. In **Pagan, J. D.** (ed.)**:** *Advances in Equine Nutrition*. University Press, Nottingham, 89–92.

7. Hambleton, P. L., Slade, L. M., Hamar, D. W.,, Kienholz, E. W., Lewis L. D., 1980: Dietary fat and exercise conditioning effect on metabolic parameters in the horse. *J. Anim. Sci.*, 51, 1330–1339.

8. Holbrook, T. C., Simmons, R. D., Payton, M. E., MacAllister, C. G., 2005: Effects of repeated oral administration of hypertonic electrolyte on equine gastric mucosa. *Equine Vet. J.*, 37, 501–504.

9. Houpt, K. A., Perry, P. J., Hintz, H. F., Houpt, T. R., 1988: Effect of meal frequency on fluid balance and behaviour of ponies. *Physiology and Behavior*, 42, 401–407.

10. Jansson, A., Dahlborn, K., 1999: Effects of feeding frequency and voluntary salt intake on fluid and electrolyte regulation in athletic horses. *Am. Physiol. Society*, 1610–1616.

11. Kaneco, J. J., 1989: *Clinical Biochemistry of Domestic Animals*. Academic Press, Inc, San Diego, New York, Boston, London, Sydney, Toronto, pp. 886–889.

12. Lopez, I., Estepa, J. C., Mendoza, F. J., Rodriquez, M., Aquilera-Tejero, E., 2006: Serum concentration of calcium, phosphorus, magnesium and calciotropic hormones in donkeys. *Am. J. Vet. Res.*, 67, 1333–1336.

13. Milhaud, G., Perault-Staub, A. M., Staub, J. F., 1972: Diurnal variations of plasma calcium and calcitonin function in the rat. *J. Anim. Physiol.*, 222, 559–567.

14. Ostrowska, Z., Kos-Kudla, B., Marek, B., Kajdaniur, D., 2003: Influence of lighting conditions on daily rhythm of bone metabolism in rats and possible involvement of melatonin and other hormones in this process. *Endocrine Regulations*, 37, 163–174.

15. Pearson, R. A., Archibald, R. F., Muirhead, R. H., 2001: The effect of forage quality and level of feeding on digestibility and gastrointestinal transit time of oat straw and alfalfa given to ponies and donkeys. *Br. J. Nu.*, 85, 599–606.

16. Piccione, G., Assenza, A., Fazio, F., Berbero, D., Caola, G., 2004: Daily rhythm of vitamin D and of the calcium and phosphate in horse. *8th International Conference of European Society of Veterinary and Comparative Nutrition*, 64–65.

17. Phillippens, K. M. H., Von Mayersbach, H., Scheving, L. E., 1977: Effects of the scheduling of meal-feeding at different phases of the circadian system in rats. *J. Nutr.*, 107, 176–182.

18. Potter, G. D., Arnold, F. F., Householder, D. D., Handen, D. H., Brown, G. D., 1992: Digestion of starch in the small or large intestine of the equine. *Pferdeheilkunde 1 (Suppl.) B.*, 107–111.

19. Raidal, S. L., Edwards, S., 2005: Pharmacokinetics of potassium in adult horses. *Aust. Vet. J.*, 83, 425–430.

20. Russel, J. E., Simmons, D. J., Huber, B., Ross, B. A., 1983: Meal timing as a Zeitgeber for skeletal deoxyribonucleic acid and collagen synthesis rhythms. *Endocrinology*, 113, 2035–2042.

21. Schott, H. C., Hinchcliff, K. W., 1998: Treatments affecting fluid and electrolyte status during exercise. *Vet. Clin. North Am. Equine Pract.*, 14, 175–204.

22. Schryver, H. F., Craig, P. H., Hintz, H. F., 1970: Calcium metabolism in ponies fed varying levels of calcium. *J. Nutrition*, 100, 955–964.

23. Shinoda, H., Stern, P. H., 1992: Diurnal rhythm in Ca transfer into bone, Ca release from bone, and bone resorbing activity in serum of rats. *Am. J. Physiol.*, 262, 235–240.

24. Simmons, D. J., Whiteside, L. A., Whitson, S. W., 1979: Biorhytmic profiles in the rat skeleton. *Metab. Bone Dis. Relat. Res.*, 2, 49–64.

25. Staub, J. F., Perault-Staub, A. M., Milhaud, G., 1979: Endogenous nature of circadian rhythms in calcium metabolism. *Am. J. Physiol.*, 237, 311–317.

26. Talmage, R. V., Roycroft, J. H. I., Andersen, J. J. B., 1975: Daily fluctuations in plasma calcium, phosphate, and their radionuclide concentrations in the rat. *Calcif. Tissue Res.*, 17, 91–97.

27. Toribio, R. E., Kohon, C. W., Hardy, J., Rosol T. J., 2005: Alterations in serum parathyroid hormone and electrolyte concentrations and urinary excretion of electrolytes in horses with induced endotoxemia. *J. Vet. Intern. Med.*, 19, 223–228.

28. Van Weyenberg, S., Buyse, J., Janssens, G. P. J., 2005: Digestibility of a complete ration in horses fed once or three times a day and correlation with key blood parameters. *Vet. J.* (In press).

29. Warmer, A. C. I., 1981: Rate of passage of digesta through the gut of mammals and birds. *Nutr. Abstract and Rev. Series B*, 51, 799–807.

Received February 12, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 142–145, 2007

IMPACT OF INBREEDING AND HERITABILITY OF CANINE HIP DYSPLASIA IN GERMAN SHEPHERD POPULATION

Kasarda, R., Mészáros, G.[1]
Kadlečík, O.[1], Buleca, J., Jr.[2]

[1]Slovak University of Agriculture, Tr. A. Hlinku 2, 949 76 Nitra
[2]University of Veterinary Medicine, Komenského 73, 041 81 Košice
The Slovak Republic

radovan.kasarda@uniag.sk

ABSTRACT

The aim of the study was genetic analysis of hip dysplasia of German shepherd breed population in Slovakia and influence of inbreeding on presence of hip dysplasia in population of inbreed animals. Based on information from German shepherds breeders association in Slovakia all purebred animals from the herd book were analyzed. Information of hip dysplasia presence and evaluation on degree of hip dysplasia was known for all parents and their progeny. For estimation of heritability of hip dysplasia mixed animal model was established where sex, father and mother of animals respectively were used as fixed effects and animal represented random effect. Sex of animals has no significant effect on presence of hip dysplasia. Effect of father was significant–high with coefficient of determination $R^2 = 0.654$. Estimated coefficient of heritability was medium, 0.564 ± 0.011. Frequency of hip dysplasia decreases with increase of inbreeding of animals. For future development of breed will be essential strict negative selection of animals with presence of hip dysplasia and increased rigorousness of selection criterions.

Key words: German shepherd; heritability; hip dysplasia; inbreeding

ADDRESS FOR CORRESPONDENCE
Kasarda, R. Ing., PhD, Slovak University of Agriculture
Dept. of Genetics and Breeding Biology, Tr. A. Hlinku 2
949 76 Nitra, The Slovak Republic
E-mail: radovan.kasarda@uniag.sk

INTRODUCTION

Canine elbow dysplasia and hip dysplasia are widespread skeletal diseases in many dog breeds. These degenerative joint diseases are characterized by conformational changes in the affected joints, and can result in clinical lameness and considerable impairment of the dog (5).

S a m o y *et al.* (11) defined incongruity at dogs as a result of bad alignment of the joint surfaces of the elbow. They suggested that the abnormal shape of the ulnar trochlear notch or a stap caused by short radius or ulna can result in loose fragments in different locations.

R e m y *et al.* (9) analyzed joint incongruity, fragmented medial coronoid process, osteochondrosis or osteochondritis of the medial humeral condyle and ununited anconeal process as the probable primary lesions of elbow dysplasia in German shepherd dogs.

The most frequent lesion was the joint incongruity, ununited anconeal process was diagnosed rarely. Also the combinations of lesions were very frequent. They stated joint incongruity and fragmented medial coronoid process as the most common lesions of elbow dysplasia in German shepherd.

The genetic correlation between canine hip and elbow dysplasia was estimated by (5) with value of $r = 0.53$. They were also the most frequent developmental orthopedic diseases in dogs during a ten year study period, done by (6). They were found at the highest number of breeds compared to other evaluated diseases. Dysplasia also occurs in humans, with frequency approximately about 4 from 1.000 births, with greater probability at girls and newborns. Higher birth weight was a special high-risk factor in humans (4). This could be also

true for dogs, while larger and heavier breeds like the German shepherd, Labrador, Rotweiler and others were considered to be most endangered.

W o o d *et al.* (12) observed strong positive relationship between offspring and parental (particularly dam) hip scores. M ä k i *et al.* (7) observed higher proportion of males with some degree of dysplasia compared to females, during estimation of genetic parameters for dysplasia for Finnish Rotweiler population. The difference between sexes was significant only for the elbow dysplasia ($p < 0.001$). W o o d *et al.* (13) also stated significantly higher occurrence of hip dysplasia at male Labradors compared to females, while (10) found no significant difference between sexes.

Although several radiographic features to diagnose dysplasia have been described, scoring is subjective because there is currently no objective method to measure the degree of dysplasia (11). According to M ä k i *et al.* (7) differences between panelists in evaluating elbow dysplasia were large. They also stated significant effect of experience of the X-raying veterinarian measured as number of X-rayed dogs. The differences between classes were small and no clear tendency was observed. As an alternative approach (4) suggested to use ultrasound visualization, when the clinical findings were uncertain. This way the need of radiography was reduced. Computer tomography and arthroscopy was suggested to measure incongruity of the elbow joints (11).

The occurrence of canine hip and elbow dysplasia in the populations suggests some kind of genetic basis for these diseases. M ä k i *et al.* (7) stated the inheritance of elbow and hip dysplasia as quantitative and close to continuous, with a major gene affecting the trait jointly with numerous minor genes. The favorable gene A1 was found as nearly completely dominant over its unfavorable counterpart A2. The heterozygote (A1A2) was more similar to the favorable homozygote (A1A1). They found at least two major genes for hip dysplasia, but the evidence for their effects on elbow joints was equivocal.

W o o d *et al.* (12) found highly significant genetic heritability $h^2 = 0.34$ from both parents, $h^2 = 0.41$ from sire alone and $h^2 = 0.30$ from dam alone in Labrador retriever breed. H a m a n n *et al.* (3) compared the heritability from the additive genetic animal ($h^2 = 0.26$) and maternal ($h^2 = 0.10$) effect. The direct animal and maternal effects were negatively correlated, with genetic correlation coefficient of $r = -0.65$. All authors emphasized the importance of selective breeding of parents to reduce the occurrence of the disease.

The aim of the study was genetic analysis of hip dysplasia of German shepherd breed population in Slovakia and influences of inbreeding on presence of hip dysplasia in population of inbreed animals.

MATERIAL AND METHODS

Based on information from German shepherd breeders association in Slovakia were into analysis included all purebred animals from the herd book. In data file were present 21.828 animal of both sexes (10.870 males and 10.958 females). Information of hip dysplasia presence and evaluation on degree of hip dysplasia was known for 7.094 parents and their progeny (animals in first and second generation).

Intensity of inbreeding (FX) in pedigree of individual was estimated using formula of (14), implemented in procedure inbreed of SAS (15), where pedigree matrix was build and diagonal elements of which present coefficients of intensity of inbreeding (FX).

Data were then split to two separate parts to inbreed and outbreed animals. Influence of intensity of inbreeding on presence of hip dysplasia was tested. Also influence of sex was tested as part of the analysis separately for inbreed and outbreed group.

For estimation of heritability of hip dysplasia mixed animal model was established where sex, father and mother of animals respectively were used as fixed effects and animal represented random effect. For estimation of heritability REML (random estimated maximum likelihood) method was used under AS REML software (2). Model equation:

$$y_{ijklm} = \mu + sex_i + father_j + mother_k + individual_l + e_{ijklm}$$

y_{ijklm} – degree of dysplasia
μ – average
e_{ijklm} – random error

Degree of hip dysplasia was indexed as follows:

(1) – Negative (dysplasia not present)
(2) – Marginal dysplasia
(3) – Light degree of dysplasia
(4) – Medium degree of dysplasia
(5) – Heavy dysplasia

RESULTS

Data file included 512 inbreed animals (282 males and 230 females) of first generation having evaluation on degree of hip dysplasia. Distribution of first generation of inbreed animals over degree of dysplasia is in Table 1. Average degree of dysplasia in group of inbreed males was 1.79 ± 1.36. Average intensity of inbreeding of males was 0.02 with minimum 0.0001 (common ancestor of parents in 3rd generation) and maximum 0.125 (mating of half-sibs). Average degree of dysplasia in group of females was 1.70 ± 1.45. Intensity of inbreeding in females was in average 0.02 with distribution from minimum 0.0001 to maximum 0.25 (mating parent – offspring).

Group of outbreed animals consists 6582 animals having evaluation of degree of hip dysplasia. Results of evaluation of outbreed animals according to degree of hip dysplasia is in Table 2. Average degree of dysplasia in group of outbreed males was 2.17 ± 1.47. Average degree of dysplasia in group of outbreed females was 2.16 ± 1.47.

Differences between males and females were small (higher in males) not significant neither in inbreed nor outbreed animals. Differences between groups of animals were tested under Student's *t*-test (15). When comparing

Table 1. Distribution of inbreed animals over degree of hip dysplasia by sex and intensity of inbreeding in their pedigree

Sex	Degree of hip dysplasia	n	$\bar{x}$	σP	Min.	Max.	% distribution
Females	1	167	0.022	0.025	0.0001	0.1250	72.61
	2	37	0.025	0.027	0.0005	0.1250	16.09
	3	22	0.016	0.018	0.0001	0.0625	9.57
	4	4	0.009	0.009	0.0002	0.0161	1.74
	5	0	0	0	0	0	0
Males	1	180	0.022	0.029	0.0001	0.2500	63.83
	2	54	0.02	0.023	0.0001	0.0800	19.15
	3	27	0.024	0.028	0.0001	0.0938	9.57
	4	19	0.013	0.016	0.0002	0.0625	6.74
	5	2	0.051	0.016	0.0400	0.0635	0.71

Table 2. Distribution of outbreed animals over degree of hip dysplasia by sex

Sex	Degree of hip dysplasia	n	% distribution
Females	1	2023	73.70
	2	471	17.16
	3	182	6.63
	4	54	1.97
	5	15	0.55
Males	1	2826	73.65
	2	657	17.12
	3	257	6.70
	4	85	2.22
	5	12	0.31

differences in degree of hip dysplasia presence between inbreed and outbreed animals, significant differences were found between groups. Where outbreed animals have higher degree of dysplasia as inbreed animals. Differences and their significances are shown in Table 3.

Table 3. Significance of *t*-test for differences in degree of hip dysplasia between groups of animals

Group 1	Group 2	Difference
Outbreed females	Outbreed males	-0.01^{-}
Outbreed females	Inbreed females	0.46^{++}
Outbreed males	Inbreed males	0.38^{++}
Outbreed animals	Inbreed animals	0.41^{++}
Inbreed females	Inbreed males	-0.09^{-}

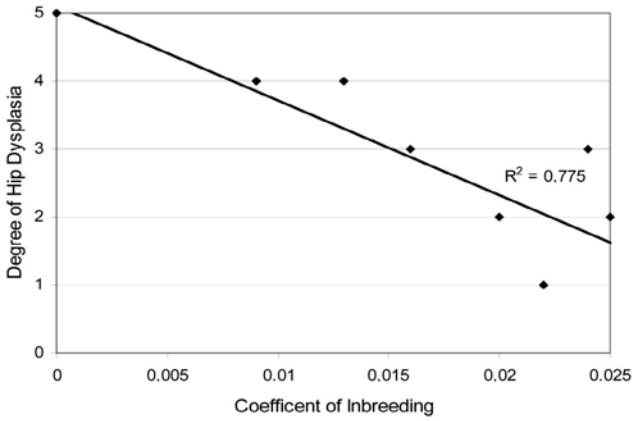


Fig. 1. Proposed trend in evolution of degree of hip dysplasia in group of inbreed animals

Figure 1 shows distribution of degree of hip dysplasia over inbreed animals and trend showing proposed general evolution of distribution over inbreed animals, that with increased inbreeding deceases presence of hip dysplasia with reliability of trend $R^2 = 0.775$.

Setting up mixed model for genetic evaluation and running AS REML, variance parameters were estimated for hip dysplasia and coefficient of heritability was computed. Several models were tested. From selected fixed effects, only father had statistically significant influence on hip dysplasia ($R^2 = 0.654$). Effect of mother and sex was not significant, which is in agreement with present state of knowledge. Analysis resulted in heritability of value $h^2 = 0.564$ with low standard error 0.011, estimated heritability is therefore of high reliability.

DISCUSSION

In analyzed population were observed 26.75 % (1.898 out of 7.094 examined) dogs with presence of hip dysplasia in some degree. When comparing to results observed

in German shepherds in USA in years 1974 to 2005, only 19 % of population there showed presence of hip dysplasia. Janutta *et al.* (5) observed in Germany presence of hip dysplasia in population of German shepherd dog to 21.3 %. Prevention of increased presence of hip dysplasia is rigorous selection of parents which is relation with different standards of breeders association in different countries.

Our results on relation of inbreeding and hip dysplasia are in agreement with paper of (8), who observed in group of inbreed dogs with inbreeding between 5–7 %, lowest presence of hip dysplasia. With inbreeding increases possibility to fix traits, therefore after accurate selection decreases chance for presence of hip dysplasia, which is in agreement also with (7) and knowledge that heritability of dysplasia shows to be polygenic affected by more genes with different expression with one of them with higher effect. This is shown also in medium height coefficient of heritability 0.564. Bliss *et al.* (1) similarly estimated medium heritability of hip dysplasia between 0.25–0.48. Lower heritability of hip dysplasia was observed by Hamann *et al.* (3).

CONCLUSIONS

Sex of animals has no significant effect on presence of hip dysplasia. Effect of father was significant-high with coefficient of determination $R^2 = 0.654$. Estimated coefficient of heritability was medium, 0.564 ± 0.011. Frequency of hip dysplasia decreases with increase of inbreeding of animals. For the future development of breed will be essential strict negative selection of animals with presence of hip dysplasia and increased rigorousness of selection criterions. Actually are allowed for mating animals with hip dysplasia up to degree 2. Because of large selection basis in Slovakia this criterion could be stricter, to eliminate possibility of inherit hip dysplasia from parents. Breeders providing inbreeding represent positive trend, fixing in animals traits connected with hip development of required quality. These trends should be confirmed by larger genetic study on molecular level.

ACKNOWLEDGEMENT

Authors acknowledge VEGA 1/3449/06, KEGA 3/5186/07 and German Shepherds Breeders Association in Slovakia.

REFERENCES

1. Bliss, S., Todhunter, R. J., Quaas, R., Casella, G., Wu, R., Lust, G., Williams, A. J. *et al.*, 2002: Quantitative genetics of traits associated with canine hip dysplasia in a canine pedigree constructed by mating dysplastic Labrador retrievers and unaffected Greyhounds. *Am. J. Vet. Res.*, 63, 1029–1035.

2. Gilmour, A. R., Cullis, B. R., Welham, S. J., Thompson, R., 1999: AS-REML *Reference Manual*. In *MSW Agriculture Biometric Bulletin*, 3. NSW Agriculture, 210 pp.

3. Hamann, H., Kirchhoff, T., Distl, O., 2003: Bayesian analysis of heritability of canine hip dysplasia in German Shepherd Dogs. *J. Anim. Breed. Gen.*, 120, 258–268.

4. Holen, K. J., Tegnander, A., Terjesen, T., Johansen, O. J., Eiknes, S. H., 1996: Ultrasonographic evaluation of breech presentation as a risk factor for hip dysplasia. *Acta paediatr.,* 85, 225–229.

5. Janutta, V., Hamann, H., Distl, O., 2006: Complex segregation analysis of canine hip dysplasia in German shepherd dogs. *J. Hered.*, 97, 13–20.

6. LaFond, E., Breur, G. J., Austin, C. C., 2002: Breed susceptibility for developmental orthopedic diseases in dogs. *J. Am. Anim. Hosp. Assoc.*, 38, 467–477.

7. Mäki, K., Liinamo, A. E., Ojala, M., 2000: Estimates of genetic parameters for hip and elbow dysplasia in Finnish Rottweilers. *J. Anim. Sci.*, 78, 1141–1148.

8. Mäki, K., Groen, A. F., Liinamo, A. E., Ojala, M., 2001: Population structure, inbreeding trend and their association with hip and elbow dysplasia in dogs. *Anim. Sci.*, 73, 217–228.

9. Remy, D., Neuhart, L., Fau, D., Genevois, J. P., 2004: Canine elbow dysplasia and primary lesions in German shepherd dogs in France. *J. Small Anim. Pract.*, 45, 244–248.

10. Rettenmaier, J. L., Keller, G. G., Lattimer, J. C., Corley, E. A., Ellersieck, M. R., 2002: Prevalence of canine hip dysplasia in a˝veterinary teaching hospital population. *Vet. Radiol. Ultrasound*, 43, 313–318.

11. Samoy, Y., Van Ryssen, B., Gielen, I., Walschot, N., Van Bree, H., 2006: Review of the literature: elbow incongruity in the dog. *Vet. Comp. Orthop. Traumatol.*, 19, 1–8.

12. Wood, J. L., Lakhani, K. H., Dennis, R., 2000: Heritability of canine hip-dysplasia score and its components in Gordon setters. *Prev. Vet. Med.*, 46, 87–97.

13. Wood, J. L., Lakhani, K. H., Rogers, K., 2002: Heritability and epidemiology of canine hip-dysplasia score and its components in Labrador retrievers in the United Kingdom. *Prev. Vet. Med.*, 55, 95–108.

14. Wright, S., 1922: Coefficients of inbreeding and relationship. *Am. Nat.*, 56, 330–338.

15. SAS V 9.1 for Windows. *SAS Institute Inc.*, Cary, NC, USA.

Received February 6, 2008

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 146–150, 2007

LUMBOSACRAL TRANSITIONAL VERTEBRA IN SOME DOG BREEDS

Ledecký, V., Gjengedal, H.
Skurková, L., Hluchý, M., Ševčík, A., Ledecká, K.

University of Veterinary Medicine, Komenského 73, 041 81 Košice
Slovak Republic

ledecky@uvm.sk

ABSTRACT

Transitional vertebra can occur in dogs throughout the vertebral column, particularly at the interface of its two anatomical parts (lumbosacral, thoracolumbar). It is a malformation of vertebrae. The lumbosacral transitional vertebra (LTV) denotes a change in the shape of the last lumbar or first sacral vertebra which can lead to asymmetry in pelvic attachment. The aim of the study was to determine prevalence of LTV in four dog breeds: German shepherd – GS, Rhodesian ridgeback – RR, Bavarian and Hannoverian bloodhound – BaHB, Bernese mountain dog – BSD, and evaluate the condition in dogs with *cauda equina* syndrome (CES). We also paid attention to determination of potential influence of LTV on prevalence of canine hip dysplasia (CHD), sex predisposition to development of LTV and comparison of incidence of asymmetric and symmetric forms of LTV. We evaluated 1964 radiographs obtained in relation to diagnostic of CHD in the mentioned four breeds of dogs over the period of 1999–2000 and 2004–2005. Of the 1964 cases, LTV was observed in 81 dogs with the highest prevalence in GS (4.44%). Sex predisposition was not observed: LTV was present in 55.55% of bitches and 44.45% of dogs. Of the total number of dogs with LTV, 17.28% suffered also from CHD; of the total number of lame dogs examined in 2005, 35 exhibited CES while 8 of them (22.5%) had also LTV. In the control group (1964 dogs), CHD without LTV was observed in 22.8% of animals and LTV without CES was recorded in 4.12% of dogs. Our observations showed no influence of LTV on prevalence of CHD. However, we observed a significantly higher occurrence of LTV in the dogs with CES in comparison with control dogs.

Key words: *cauda equina* syndrome; canine hip dysplasia; lumbosacral transitional vertebra

INTRODUCTION

Transitional lumbosacral vertebra is a non-physiologically formed vertebra between the lumbar and sacral part of the vertebral column acquiring lumbar and sacral characteristics. It occurs frequently between the last lumbar and first sacral vertebra. The shape of this transitional vertebra may vary and the abnormality may be bilaterally symmetric or asymmetric. Physiological formation of the lumbosacral zone in dogs is presented in Fig. 1.

Asymmetric transitional vertebra (on one side there is a typical *processus transversus* and on the other side the *processus transversus* resembles ala *osis ilii*) is visible in ventrodorsal radiograph which is commonly used to screen for canine hip dysplasia (CHD). To be able to determine the numerical abnormalities of vertebrae, lateral projection is necessary in addition to the ventrodorsal radiograph.

Morphology of LTV varies, particularly regarding the *processus transversus* which can present as "right" transverse process not fused to *os ilium* or *os sacrum*, or it can appear as *processus transversus* partially or completely attached to *os ilium* or *os sacrum* (Breit *et al.* – 2; Larsen – 8; Morgan – 10; Winkler and Loeffler – 14). According to the way of attachment of transitional lumbosacral vertebra to *os ilium*, we recognise three types of LTV.

Type 1: lumbar type – not attached to *os sacrum* and *os ilium*. It can appear as a normal lumbar transverse process

but can also have a wider and shorter base and deformed tip pointed at any direction.

Type 2: transitional type – the base is shorter and wider than that with the type 1 and the vertebra is partially attached to *os ilium* and frequently to *os sacrum* but the tip is always free.

Type 3: sacral type – it has a completely formed "wing" of *os sacrum* with wide attachment to *os ilium* and frequently also to *os sacrum*; the tip is not free.

On the basis of previous description we recognise three forms of symmetric transitional vertebra (1/1, 2/2, 3/3) and six forms of asymmetric transitional vertebra (1/2, 1/3, 2/1, 2/3, 3/1, 3/2) (D a m u r - D j u r i c *et al.* – 4) (Figs. 2, 3, 4).

The lumbosacral transitional vertebra (LTV) can allegedly accelerate development of degenerative disease of intervertebral discs. Should this thesis appear to be true, the presence of transitional vertebral segment could induce development of degenerative disease of intervertebral discs. This can subsequently lead to development of *cauda equina* syndrome (CES) (M o r g a n *et al.* – 11). The *cauda equina* syndrome is a complex of neurological symptoms caused by compression of nerve trunks running through the lumbosacral part of the spinal canal.

In the present study we evaluated the prevalence of lumbosacral transitional vertebra in 4 breeds of dogs after radiographical examination for CHD. Another objective of our study was to investigate the potential influence of LTV on prevalence of CHD and CES and potential sex predisposition to LTV.

MATERIAL AND METHODS

In order to monitor canine hip dysplasia in dogs of breeds predisposed to this condition from 12 months of age we evaluated 1964 radiographs which were taken in ventrodorsal extension position (Fig. 4).

The dogs were sedated before examination (butorphanol – 0.2 $mg.kg^{-1}$ i.m., xylazin – 1 $mg.kg^{-1}$ i.m., pre-medication with atropine – 0.03 $mg.kg^{-1}$ i.m.). We evaluated radiographs obtained for the period of 4 years.

Our evaluations included four breeds of dogs: German shepherd, Bernese mountain dog, Rhodesian ridgeback, Bavarian and Hannoverian bloodhound (bloodhounds were evaluated as one breed).

The results obtained were evaluated statistically by χ^2-test (with Yates correction).

RESULTS

Evaluation of 1964 radiographs obtained in ventrodorsal position when screening for CHD provided the following results (Tab. 1).

Analysis of prevalence of LTV in relation to breed

Of the total number of evaluated dogs 81 (4.12 %) exhibited transitional vertebra (LTV) ranging between 0 and 7.54 %. Evaluation according to breeds showed the highest prevalence of LTV in German shepherd (the mean for 4 years = 4.44 %) ($p < 0.001$, highly significant (**)) (Tab. 2).

In the 81 animals with lumbosacral transitional vertebra we diagnosed most frequently pelvic asymmetry with shallower acetabulum in one hip joint and also shift of femoral head from the dorsal acetabular margin in the lateral direction.

Analysis of prevalence of LTV in relation to sex

Analysis of sex predisposition to transitional vertebra showed that of the total number of 1964 animals examined for CHD 992 were bitches (50.5 %) and 972 dogs (49.5 %). Of the total number of unaffected animals (1883 LTV-free) 947 were bitches (50.3 %) and 936 dogs (49.7 %). LTV was observed in 45 bitches (55.55 %) and 36 dogs (44.45 %) ($p > 0.05$, non-significant – ns) therefore no relationship was observed between the prevalence of LTV and sex of dogs.

Analysis of prevalence of LTV in relation to CHD

Of the total number of dogs with LTV, hip dysplasia was diagnosed in 14 dogs, i. e. 17.28 %. According to the FCI scale for evaluation of CHD, the dogs examined exhibited classes B and C of dysplasia. It should be mentioned that the prevalence of CHD (classes B to E) in German shepherd dogs in the years 2004 and 2005 ranged between 20.3 and 21.55 %, regardless to the presence of LTV ($p > 0.05$, non-significant – ns), i. e. there was no relationship between the prevalence of LTV and CHD in the investigated dogs.

Analysis of prevalence of LTV in relation to CES

On the basis of analysis of lame dogs of large breeds carried out in 2005 the diagnosis *cauda equina* syndrome was made in 35 dogs. Eight of them exhibited transitional vertebra or supernumerary vertebra, i. e. 22.5 % of dogs were positive for this condition. In the control group (1964 dogs) without CES symptoms LTV was detected only in 4.12 % (81 dogs), i. e. the proportion of dogs with LTV symptoms was significantly higher in the CES group than in the control one (highly significant – ***, $p < 0.001$). The influence of sex, age and breed predisposition to CES was not evaluated in our study.

Analysis of prevalence of LTV according to its type

Of 81 dogs with symptoms of LTV 49 animals (60.49 %) showed transitional vertebra of the symmetrical type while the asymmetric type was detected in 32 (39.5 %) dogs ($p > 0.05$, non-significant – ns).

Summary evaluation of prevalence of LTV and the number of evaluated dogs according to breeds is presented in Table 3.

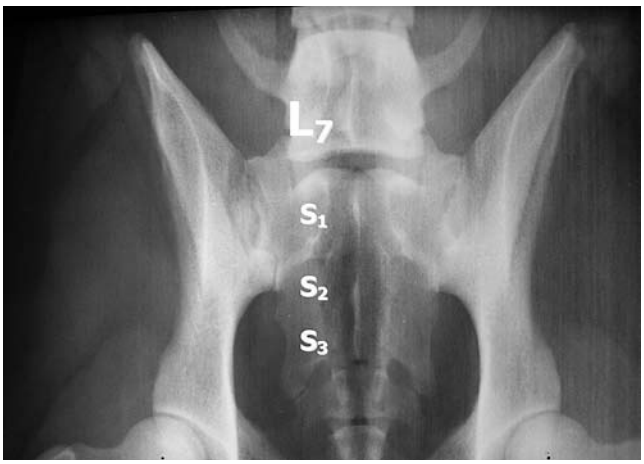


Fig. 1. Ventrodorsal pelvic radiograph (no finding) – visible last lumbar vertebra, *os sacrum* was formed by fusion of three vertebrae, the lumbosacral junction is symmetrical without sign of morphological deviations

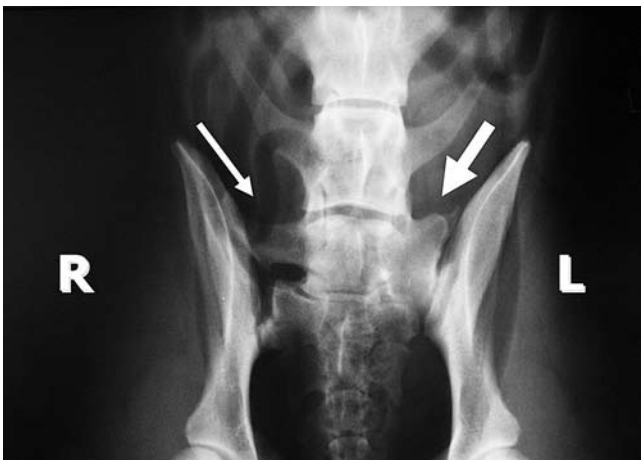


Fig. 2. Ventrodorsal pelvic radiograph (R – right side of the dog, L – left side of the dog) – asymmetric type of transitional vertebra (2/3): On the left side of *os sacrum* – *processus transversus* of the sacral type (type 3) with a wide area of contact with *os ilium* (wider arrow). On the right – the process bears signs of lumbar transverse processes (narrow arrow) but there is still some attachment to *os ilium* on the basis of which it is classified as type 2 (transitional type)

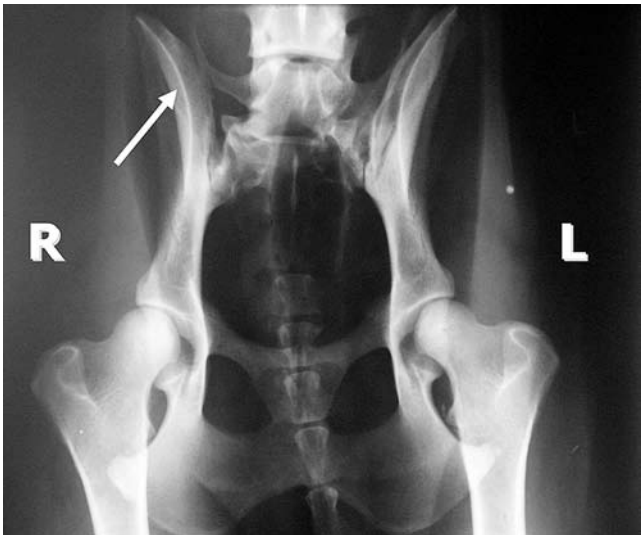


Fig. 3. Ventrodorsal pelvic radiograph (R – right side of the dog, L – left side of the dog) – asymmetric type of transitional vertebra (1/2). *Processus spinosus* of the first sacral vertebra is separated from crista mediana of *os sacrum*. On the right side there is a free *processus transversus* (arrow) (type 1), the process on the left side resembles more the *os sacrum* wing. The contact area with *os ilium* is wider but its tip remains free (type 2). The transitional segment is rotated slightly to the left, *os sacrum* to the right

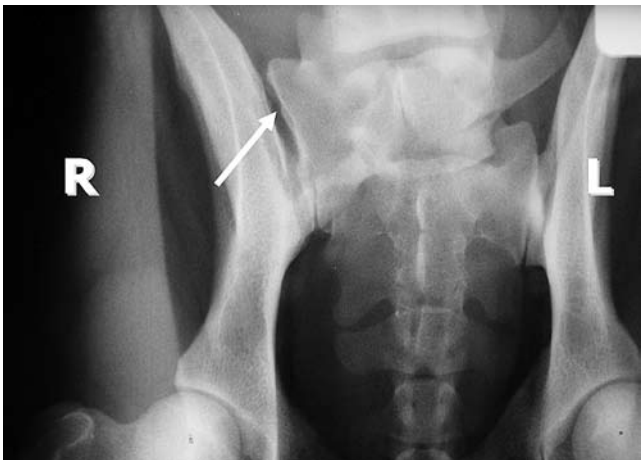


Fig. 4. Ventrodorsal pelvic radiograph (R – right side of the dog, L – left side of the dog) – asymmetric type of transitional vertebra (3/1). The LTV *processus spinosus* is not fused with processes forming *crista sacralis mediana*. The LTV process on the left side resembles *processus transversus* without contact with *os sacrum* or *os ilium* (type 1 – lumbar), the process on the right side (arrow) is shaped as *os ilium* wing (type 3 – sacral). LTV is rotated to the right

DISCUSSION

In the study conducted by Damur-Djuric *et al.* (4) in 2006, 3.5 % prevalence of LTV was observed when evaluating 4000 radiographs taken for the purpose of CHD screening. Considering the fact that the incidence of lumbosacral abnormalities depends to a considerable degree on the purpose of relevant examination, our result (4.12 %) is comparable with the result of the above mentioned authors.

Our study showed that German shepherd was the breed with the highest prevalence of LTV (4.44 %). Previous studies also described high prevalence of LTV in German shepherds, namely 4.3 % (Larsen – 8), and 5.7 % (Flückiger *et al.* – 6).

In another study which evaluated radiographs taken in both ventrodorsal and lateral projection as high as 8% prevalence of LTV was observed in German shepherd dogs (Morgan and Stevens – 12). Higher prevalence of LTV in the latter study can be explained by the fact that the ventrodorsal projection makes it more difficult to uncover some symmetrical or numerical abnormalities of vertebrae. The prevalence of LTV generally reported in German shepherd dogs ranges between 4.3 and 20.5 %.

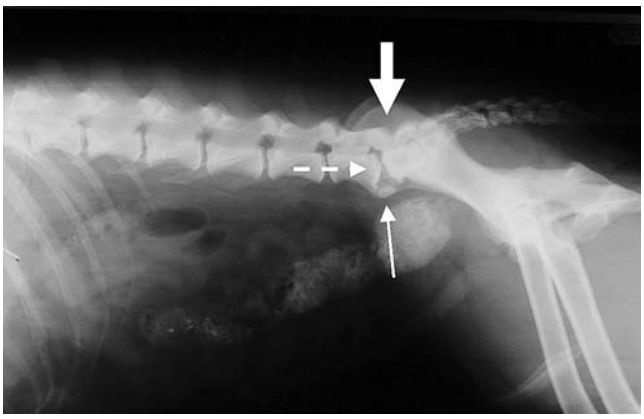


Fig. 5. Lateral projection – symmetrical type of LTV with degenerative changes at the site of lumbosacral junction: presence of spondylosis (thin arrow), sclerosis of intervertebral disc L7 (dashed arrow), narrowing of the spinal canal in the zone S1 (wider arrow)

The higher prevalence of LTV observed in German shepherd dogs in comparison to other breeds has probably a genetic basis. Ziegler (15) explained the higher prevalence of lumbosacral transitional vertebra by unsuitable breed selection in this particular breed. Apparently, selection for long back was carried out in both German shepherd and Basset. This was evidently the reason of an unconscious selection for long vertebrae or higher number of vertebrae. However, increased number of vertebrae leads to shortening of vertebra bodies (Mayer and Lindfeld – 9). This indicates that the vertebral length in dogs ranges within certain limits. After exceeding this range there is a change in the number of vertebrae (Breit and Künzel – 3).

As far as the incidence of LTV in Bernese mountain dogs is concerned, Damur-Djuric *et al.* (4) reported 9.4% prevalence of LTV. In our study we examined 72 dogs of this breed and the highest prevalence of LTV reached 4.34% (in 2004).

Our analysis of the relationship between CHD and LTV showed that 17.28% dogs with CHD (class B or C) were positive also for LTV. This is a relatively low number in comparison with the results of Lanting (7) who detected LTV in 69% of dogs with CHD.

Flückiger *et al.* (6) observed 16.3% prevalence of LTV in dogs with CES. Results obtained in our study were somewhat higher, 22.5% of dogs with CES exhibited LTV. This author postulated that dogs with LTV show in the later age 8-times higher susceptibility to development of CES than dogs without LTV. Similar to this, German shepherds as breed with this predisposition also showed approx. 8-times higher susceptibility to development of CES than other breeds. The author also assumed that the onset of development of CES can be expected 1 to 2 years earlier in dogs with LTV. Because of that owners of dogs with LTV are not recommended to subject them to financially and time-consuming training as the risk of development of CES in these dogs is higher (Fig. 5).

Morgan *et al.* (11) presented observations about percentage proportion of dogs with occurrence of both LTV and CES. Their incidence was observed in 77.8% of dogs: 14 of 18 dogs examined for degenerative disease of intervertebral discs.

The results presented indicate that the proportion of dogs with LTV is significantly higher in the groups with CES. This at the same time supports the theory of higher

Tab. 1. Proportion of evaluated dog breeds

Breed	Number of dogs	% Proportion of breeds
German shepherd	1767	89.97
Rhodesian ridgeback	47	2.39
Bernese mountain dog	72	3.76
Bavarian and Hannoverian bloodnound	78	3.97

Tab. 2. Prevalence of lumbosacral transitional vertebra in investigated dog breeds

Breed	% Prevalence of LSTV accoring to breed
German shepherd	4.44 (1.46–7.54)
Rhodesian ridgeback	2.12. (0–3.8)
Bernese mountain dog	2.77 (0–4.34)
Bavarian and Hannoverian bloodnound	0

Tab. 3. Summary of prevalence of LSTV during the investigated period

Period	German shepherd			Bernese mountain dog			Rhodesian ridgeback			Bavarian and Hannoverian bloodhound		
	No.	Posit.	%	No.	Posit.	%	No.	Posit.	%	No.	Posit.	%
1999+	820	12	1.46	–*	–*	–*	–*	–*	–*	–*	–*	–*
2000												
2004	483	21	4.34	46	2	434	21	0	0	33	0	0
2005	464	35	7.54	26	0	0	26	1	3.8	45	0	0
Total	**1967**	**78**	**4.44**	**72**	**2**	**434**	**47**	**1**	**3.8**	**78**	**0**	**0**

* – breeds Bernese mountain dog, Rhodesian ridgeback and Bavarian and Hannoverian bloodhound were evaluated from year 2004

prevalence of CES in dogs with LTV (F l ü c k i g e r *et al.* – 5; L a r s e n – 8; M o r g a n *et al.* – 10, 13).

According to B a i l e y and M o r g a n (1) the transitional segment may predispose the adjacent intervertebral discs to early degeneration and subsequent protrusion and can potentially lead to instability of vertebrae and to compression of spinal nerves after hypertrophy of surrounding soft tissues.

It is known that development of CES in dogs with LTV occurs at early age. It appears that LTV accelerates degeneration of the intervertebral discs located cranially in front of LTV or the disc itself is dysplastic and because of that predisposed to degeneration. F l ü c k i g e r *et al.* (5) also stated that LTV decreases range of movement at the site of lumbosacral junction. This results in an increased range of movement between the last lumbar disc and LTV with subsequent acceleration of intervertebral disc degeneration. This explains the predominance of type 2 (transitional type of LTV) in the dogs with CES. Processes of this type are therefore in contact with pelvis and thus can limit the range of movement.

F l ü c k i g e r *et al.* (5) also evaluated the influence of sex on prevalence of CES. Although their results indicated that bitches with LTV are more susceptible to the development of CES, regression analysis showed that it is the presence of LTV and not the sex that increases the risk of CES. In reality the risk of development of CES is higher in dogs than in bitches (approx. twofold) (however, the study mentioned evaluated only 15 dogs with both CES and LTV).

The part of our study investigating the differences in sex predisposition provided negative results for LTV (50.50% in bitches, 49.50% in dogs). These results correspond to those presented in other studies: D a m u r - D j u r i c *et al.* (4) reported 47.8% prevalence of LTV in dogs and 52.2% in bitches. Despite that we should mention that some other authors observed slightly higher prevalence of LTV in dogs or, on the contrary, in bitches (M o r g a n – 10; Z i e g l e r – 15; W i n k l e r *et al.* – 14).

In our study the symmetric type of LTV was observed in 39.5% of examined dogs and the asymmetric one in 60.49% of examined animals. Other authors reported approximately equal incidence of both types of LTV. D a m u r - D j u r i c *et al.* (4) reported 49% symmetric LTV and 51% asymmetric while B r e i t *et al.* (2) observed 44% symmetric LTV and 56% asymmetric.

Our results failed to prove sex predisposition to LTV in the examined dogs but indicated breed predisposition (German shepherd). At the same time it was observed that the influence of LTV on prevalence of CHD was not confirmed but LTV could lead to abnormal rotation of pelvis and subsequently to insufficient covering of femoral head by the acetabulum (usually unilateral). In addition to that dogs with LTV were at greater risk for CES.

REFERENCES

1. Bailey, S., Morgan, J. P., 1992: Congenital spinal malformations. Veterinary Clinics of North America. *Small Animal Practice*, 22, 985–1015.

2. Breit, S., Knaus, I., Künzel, W., 2003: Differentiation between lumbosacral transitional vertebrae, pseudolumbarization and lumbosacral osteophyte formation in ventrodorsal radiographs of the canine pelvis. *Veterinary Journal*, 165, 36–42.

3. Breit, S., Künzel, W., 2002: The diameter of the vertebral canal in dogs in cases of lumbosacral transitional vertebrae or numerical vertebral variations. *Anatomy and Embryology*, 205, 125–133.

4. Damur-Djuric, N., Steffen, F., Hässig, M., Morgan, J. P., Flückiger, M. A., 2006: Lumbosacral transitional vertebrae in dogs: classification, prevalence and association with sacroiliac morphology. *Veterinary Radiology and Ultrasound*, 47, 32–38.

5. Flückiger, M. A., Djuric, N., Hässig, M., Morgan, J. P., Steffen, F., 2006: A lumbosacral transitional vertebra in the dog predisposes to *cauda equina* syndrome. *Veterinary Radiology and Ultrasound*, 47, 39–44.

6. Flückiger, M. A., Djuric, N., Hässig, M., Steffen, F., Morgan, J. P., 2003: Lumbosacral transitional vertebra (LTV) in the dog: Breed prevalence and correlation with *cauda equina* syndrome (CES). *International Veterinary Radiology Association Meeting*, Midrad, SA. August 19th–22nd.

7. Lanting, F., 2001: Transitional vertebral segment (TVS). www.dogstuff.info/transitional-vertebral-segment-lanting.html

8. Larsen, J. S., 1977: Lumbosacral transitional vertebrae in the dog. *Journal of the American Veterinary Radiology Society*, 18, 76–79.

9. Mayer, H., Lindfeld, A., 1969: Number and length of vertebrae in the improved German county-pig. *Dtsch. Tierärztl. Wschr.*, 76, 448–453.

10. Morgan, J. P., 1999: Transitional lumbosacral vertebrae anomaly in the dog: a radiographic study. *Journal of Small Animal Practice.*, 40, 167–172.

11. Morgan, J. P., Bahr, A., Franti, C. E., Bailey, C. S., 1993: Lumbosacral transitional vertebrae as a predisposing cause of *cauda equina* syndrome in German Shepherd Dogs: 161 cases (1987–1990). *Journal of American Veterinary Medical Association*, 202, 1877–1882.

12. Morgan, J. P., Stevens, M., 1985: *Radiographic Diagnosis and Control of Canine Hip Dysplasia*. Ames, IA: Iowa State University Press.

13. Morgan, J. P., Wind, A., Davidson, A. P., 2000: Lumbosacral disease. In **Morgan, J. P.** (Ed.)**:** *Hereditary Bone and Joint Disease in the Dog*. Hannover: Schlütersche, 209–230.

14. Winkler, W., Loeffler, K., 1985: *Lumbosakrale Übergangswirbel beim Hund*. Thesis in Veterinary Medicine. Freie Universität, Berlin.

15. Ziegler, G., 1989: *Assimilationsstörungen im lumbosakralen Übergangsbereich der Wirbelsäule bei Hund und Katze*. Thesis in Veterinary Medicine, Ludvig-Maximilians-Universität, München.

Received October 1, 2007

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 151–155, 2007

INCISOR ROOT CANAL ANATOMY *VERSUS* GUTTA-PERCHA OBTURATION TECHNIQUES IN DOGS

Capík, I.

Clinic of Small Animals
University of Veterinary Medicine, Komenského 73, Košice
The Slovak Republic

capik@uvm.sk

SUMMARY

An *in vitro* study was conducted to compare gutta-percha selected obturation techniques of the developing incisors in dogs. Radiographic examination was used for evaluation of apical and overall appearance of root canal obturation. Apical leakage technique was used to evaluate ability of each method to provide adequate apical obturation. The warm vertical condensation with chemically softened gutta-percha in the apical region provided the best radiographic appearance without apical dye leakage. The same vertical obturation technique without the use of chemically softened gutta-percha in the apical region showed 33% dye leakage incidence. Lateral obturation techniques provided the least qualitative radiographic appearance to the endodontic fill and dye leakage incidence.

Results of evaluation resulted in the following conclusions. Lateral obturation techniques used in this study have their limitations resulting from irregular root canal anatomy. Vertical obturation techniques showed better hermetic apical sealing, mainly in the case of vertical condensation of the chemically softened gutta-percha in the apical part of the root canal.

Full correspondence address:
Doc. MVDr. Igor Capík, PhD.
Klinika chirurgie, ortopédie a röntgenológie
Univerzita veterinárskeho lekárstva,
Komenského 73, 040 01 Košice
Slovenská republika
Tel: +421 915 984 607

Key words: dog; endodontic; maturing incisors; obturation; root canal

INTRODUCTION

Bacteria in the root canal system are the primary cause of periapical periodontitis (5, 7). Healing of apical periodontitis involves a combination of disinfection of the root canal space through chemo-mechanical means (2, 3) and sealing both the root canal and access cavity with materials that will prevent reinfection (8).

The correct approach to therapy requires not only good orientation in a range of different types of available materials and techniques involving obturation of root canals but also perfect knowledge of anatomy and physiology of the endodontic system and good understanding of respective pathological processes.

Incisors, premolars and molars are possible radiologically viewed from labio-lingual/ palatal view but not from mesio-distal one. Our experience using extracted developing incisors (i. e. teeth in dogs of 8 to 14 months of age) confirms the following anatomical characteristics:

1) The crown size is small in relation to labio-lingual diameter of root canal, thus preventing sufficient access cavity preparation.

2) The walls of root canal from mesio-distal view create approximately elliptical shape with the widest diameter in the middle third of root canal.

3) The apical area of the root canal is divergently separated in some incisors below 1 year of age.

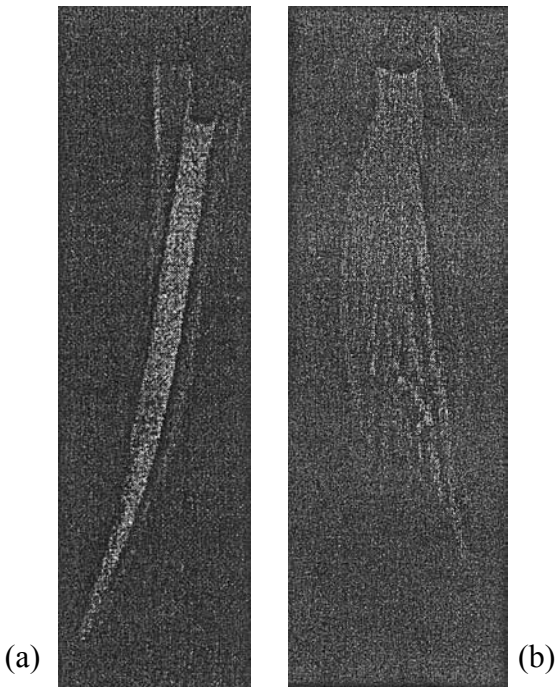


Fig. 1. Cold lateral obturation technique
Labio-lingual view (a) with the score 2 in apical region and 2 in the whole root canal; mesio-distal view (b) with the score 4 in the apical region and 3 in the whole root canal

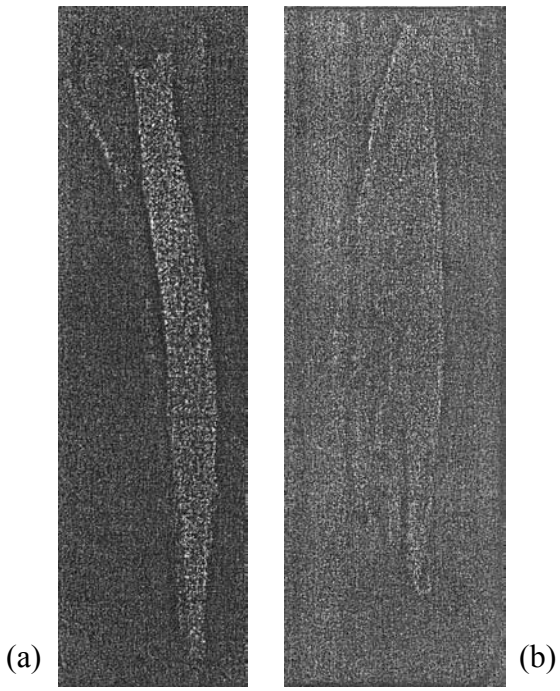


Fig. 2. Warm lateral condensation technique
Labio-lingual view (a) with the score 1 in apical region and the whole root canal; mesio-distal view (b) with the score 3 in the apical region and 2 in the whole root canal

The purpose of this study was to evaluate and compare radiologically effectiveness of root canal obturation in extracted incisors from two perpendicular views and the apical dye leakage of obturation techniques using gutta-percha as a root filling material.

MATERIAL AND METHODS

The study was conducted on incisors and canine teeth obtained from cadavers of 7–12 months old dogs. Only teeth with a wide root canal, closed apex and absence of root fractures or any other pathological changes were included into the study. Altogether 36 teeth were used. To exclude any variability of procedures, the same person took all X-ray pictures and made all the fillings. The teeth were divided to 5 groups, six in each, and were obturated with gutta-percha (GP) cones.

In the 1st group we used the technique of cold lateral condensation (LC). Standardised GP cones of sizes 40–90 according to the real width of the root canal were used as master cones. The lateral condensation was ensured by spreaders of size 15 or 20 ISO. The surplus GP was removed from the crown zone by a warmed device and vertically condensed by a wide plugger.

The 2nd group consisted of teeth obturated also by lateral condensation but a combination of GP and root canal cement was used (LC-C). The procedure was the same as that used in the 1st group but before the obturation we applied a thin cement layer to the canal and to individual GP cones. Comparison of the results obtained in the 1st and 2nd group allowed us to determine the influence of the cement layer on the tightness achieved by lateral condensation technique.

In the 3rd group we used lateral condensation of heat-softened GP (WLC – warm lateral condensation). This group comprised teeth contralateral to those used in the 1st group in which we expected very similar structure of the canal. We observed differences in the quality of tightness achieved by respective techniques.

In the 4th group we used the technique of warm vertical condensation-back packing (WVC). The GP cones were shortened to 4 mm, inserted into the root canal and condensed using warm plugger of appropriate diameter.

Obturation of root canals of teeth from the 5th group was carried out by the technique of back packing by halothane-softened GP (BP-H). The principal GP cone was shortened to 5 mm and the apical 4 mm were submerged in halothane for 10 s. Immediately after that the cone was inserted and condensed vertically by a plugger of appropriate diameter. Subsequently, the remainder of root canal was obturated by warm vertical condensation as in the 4th group.

After obturation of root canals, two X-ray projections in directions perpendicular to each other were obtained of all teeth from individual groups. They were scanned and divided by a computer to four groups according to the quality of root obturation.

The cement layer on roots of all teeth was coated with a lacquer film with the exception of the apical zone which was left uncoated. Then the teeth were fixed in a dental wax plate and their apical parts were submerged in gentian blue up to the cement-enamel junction and left there for two weeks. This allowed us to assess the extent of penetration of fluid through

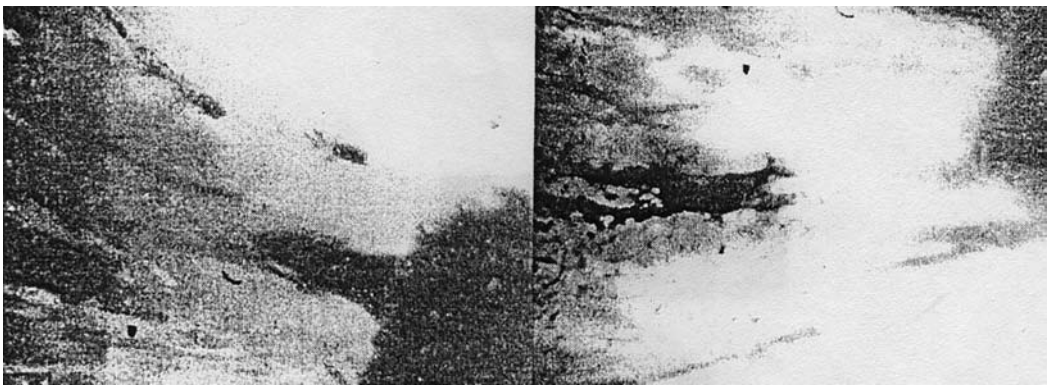


Fig. 3A. Section of the apical region showing excelent obturation of the apical delta and apical part of root canal using chemically (halo tan) softened gutta-perča

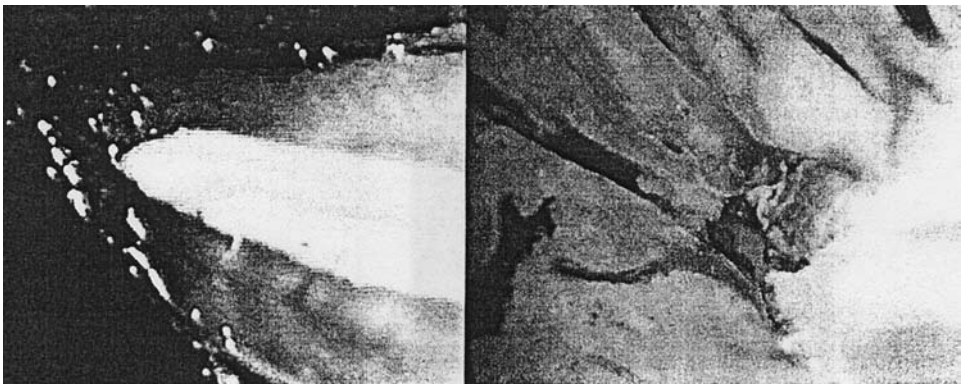


Fig. 3B. Root sections of tooth obturated using cold lateral condensation. Apical dye leakage seen in the apikal delta (a). Dye leakage in the apical part of the root (b)

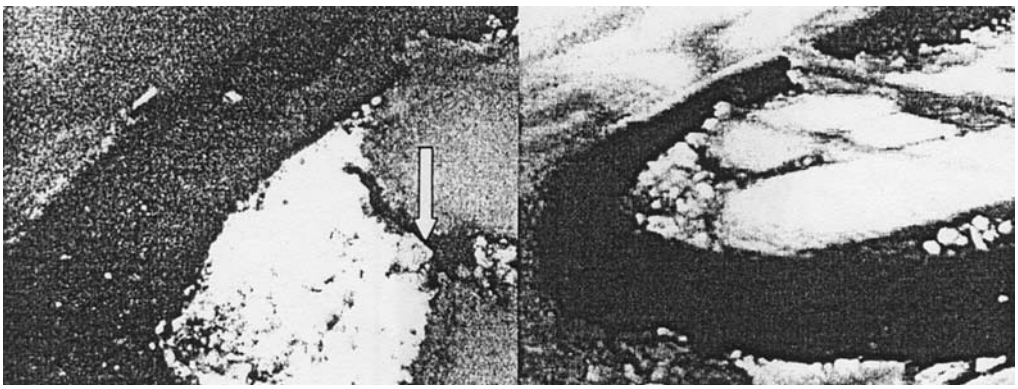


Fig. 3C. Cold lateral condensation using gutta-percha and root cement: a – Root canal fill containing considerable volume of root canal cementum among gutta-percha cones with the presence of microgaps (arrow), b – Root cement colored by dye confirms insufficient obturation

the apical delta and adjunct canals in the apical third into root canals at various ways of obturation. The extent of passage of liquid was evaluated on sections of the roots. The teeth were cut longitudinally using a diamond saw. The sections were photographed and the extent of penetration of the dye to the root canal was assessed.

The quality of obturation was evaluated on the basis of the degree of replication of canal working length, surface adaptation and homogeneity of gutta-percha and resistance of obturation to penetration of the dye.

RESULTS

The tables 1 to 5 present evaluation of X-ray quality of obturation using a four-grade scale (1 – excellent, 2 – very good, 3 – good, 4 – poor).

Results of evaluation of quality of apical obturation of root canals based on X-ray examination resulted in the following conclusions:

When evaluating the quality of obturations from the labio-lingual projection, BP-H obturation technique reached 1,

Table 1. Lateral condensation

Evaluated part	View	View evaluation*						Mean value
Apex	L-L	2	2	2	2	1	2	1.83 ± 0.41
	M-D	2	4	3	3	3	2	2.83 ± 0.41
Root canal	L-L	2	2	1	2	1	2	1.67 ± 0.52
	M-D	2	3	3	2	2	2	2.33 ± 0.52

Table 2. Warm lateral condensation

Evaluated part	View	View evaluation*						Mean value
Apex	L-L	1	2	2	2	2	2	1.83 ± 0.41
	M-D	3	2	3	2	3	3	2.17 ± 0.36
Root canal	L-L	1	2	2	1	2	2	1.67 ± 0.52
	M-D	2	2	2	2	2	1	1.83 ± 0.41

Table 3. Lateral condenstion using root canal cement

Evaluated part	View	View evaluation*						Mean value
Apex	L-L	2	1	1	2	1	2	1.5 ± 0.55
	M-D	2	2	1	2	1	1	1.5 ± 0.55
Root canal	L-L	2	2	1	1	1	1	1.33 ± 0.52
	M-D	2	2	1	2	2	1	1.67 ± 0.52

Table 4. Warm vertical-back packing

Evaluated part	View	View evaluation*						Mean value
Apex	L-L	2	1	1	1	1	1	1.17 ± 0.28
	M-D	2	2	1	1	2	1	1.50 ± 0.17
Root canal	L-L	1	1	1	1	1	2	1.17 ± 0.28
	M-D	2	2	2	1	1	2	1.67 ± 0.22

Table 5. Halotan softened GP/warm vertical-back packing

Evaluated part	View	View evaluation*						Mean value
Apex	L-L	1	1	1	1	1	1	1
	M-D	2	1	1	1	1	1	1.17 ± 0.28
Root canal	L-L	1	1	2	1	1	1	1.17 ± 0.28
	M-D	1	2	2	2	1	1	1.50 ± 0.28

Table 6. Apical dye leakage in the different obturation techniques

Obturation technique	Positive/negative	% leakage
LC	4/2	66
LC-C	3/3	50
WLC	3/3	50
WVC	2/4	33
BP-H	0/6	0

* 1 – excellent; 2 – very good; 3 – good; 4 – poor
L-L – labio-lingual; M-D – mesio-distal

WVC – 1.17 and LC-C provided a mean grade equal to 1.5 on the mentioned scale. With the remaining two techniques (LC, WLC) the mean grade reached was equal to 1.83 significantly different from BP-H and WVC obturation techniques ($P < 0.05$).

The mesio-distal projection of treated root canals indicated similar variability of the quality of apical obturation of root canals. The highest quality (mean grade 1.17) was reached with root canals obturated with chemically softened gutta-percha (BP-H). This technique was followed by LC-C, WVC – 1.5, WLC – 2.17 and LC – 2.83. The significant difference was confirmed between WLC, LC and other condensation techniques.

Apical obturation from both views. The best general quality of the apical obturation was achieved in the BP-H group (1.09) followed by LC-C, WVC (1.5), WLC (2) and LC (2.33).

Evaluation of X-ray quality of obturation of the entire canal indicated higher variability between the techniques in comparison with apical obturation. The labio-lingual projection showed the highest mean value (1.67) in the LC and WLC groups. LC-C group reached the mean value 1.33. WVC and BP-H (1.17) groups confirmed significantly better obturation results ($P < 0.05$) than LC and WLC groups.

The mesio-distal X-ray view to assess obturation quality of the whole root canal confirmed better radiological signs of obturation in the BP-H (1.5), WVC and LC-C (1.67) groups in comparison with the WLC (1.83) and LC (2.33) groups. The values in the BP-H have been significantly differing from LC and WLC groups (Figs. 1, 2)

The best general quality of the whole root canal obturation was achieved in the group BP-H group (1.33). LC group showed significantly lower success of obturation ($P < 0.05$).

Sections of individual teeth showed a penetration of gutta-percha into apical delta canals only in case of halothane-softened gutta-percha (Fig. 3)

Comparison of apical dye leakage in different obturation techniques is presented in Table 6.

DISCUSSION

According to the opinion prevailing in professional literature, higher clinical successfulness is ascribed to root canal obturations containing higher proportion of gutta-percha and lower proportion of cement (6). It has been explained by closer adaptation of gutta-percha to the canal wall. The good adaptation of gutta-percha to the canal wall increases the degree of complete ob-

turation of the root space. Warm gutta-percha adapts better to the wall than the cold one and the obturation techniques involving warm gutta-percha are still subject to development.

Techniques using heat-softened gutta-percha include its vertical condensation, injection of thermoplastic GP, thermo-mechanical obturation and thermoplastic GP on a hard core – TermaFil (4). Some risk arising from the use of the mentioned techniques involves increased temperature in the treatment zone which can result in irreversible damage to periodontal tissues.

Our results point to some anatomical characteristics of developing dog teeth which affect the quality of root obturation employing the techniques used in our study. In the labio-lingual/palatal projection the walls of pulpal cavities and root canals tapered toward the apex. In the mesio-distal projection the width of pulpal cavity and root canals of incisors showed more pronounced changes compared to the labio-lingual projection. Canals of developing incisors and canines have elliptical shape and their width is the highest in the central portion of the root canal.

Due to the respective anatomical structure of the crown of developing teeth, the preparation of sufficiently wide opening to the canal is impossible without marked destruction of the crown. Dilaceration of the apex was observed in some roots of developing incisors. The mentioned anatomical structure resulted in lower quality of obturation of the root canal particularly when using lateral condensation techniques.

One study reported that vertical condensation with the use of shortened GP conuses (Simplifill technique) was the best in comparison with endodontic technique using manual and machine filling at condensation (9). According to results of this study this approach resulted in 0 % leakage in comparison with other techniques.

According to results of our X-ray examination, the warm vertical condensation using shortened GP conuses (Simplifill technique) provided statistically confirmed better results of obturation of the apical root portion in comparison with cold and warm lateral condensation.

Cold lateral condensation without the use of root cement provided worse results in comparison with its presence, however, the X-ray detected differences in the quality of obturation of the apical third of root canal were insignificant.

The best obturation was achieved using the techniques based on vertical condensation of halothane-softened GP in the apical part and vertical back-packing obturation with heat-softened gutta-percha in the remaining part of root canal.

Successfulness of the latter techniques was markedly better than that of cold and warm lateral condensation. Comparison with other obturation techniques failed to show significant differences.

Observation of sections for penetration of the stain through the apical delta into the root canal confirmed the best hermetic sealing with halothane-softened gutta-percha. Similar results were presented in the study by B e a t t y *et al.* (1) who reported considerably lower penetration of liquid through fillings with chemically and heat softened GP. Only the chemically softened gutta-percha allowed us to achieve hermetic sealing of the apical delta of all treated root canals. All other techniques showed various degrees of leakage in the range of 33–66 %. Obturation of apical delta canals was also achieved by this obturation technique.

ACKNOWLEDGMENT

This study was supported by Scientific Grant Agency of Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences No. 1/0566/03.

REFERENCES

1. Beaty, R. G., Zakariasen, K. L., 1984: Apical leakage associated with three obturation techniques in large and small root canals. *Int. Endod. J.*, 17, 67–72.

2. Byström, A., Sundquist, G., 1983: Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. *Oral Surg., Oral Med., Oral Pathol.*, 55, 307–312.

3. Byström, A., Sundquist, G., 1985: The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *Int. Endod. J.*, 18, 35–40.

4. Genoglu, N., Garip, Y., Bas, M., Samani, S., 2002: Comparison of different gutta-percha root filling techniques: TermaFil, Quick-Fill, system B and lateral condensation. *Oral Surg., Oral Med., Oral Pathol.*, 3, 333–336.

5. Kakehashi, S., Stanley, H., Fitzgerald, R., 1965: The effect of surgical exposures of dental pulpsin germ-free and conventional laboratory rats. *Oral Surg., Oral Med., Oral Pathol.*, 20, 340–349.

6. Kontakiotis, E. G., Wu, M. K., Wesselnik, P. R., 1997: Effect of sealer thickness on long-term sealing ability: a 2-year follow-up study. *Int. Endod. J.*, 30, 307–312.

7. Möler, A. J., Fabricius, L., Dahlen, G., Hezden, G., 1981: Influence of periradicular tissues of indigenous oral bacteriaand necrotic pulp tissues in monkeys. *Scand. J. Dent. Res.*, 89, 475–484.

8. Ray, H. A., Trope, M., 1995: Periapical status of endodontically treated teeth in relation to the technical quality of the root filling and coronal restoration. *Int. Endod. J.*, 28, 12–18.

9. Stein, K. E., Marreta, S. M., Siegel, A., Vitoux, J., 2004: Comparison of hand-instrumented, heated gutta-percha and engine-driven, cold gutta-percha endodontic techniques. *J. Vet. Dent.*, 21, 136–145.

Received November 2, 2007

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 156–161, 2007

THE USE OF PRESERVED CANINE RENAL CAPSULE AND COSTAL PLEURA TO REPAIR LAMELLAR CORNEAL LESION IN NORMAL RABBITS

Trbolová, A.[1], Kozák, I.[2], Balicki, I.[3], Ševčíková, Z.[1]

[1]University of Veterinary Medicine, Košice
[2]Department of Ophthalmology, P. J. Šafárik Medical School, Košice
The Slovak Republic
[3]Katedra i Klinika Chirurgii Zwierząt Wydziału Medycyny Weterynaryjnej AR, Lublin
Poland
talex@post.sk

SUMMARY

The purpose of this study was to evaluate the use of canine renal capsule and pleura preserved in 98% glycerine to repair lamellar corneal lesion in experimental rabbits. Fifteen white New Zealand rabbits divided into 3 groups were used (5 rabbits with renal capsule graft, 5 with costal pleura graft and 5 control rabbits the corneal lesions of which were covered with a third eyelid flap). A central, half-thickness keratectomy of the stroma was created by using a 6.0 mm trepine. The donor implant of canine renal capsule and costal pleura of the same shape and 6.5 mm size was sutured in place with simple interrupted suture using 10–0 nonresorbable material (Ethilon). In all groups chloramphenicol eye drops were administered 4 times a day for 14 postoperative days. In the control group the corneal lamellar defect was covered for 14 days with a third eyelid flap. Clinical evaluation was carried out on days 1, 7, 15, 30 and 60 after surgery. The donor corneas of each group were subjected to histological evaluation on days 30, 45 and 60 post surgery. Blepharospasm /photophobia were more intense on days 1 to 7 after surgery and regressed on day 15 after surgery. Ocular discharge was present in all groups till day 7, and in the group with grafts it persisted at a low intensity until day 30 after surgery. Corneal oedema near the implant was observed on day 7 after surgery; the area of the former oedema increased until day 30 (in groups with a graft) but afterwards it decreased again and persisted till the end of the experiment at an intensity that was identical with that observed at the beginning. Corneal vascularization started 7 days after surgery and persisted until the end of the experiment. No vascularization was present in the control group. There was no fluorescein retention by postoperative day 15. In the control group, in addition to the normal course of healing the histological findings on day 60 after surgery revealed a thinner epithelial cell layer, epithelial detachment and fibrotization in the stroma region. In the group with the renal capsule the incorporation (grown-in) into the stroma region was present; in addition to that vascularization, macrophages (Ma), lymphocytes (Ly) and stromal fibrotization were observed. In the „pleura group“ no implantation of the graft could be observed; histology revealed vascularization, local absence of epithelium, occurrence of fibrotic tissue, Ma and Ly. The clinical and histological evidence indicates that the xenologous renal capsule and costal pleura can be useful as alternative tissues to repair lamellar corneal lesions.

Key words: canine renal capsule and costal pleura; cornea; keratoplasty; rabbit; xenogenic graft

INTRODUCTION

Ulcerative corneal lesions are common in small animals and may progress to the complete perforation. Deep corneal ulcers, especially with descemetocele are ophthalmic emergencies and require specific therapy to prevent perforation. Prompt and effective repair of deep or large-diameter corneal defects

is desirable in order to maintain or restore vision. Surgical techniques employed for corneal ulceration include conjunctival allografts (1, 2, 3, 4, 5), cyanoacrylate adhesive tissue (5, 6, 7, 8), corneal – scleral – conjunctival transposition (9, 10), lamellar keratoplasty (11, 5, 12), penetrating keratoplasty (5, 13) and tectonic corneal grafts (12, 14).

Conjunctival pedicle grafts are practical and versatile and as such they are widely and successfully used in veterinary ophthalmology (2, 3). However, conjunctival grafts may ultimately result in corneal opacities that may impair vision when the lesion is in the axial cornea. Preserved biological membranes of various tissue types have been used for many surgical purposes including repair of the thoracic wall (15) and the oesophagus (16) in dogs. There are only a few reports on xenogenic grafts in ophthalmic surgery; these include split-thickness dermal grafts (17), equine pericardium (18), peritoneum (19), equine amniotic membrane (20), human amniotic membrane in rabbits (21), equine renal capsule (22), small intestinal submucosa (23) and expanded polytetrafluoroethylene (Gore tex) (24, 25). The ideal biomaterial for the repair of corneal defects should comply with strict specifications for optical clarity, support of epithelial migration and adhesion, permeability to solutions and resistance to corneal proteases (23, 26). These potential biomaterials include collagen, collagen-hydrogel copolymers, bioactive synthetics, and coated hydrogels (23, 26).

The purpose of this study was evaluate the canine renal capsule and costal pleura to repair lamellar corneal lesions in experimental rabbits.

MATERIALS AND METHODS

Fifteen New Zealand white male rabbits aged 8 months and weighing 2.5–3.0 kg were used in the experiment. All rabbits were handled in compliance with the Association for Research and Vision in Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic Research. Prior to acceptance for use, all rabbits underwent a baseline ocular examination to rule out pre-existing anterior segment abnormalities.

All surgical procedures were preceded by general anaesthesia. For premedication 0.05 $mg.kg^{-1}$ atropin (Atropin Spofa inj. 0.5 $mg.ml^{-1}$) was administrated s.c. and after 15 min we applied 5 $mg.kg^{-1}$ xylazin (Rometar 2 %, a.u.v., Spofa) together with 40 $mg.kg^{-1}$ ketamine i.m. (Narkamon 5 % Spofa, a.u.v.) in one syringe.

In order to study the postoperative course three groups of five animals were created. After keratectomy the renal capsule and pleura were sutured to the cornea in the first and the second group, respectively whereas in the third group the defect was covered with a third eyelid flap. In the postoperative period the rabbits received Azidamphenicol (chloramphenicol, Unimed) eye drops 4 times daily for 2 weeks.

Biological prosthesis

The canine renal capsule and costal pleura were preserved in 98 % glycerine. The biological prostheses were harvested from dogs that were euthanized for untreatable spinal diseases but were otherwise healthy. Both biological materials were preserved in a refrigerator at 5 °C for a maximum of 14 days. Prior to surgery the renal capsule as well as the pleura were rinsed with sterile saline.

Surgical technique

By means of corneal trephine with diameter of 6 mm keratectomy was carried out in the centre of the cornea into one half of the depth of the stroma. After trepanation the cornea was removed using the blade of a scalpel (size 11). The donor graft (renal capsule and costal pleura) had the size of 6.5 mm that was obtained by using the donor corneal trephine. Both xenografts were attached to the donor "bed" using single interrupted suture, non-absorbable material 10–0 (Ethilon).

In the third group the keratectomy defect was covered with a third eyelid flap for 14 days.

Clinical and morphological evaluation

Clinical observations were carried out on days 1, 3, 7, 15, 30 and 60. The following ocular signs were evaluated: blepharospasm/photophobia, ocular discharge, vascularization, corneal oedema and degree of healing (fluorescein test). For evaluation the following scale was used: (-) absence of sings; (+) light; (++) moderate and (+++) intense. On days 30 and 45 one rabbit of each group was euthanized, for histological examination. After termination of the experiment (day 60), all rabbits were euthanized and their eyes were submitted for histologically examination.

The samples for histology were processed using a standard procedure with fixation in 10 % neutral formaline and embedding in paraffin. Sections of 5–6 μm thickness were prepared and stained with haematoxylin-eosin.

The corneas of the control group and of those animals that received grafts were evaluated for vascular congestion, oedema, haemorrhage, infiltration of polymorphonuclear and mononuclear leukocytes as well as for vascularization and fibrosis from the grafts, the receptive area and its surroundings.

RESULTS

Clinical evaluation

The corneas of the "renal capsule" and the "pleura" groups retained the fluorescein stain around the graft till day 7 after surgery. In the control group evaluation of the fluorescein test was possible on day 14 after surgery because of covering the corneal defect by a third eyelid flap. The controls were still fluorecsein positive on day 15.

Blepharospasm/photophobia (Fig. 1) in the grafted corneas was moderate (++) in the first postoperative days following which the sings reduced until they completely disappeared 15 days after surgery. Ocular discharge (Fig. 2) was present in all groups and it was of moderate intensity (++) for the first three days. The intensity of discharge diminished in all groups (+) seven days after surgery, however, in the renal capsule and pleura group discharge was present up to day 30 after surgery.

HISTOLOGICAL PICTURES

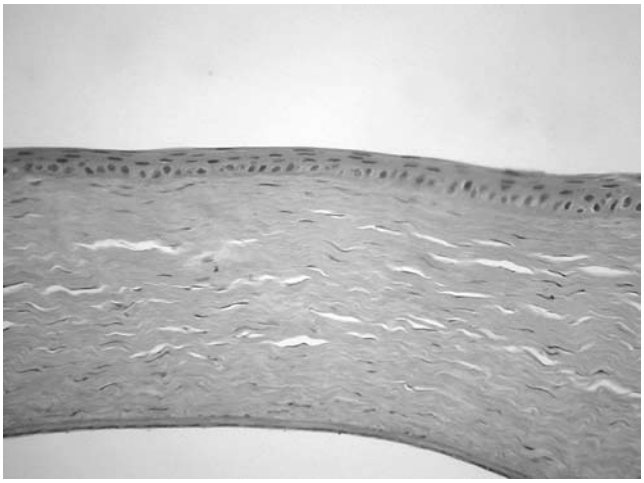


Fig. 5. Pleura 20 × – no changes in epithelium and corneal stroma, H&E on day 30

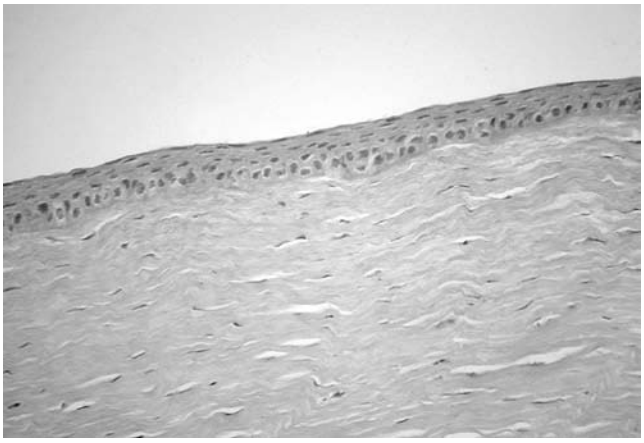


Fig. 6. Renal capsule 20 × – no changes in epithelium and corneal stroma, H&E on day 30

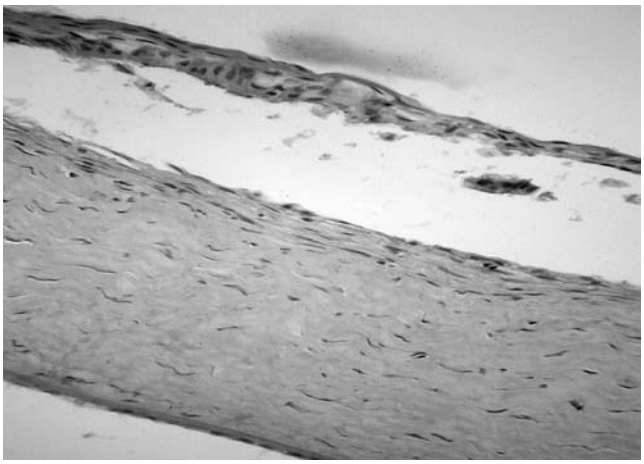


Fig. 7. Control 20 × – damaged epithelium (detachment) and fibrotisation, H&E day 30

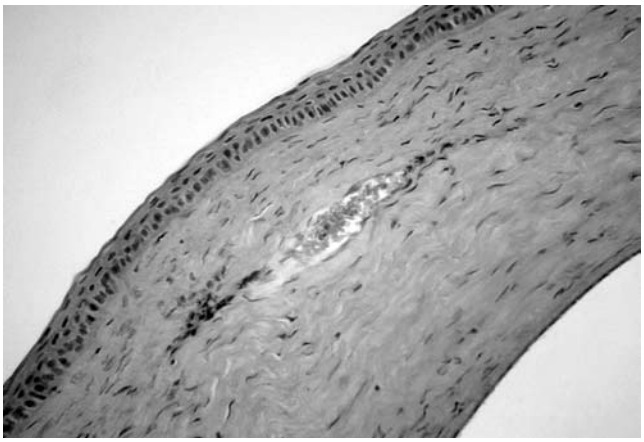


Fig. 8. Renal capsule 20 × – well developed epithelium, vascularisation with slight infiltration with macrophage, lymphocyte, fibroblast and fibrocyte (reparative process), H&E at day 60

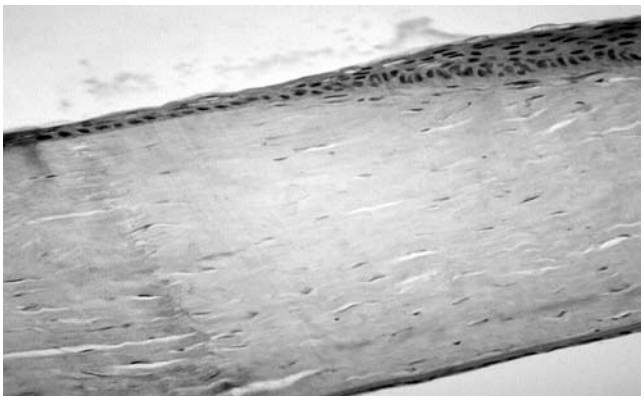


Fig. 9. Control 20 × – different thickness of epithelium, H&E on day 30

Fig. 10. Control 20 × – thickening of epithelial layers, H&E on day 60

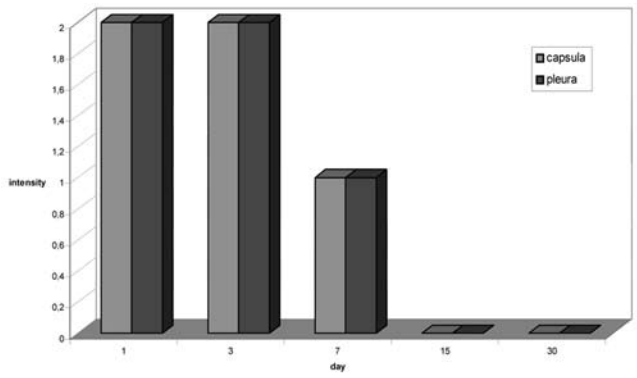


Fig. 1. Occurence of blepharospasm (intensity 1 = +, intensity 2 = ++)

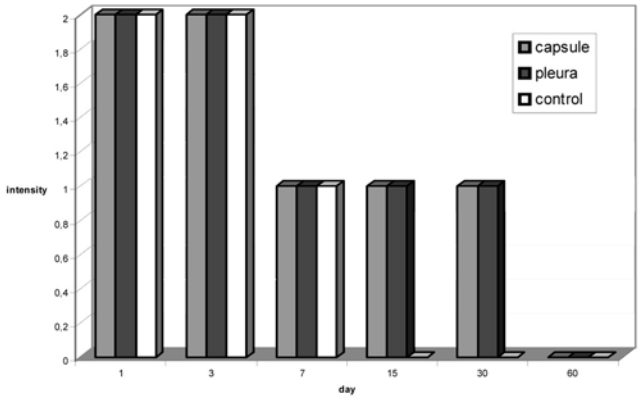


Fig. 2. Occurrence of discharge (intensity 1 = +, intensity 2 = ++)

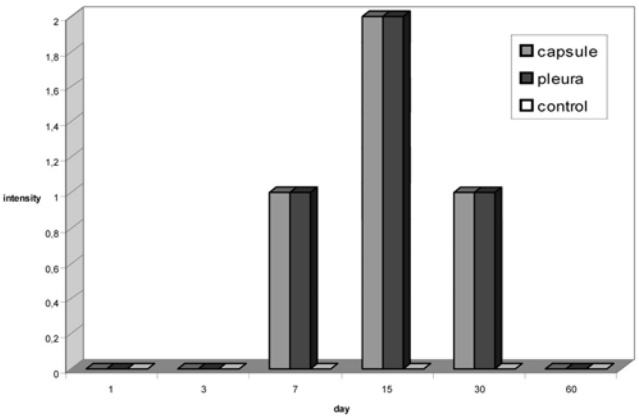


Fig. 3. Occurrence of vascularisation (intensity 1 = +, intensity 2 = ++)

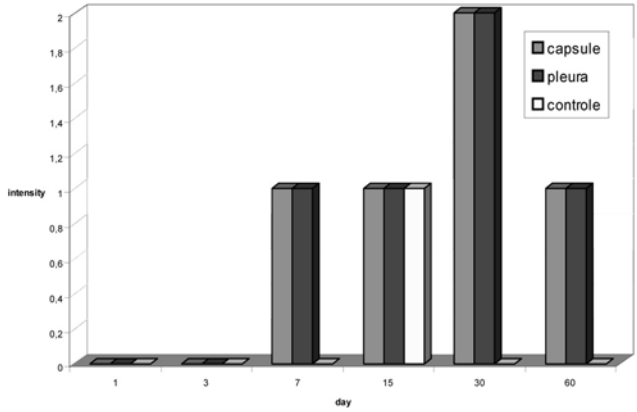


Fig. 4. Occurrence of oedema (intensity 1 = +, intensity 2 = ++)
The control group can be not expressed untill day 15 – up to this day the cornea was covered by the third eyelid. On day 30 and 60 control group has 0 value

Seven days after surgery, vascularization (Fig. 3) in the grafted groups was of low intensity (+) and superficial as well; the blood vessels were coming from the limbus and reaching the grafts. Vascularization intensified fifteen days after surgery in both keractemised groups (++). There was no vascularization in the control group. Both grafted groups showed mild corneal oedema on day 7 after surgery. Oedema (Fig. 4) was present in all groups 15 days after surgery but disappeared in the control group on day 30 after surgery. In the grafted groups a mild oedema persisted until termination of the follow up.

Histopathological evaluation

Histopathological findings on day 30 (one cornea of each group) after surgery showed slight detachment of the epithelial membrane, and flattering of epithelial cells and finally a different thickness of the epithelium in the controls groups (Figs. 7, 9). In the "pleura" group histology on day 30 after surgery (Fig. 5) revealed different thickness of the epithelium without changes in the deep part of the stroma. The "renal capsule" group showed different thickness of the epithelium (Fig. 6). On day 45 (one cornea of each group) postoperatively vascularization could be observed in both groups with grafts.

On day 60 (one cornea of each group) the following changes were observed in the control group: thickening of epithelial layers, detachment of epithelium and stromal fibrotization (Fig. 10). In the renal group vascularization was present as well as a small necrosis in the renal transplantation area (Fig. 8). The corneal surface was not damaged. A complete epithelial cell line was observed as well as "incorporation" of the renal capsule into the stroma. The group with the pleura graft displayed vascularization and mononuclear inflammation of the surface corneal layers, and fibrotic tissue with some Ly at the end of the experiment.

DISCUSSION

Many surgical procedures have been described in both veterinary and human ophthalmology that use various biologic and synthetic materials in order to re-

construct deep or imminent perforating corneal ulcers. Their therapeutic goal is to restore globe integrity, to stabilize the inner contents, to preserve vision, and to prevent endophtalmitis and glaucoma. In humans, corneal grafting may be performed to provide a clear optical axis for vision.

Reconstructive surgery successfully used many different biological mambranes in damaged organs. Peritoneum, pericardium, small intestinal submucosa, renal capsule as well as amniotic membrane have been successfully used both experimentally and clinically in the management of deep, perforating corneal injuries.

Partial corneal opacification that was present in both xenograft groups has been described in the management of corneal lesions using renal capsule, pericardium, peritoneum, amniotic membrane or porcine small intestine. In all patients the ocular fundus was visible. Blepharospasm/photophobia and ocular discharge were significant both in the "renal capsule" and in the "pleura group" and lasted for two weeks after surgery (discharge disappeared after 30 days). This finding probably resulted from epithelial nerve ending stimulation and/or reaction in the suture areas. The sutures were left *in situ* for the whole follow–up period. Vascularization of the cornea was present in both groups until seven days after surgery.

Corneal vascularization is beneficial in the early healing stages of ulcerative keratitis, however, excessive vascularization may cause ocular discomfort and corneal opacity. Vascularization together with graft size (more than 8 mm) and proximity to the limbus are important factors that adversely affect graft survival in penetrating keratoplasty (27).

Possible complications associated with the use of xenologous grafts after lamellar keratoplasty include postoperative infections and progression of the autolytic process caused by leukocyte collagenase or bacterial protease which may be present (28).

None of these complications was observed in our patients. Human medicine uses various solutions for storing corneal tissue like Mc Carey-Kaufman or Optisol (29, 30, 31), however, due to their price these solutions are not very wide-spread in veterinary medicine. Tissue storage in 98% glycerine for up to 30 days (18, 20) has been described as a good alternative; the latter was also used in our study.

Histopathological changes at the end of the follow-up period revealed thinned epithelium, in some cases epithelial detachment, and fibrotization of the stroma. The latter probably resulted from destruction of the epithelial basement membrane during lamellar keratoplasty. Inflammatory reaction of various degree was evidenced by the presence of mononuclear cells, macrophages and lymphocytes. Most of the inflammatory cells combined the morphological characteristics of macrophages/histiocytes phagocyting cellular debris (32). Histologically, fibrotization in the graft groups was proved from day 60 on. This finding coincided with that obtained when using amniotic membranes, pericards or small intestinal submucosa (23). Vascularization in the graft group pointed at some degree of graft rejection, with clinical and histological evidence indicating that the xenologous renal capsule and pleura might be useful in repairing lamellar lesions of the cornea.

According to the histological findings the epithelium was mainly found detached from the basal membrane in the control group. On the contrary, in the transplantation groups epithelial adherence to the basal membrane was improved, however, optical transparency of the cornea was decreased due to fibrotization and vascularization.

Xenologous transplants are always connected with the risk of transmitting infectious or viral diseases, and prevention of transplant rejection due to immunological reactions of the cornea also remains a question of great importance. Thus the use of xenologous transplants in ophthalmology provides several topics for further studies.

REFERENCES

1. Alvarenga, J., Barros, P. S. M., Iwasaki, M., Stopiglia, A. J., Guerra, J. L., 1986: Oesophagoplastie lors de l'ablation de nodules parasitaires chez le chien. *Le Point Vet.*, 17, 701–702.

2. Andrade, A. L., Laus, J. L., Figueirido, F., Blogg, J. R., 1999: The use of preserved equine renal capsule to repair lamellar corneal lesions in normal dogs. *Vet. Ophthalmol.*, 2, 79–82.

3. Barros, P. S. M., Safatle, A. M. V., Rigueiro, M., 1993: Xenologous pericardium as keratoprothesis in the dog (Abstract). *24th Ann. Meet. ACVO*, 23.

4. Barros, P. S. M., Safatle, A. V. M., Melabra, T. A., Dice, P. F., Wyman, M., 1993: Xenologous pericardium as a keratoprothesis in the dog. An experimental study (Abstract) *24th. Ann. Meet. ACVO*, 23.

5. Barros, P. S. M., Garcia, J. A., Laus, J. L., Dice, P. F., 1995: Preserved equine amniotic membrane used in the repair of the cornea of the dog. *Invest. Ophthalmol. Vis. Sc.*, 36, 982–985.

6. Barros, P. S. M., Garcia, J. A., Laus, J. L., Dice, P. F., 1998: The use of xenologous amniotic membrane to repair canine corneal perforation created by penetrating keratectomy. *Vet. Ophthalmol.*, 1, 119–123.

7. Brightman, A. H., McLaughlin, S. A., Brogdon, J. D., 1989: Autologous lamellar corneal grafting in dogs. *J. Am. Vet. Med. Assoc.*, 195, 469–475.

8. Fearstone, H. J., Sansom, J., Heinrich, Ch. L., 2001: The use of porcine small intestinal submucosa in ten cases of feline corneal disease. *Vet. Ophthalmol.*, 4, 147–153.

9. Garcia, J. A., Barros, P. S. M., Laus, J. L., Dice, P. F., 1996: Preserved homologous peritoneum graft after lamellar keratectomy in dogs. *Braz. J. Vet. Res. Anim. Scien.*, 33, 290–294.

10. Gasset, A. R., Hood, C. I., Ellison, E. D. 1970: Ocular tolerance to cyanoacrylate monomer tissue adhesive analogues. *Invest. Ophthalmol.*, 9, 3–11.

11. Gelatt, K. N., Gelatt, J. P., 1995: *Handbook of Small Animal Ophthalmic Surgery*, Vol. 2: *Corneal and Intraocular Procedures*. Pergamon, New York.

12. Giminez, M. T. P., Farina, I., 1998: Lamellar keratoplasty for the treatment of feline corneal sequestrum. *Vet. Ophthalmol.*, 1, 162–166.

13. Hacker, D., 1991: Frozen corneal graft in dogs and cats: a report on 19 cases. *J. Am. Anim. Hosp. Assoc.*, 27, 387–398.

14. Hakanson, N., Meredith, R. E., 1987: Conjunctival pedicle graft in the treatment of corneal ulcers in the dog and cat. *J. Am. Anim. Hosp. Assoc.*, 23, 641–648.

15. Hakanson, N., Lorimer, D., Meredith, R. E., 1988: Further comments on conjunctival pedicle grafting in the treatment of corneal ulcers in dog and cats. *J. Am. Anim. Hosp. Assoc.*, 24, 602–605.

16. Hansen, P. A., Guandalini, A., 1999: A retrospective study of 30 cases of frozen corneal grafts in dogs and cats. *Vet. Ophthalmol.*, 2, 233–241.

17. Kern, T. J., 1990: Ulcerative keratitis. *Vet. Clin. N. Am. Small Anim. Pract.*, 20, 643–666.

18. Kim, C. J., Tseng, S. C. G., 1995: Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea*, 14, 473–484.

19. Legeais, J. M., Renard, G. D'Hermies, F., Kaufman, H. E., 1991: Surgical management of corneal perforation with expanded polytetrafluoroethylene (Goretex). *Ophthal. Surg.*, 22, 213–217.

20. MacCarey, B. E., Kaufman, H. E., 1974: Improved corneal storage. *Invest. Ophthalmol.*, 13, 165–173

21. Maurellio, J. A. Jr., Pokorny, K., 1993: Use of split-thickness dermal grafts to repair corneal and scleral defects – a study of 10 patients. *Brit. J. Ophthalmol.*, 77, 327–331.

22. Mochos, M., Droutsas, D., Boussalis, P., Nichols, B. D., 1997: Clinical experience with cyanoacrylate tissue adhesive. *Doc. Ophthalmol.*, 93, 237–245.

23. Parshall, C. J., 1973: Lamelar corneal-scleral transposition. *J. Am. Anim. Hosp. Assoc.*, 9, 270–277.

24. Scagliotti, R. H., 1988: Tarsoconjunctival island graft for the treatment of deep corneal ulcers, descemetoceles and perforations in 35 dogs and 6 cats. ***Seminars in Vet. Med. and Surg. (Small Animal)***, 3, 69–76.

25. Stopiglia, A. J., Alvarenga, J., Barros, P. M. S., Guerra, J. L., Iwasaki, M., 1986: Reparation chirurgicale de la paroi thoracique du chien. *Le Point Vet.*, 18, 239–243.

26. Thompson, K. P., Hanna, K., Waring, G. O., Nobe, J. R., 1991: Current status of synthetic epikeratoplasty. *Refract. Corn. Surg.*, 7, 240–248.

27. Webster, R. G., Slansky, H. H., Refojo, M. F., Wyman, M. K., 1968: The use of adhesive for the closure of corneal perforation: report of two cases. *Arch. Ophthalmol.*, 80, 705–709.

28. Whittaker, C. J. G., Smith, P. J., Brooks, D., Wilson, S. E., 1997: Therapeutic penetrating keratoplasty for deep corneal stromal abscesses in 8 horses. *Vet. Comp. Ophthalmol.*, 7, 19–28.

29. Wilkie, D. A., Wolf, E. D., 1991: Treatment of epibulbar melanocytoma in a dog using full-thickness eye wall resection and synthetic graft. *J. Am. Vet. Med. Assoc.*, 198, 1019–1022.

30. Wilkie, D. A., Whittaker, C. J. G., 1997: Surgery of the cornea. *Vet. Clin. N. Am.: Small Anim. Pract.*, 27, 1067–1107.

31. Williams, K. A., Roder, D., Esterman, A., Bourne, W. M., 1992: Factors predictive of corneal graft survival (Reports from the Australian Graft Registry). *Ophthalmol.*, 99, 403–414.

32. Yau, C. W., Kaufman, H. E., 1986: A medium-term corneal preserving medium (K-Sol). *Arch. Ophthalmol.*, 104, 598–601.

Received October 22, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 162–168, 2007

THE USE OF CHONDROCYTE IMPLANTS FOR REPAIR OF ARTICULAR CARTILAGE DEFECTS IN RABITS

Horňák, S., Ledecký, V.

University of Veterinary Medicine, Komenského 73, 041 81 Košice
The Slovak Republic

hornak@uvm.sk

ABSTRACT

The aim of the study was to verify the *in vivo* survival of cultured chondrocytes without periosteal flap coverage, used in the form of fibrinous suspension of chondrocytes or solid kol I-HYA chondrograft as a replacement of the damaged segment of articular cartilage by biologically and mechanically full-value tissue. The study was carried out on 9 rabbits. In the first stage, under general anaesthesia with Narcamon, after Rometar pre-medication, a deep chondral defect was created in the medial femoral condyle. The cartilage harvested at the time of defect creation was used for expanding chondrocytes *in vitro* and for preparation of a chondrocyte implant. After its preparation the second arthrotomy was performed involving insertion of an implant into the defect artificially created on articular surface. The repair of the chondral defect was investigated in three experimental groups of rabbits, each consisting of three rabbits: Group 1: repair of the chondral defect by autologous *in vitro* cultured chondrocytes with l I-HYA membrane, Group 2: repair of the chondral defect by suspension of autologous *in vitro* cultured chondrocytes which were "introduced" immediately before application into the fibrin glue Beriplast® (Centeon Pharma GmbH) and overlaid with a kol I-HYA membrane, Group 3: no specific repair of artificially produced chondral defect was attempted in these animals. The animals were euthanized in 3 stages according to a predetermined time schedule: 3, 6 and 12 months following the defect treatment, and a portion of the joint with an implant was withdrawn for subsequent histological and histochemical examination. Both types of implants used for the repair of the damaged cartilage segment showed a stimulative effect on production of articular hyaline cartilage in the place of the defect. The chondrocytes were capable of proliferation and secretion of proteoglycans at both treatment modalities.

Key words: articular cartilage; chondral defect; chondrocyte implants

INTRODUCTION

Articular hyaline cartilage is a specialised connective tissue important for distribution of pressure and sheer forces within joints with a specific role in animal locomotion (1). Articular cartilage is known for the absence of perichondrium, it is avascular, alymphatic and aneural (2).

Articular cartilage has very limited self-regeneration ability. The repair of articular cartilage lesions is insufficient. The response of the normal articular cartilage to damage or arthrotic degeneration is frequently partial and repair of the damaged tissue is incomplete. Surface defects of articular cartilage do not heal spontaneously and usually turn to extensive degeneration (9). Specialised cartilage cells (chondrocytes) synthesize components of intercellular matter. It was observed that despite limited ability of chondrocytes to migrate from marginal zones into the defective site, they cannot multiply in these locations and produce macromolecules needed for repair of the organised intercellular matrix typical of normal articular cartilage (11).

Cartilage properties result from its microscopic structure. Detailed studies of morphology and biology of mature cartilage showed that it is a highly organised structure. Complex connections between chondrocytes and the matrix are maintained

actively and are responsible for preservation of the shape and function of the tissue owing to the structural character of the solid component (collagen, proteoglycans and glycoproteins). This means that the tissue that would reconstruct correctly the damaged surface of articular cartilage should replicate these structures very precisely so as to sustain the same load the physiological cartilage can withstand.

The present bioengineering technologies offer new possibilities for repair of cartilage defects. Transplantation of autologous chondrocytes, allogenic chondrocytes, perichondral grafts and biodegradable membranes are the methods that have been tested experimentally and clinically. An advantage of transplantation of autologous chondrocytes is that it can presumably induce repair of defects by full-value hyaline cartilage. The beginnings of experiments on animals involving the culturing of chondrocyte cells date back to the sixties of the past century (4, 10). A method described in 1989 used an animal model (7). Chondrocytes cultured in collagen gels produced a three-dimensional network. Some authors (1, 12) used implants obtained by culturing experimental chondrocytes in collagens. They evaluated the three-dimensional (3-D) culturing as a method suitable for preparation of solid, transplantable matrices.

Mature chondrocytes are harvested by biopsy of articular cartilage. Freshly isolated chondrocytes preserve their original phenotype during monolayer cultivation for several days up to weeks (11). In case of longer cultivation and sub-cultivation, the cells tend to change production of collagen of type II to types I and III. Their original phenotype changes. The original phenotype is preserved at 3-D cultivation (2).

MATERIAL AND METHODS

Animals. The experiment was carried out on 9 domestic rabbits. They were assigned at random to three groups and subjected to different treatment of artificially created chondral defect. Each group consisted of three animals:

Group 1: repair of the chondral defect by autologous *in vitro* cultured chondrocytes with a kol I-HYA membrane.

Group 2: repair of the chondral defect by suspension of autologous *in vitro* cultured chondrocytes which were "inserted" immediately before application into a fibrin glue Beriplast® (Centeon Pharma GmbH) and overlaid with a kol I-HYA membrane.

Group 3: no specific repair of the artificially produced chondral defect was attempted.

Experimental procedure. Each rabbit underwent two operations except for rabbits in the control group 3 which were operated only once to create a deep chondral defect. All operations were performed under general anaesthesia. The anaesthesia was initiated with a combination of intramuscularly administered Narkamon (ketamin hydrochloride) 1 % inj. (Léčiva, CZ) at a dose of 40 mg.kg^{-1} and Rompun 2 % (xylasin hydrochloride) amp. (Bayer, Turkey) at a dose of 4 mg.kg^{-1}. In the subsequent phase, animals were anaesthesiszed by inhalation of 3 % mixture of Isofluran (Baxter) and air through a modified mask, under sterile conditions and with coverage by Neloren inj. (Lek, Slovenia) at a dose of 0.5 ml.

The first operation was performed after 2-week quarantine. During arthrotomy of the knee joint a sample of articular cartilage was harvested and an artificial chondral defect was created which was then treated in the second stage of the experiment. In each rabbit we operated only one pelvic limb at the knee joint. A lateral parapatelar approach was used to gain access to the femoral medial condyle. After careful luxation of the patella a deep chondral defect was created at the medial femur condyle using a metal puncture needle (Chirana, SR). The cartilage harvested at defect creation was used for *in vitro* cultivation of chondrocytes. While creating the desired defect we made an effort to prevent its penetration into the vascularised subchondral zone so it reached maximally the zone of calcified cartilage. Exposure of the vascularised layer and the associated discharge of blood could induce a non-specific healing process. After reposition of the patella, the operation wound was closed in three layers.

The second operation was performed after the successful expanding of chondrocytes and preparation of the chondrocyte graft in the Associated cell bank (ZTB) LF UPJŠ and FNsP Košice, after 20 days on average from harvesting the cartilage (18–23 days). This operation focused on repair of the artificial chondral defect and was performed on 6 rabbits from Groups 1 and 2. The operation was performed under identical conditions, using the same procedure as in the first operation, i. e. lateral parapatellar arthrotomy in the original scar. After gaining access to the medial femoral condyle, the chondral circular defect was adjusted (deepened or enlarged) if necessary to ensure that its diameter was 3 mm on average. Its depth ranged between 1 and 1.5 mm so it just about reached the surface of the calcified zone. This way prepared deep defect was treated subsequently using one of the procedures described below:

Group No. 1: The defect was filled with an autologous 3D kol I-HYA chondrograft. The chondrograft was fixed to the defect base and the surrounding chondrium by a two-part fibrin glue Beriplast® (Centeon Pharma GmbH). The diameter of chondrograft was approx. 3 mm and its thickness 1–1.5 mm, which corresponded to the size, shape and depth of the defect. The chondrograft was inserted in a way so as the surface with predominance of chondrocytes was directed downwards toward the defect base and the surface with predominance of collagen fibres was oriented upwards. In this way the layer with predominance of collagen fibres formed articular surface and the layer with predominance of cells the deep chondral zone.

Group No. 2: The deep chondral defect was filled with a suspension of autologous culture-expanded chondrocytes in a fibrin glue Beriplast®, overlaid with a covering collagen I-HYA membrane. The membrane was 0.3–0.4 mm thick and its shape corresponded to that of the chondral defect. The edges of the covering membrane were fixed on the surface by a thin layer of pure fibrin glue Beriplast® without admixture of chondrocytes. This fixation of the junction with chondrium should prevent early detachment of the membrane.

Group No. 3: This was the control group consisting of 3 rabbits which underwent only one operation that produced a deep chondral defect and this defect was left to heal spontaneously without any further treatment.

After the operation, the limb was immobilised by a strengthened elastic bandage. The rabbits tolerated this limb immobilization very well.

The animals were euthanized in 3 stages according to the predetermined schedule: 3, 6 and 12 months after treatment of the defect. In the specified intervals one rabbit from each group was sacrificed by intrapulmonary administration of 2 ml of preparation T 61. The tissue withdrawn from the animals was fixed in 10 % formaldehyde before additional processing.

Evaluation. Evaluation of the experiment was based on several parameters which reflected successfulness of the repair. The time needed for the limb to sustain full load and for recovery of the original gait stereotype was evaluated clinically.

The principal part of evaluation focused on histological examination of the obtained samples. The osteochondral samples were subjected to preliminary processing and decalcification in Chelaton (Bioptika, Italy). This way prepared material was cut with a microtome to obtain 15 μm series of thin section. The sections were stained with haematoxylin-eosin, safranin O, and monoclonal antibody to human collagen II with cross-reactivity to rabbit collagen type II. The microscopic evaluation of thin sections was qualitative (character of tissue in repair location) and semi-quantitative (extent of repair and relative number of cells in repair location).

RESULTS

Locomotory activity. Despite repeated arthrotomy none of the animals showed complications in terms of patella luxation, septic arthritis, knee joint exsudate, dehiscence or surface infection of wounds. After the operation we observed that the rabbits spontaneously put less weight on the operated limb for approximately 10–14 days. The locomotory stereotype, full load and extent of locomotion recovered completely by the end of the second week. The standard evaluation of locomotion outside the cage, carried out on day 14 after the treatment of the chondral defect, showed no signs of minimizing the use of the operated limb in comparison with the non-operated one.

Histological findings. 15 μm series of thin sections were stained by the standard procedure with haematoxylin-eosin (HE), specific stain for proteoglycans with safranin O (both staining products were evaluated under a light microscope) and monoclonal anticollagen II antibody (fluorescence evaluation). In the subsequent paragraphs we present description of histological findings in rabbits from respective groups. In Group No. 1 (Fig. 1) in the location of the defect repair by autologous kol I-HYA chondrograft we observed predominance of hyaline-like cartilage tissue (HyLCa), particularly in rabbit No. 3. In rabbits No. 1 and 2 we observed foci of HyLCa (located in deep layers of repair focus) and sporadic segments of fibrin-like cartilage tissue (FiCa) 3 and 6 months after the treatment. This involved particularly the surface zones of chondrografts with predominance of collagen I membrane fibres and due to its spindle shape the cellular population resembled more the fibroblasts. However, antibody staining of anticollagen II confirmed preservation of the chondrocyte phenotype also in the surface layer.

Attachment of reparative tissue to the original deep chondrium layer or zone of calcified cartilage was sufficiently strong which prevented detachment of chondrografts while cutting the thin layers. After 12 months following the treatment, when preparing thin sections from samples withdrawn from rabbit No. 3, we observed a partial detachment at the edges of the chondrograft, however, in the central zone the attachment of chondrograft was strong without any tendency to separate during the cutting.

Staining with safranin O revealed relatively high production of PG, particularly in the deep zones, and smaller intensity of staining in the surface zones of regeneration. In rabbit No. 3 the intensity of staining with safranin O was high even in surface zones except for a focus of weakened adherence to the surrounding cartilage. Cells in the deep layer were round or polygonal. The density of cells in the surface and articular zones was lower and production of PG was restricted more to the pericellular zone. Such formation of layers resembled very much the layers of healthy, undamaged cartilage.

Staining with rabbit anticollagen II antibody verified the chondrocyte phenotype of cells in the focus of implanted chondrograft. The density of cells was comparable with that in the normal cartilage and there were sporadic locations with even higher cellular density. The surface of chondrograft was smooth, continuous and showed no signs of degeneration even after one year after implantation. Complete filling of the defect was observed in rabbits No. 1 and No. 3 although in rabbit No. 1 we detected slight symptoms of hypertrophy in one segment resulting probably from insertion of primary thicker chondrograft in the stage of defect reparation. In rabbit No. 2, in the marginal zone, we observed a detachment of the surface portion of chondrograft, caused most likely by damage to the chondrograft during manipulation and insertion into the defect focus. However attachment of chondrograft to the surrounding chondrium as well as to the base was strong and no separation of chondrograft during cutting of respective sections was observed. On the whole, repair of the defects in rabbits No. 1 and 2 was evaluated as very good and that in rabbit No. 3 as excellent.

Very good results were obtained in Group 2 of rabbits in which we treated the defect by cellular suspension of autologous chondrocytes overlaid with kol I-HYA membrane (Fig. 2). In two rabbits (No. 5 and 6), 6 and 12 moths after the treatment, the defect was filled completely, the articular surface was smooth and showed no signs of hypertrophy. In the focus of the applied cellular population, we observed clear signs of repair with considerable proportion of cellular population of chondrocytes. In the deep zones, the chondrocytes were round or polygonal and their dimensions almost reached those of chondrocytes of normal cartilage. The surface

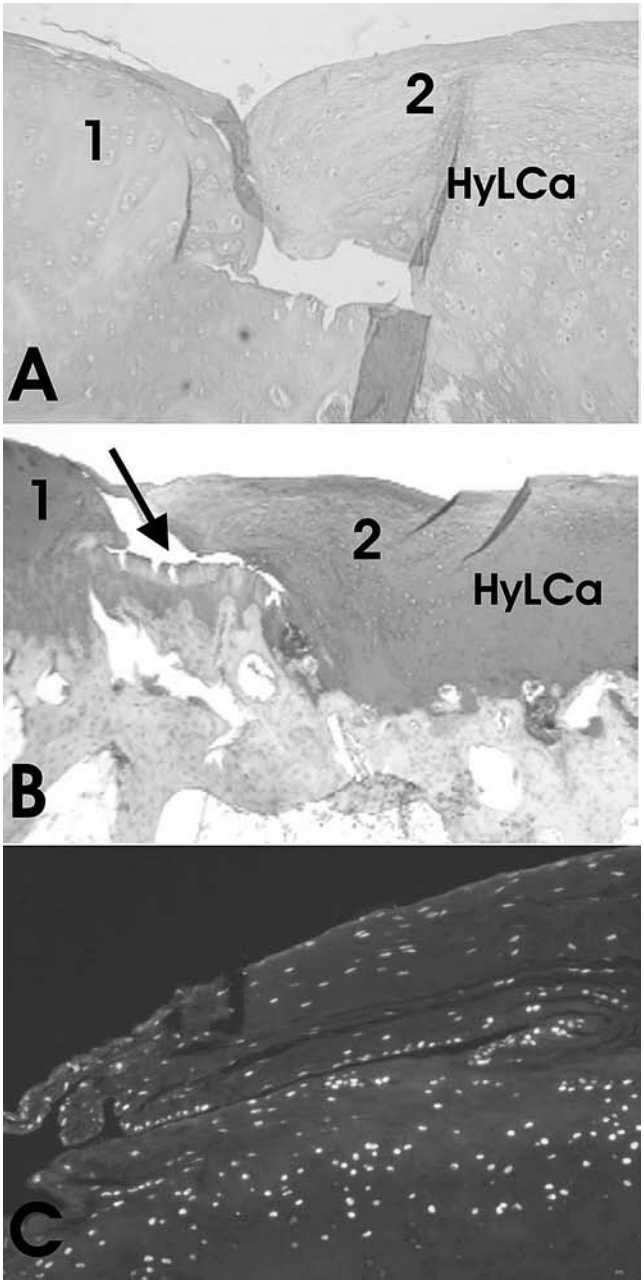


Fig. 1. Rabbit No. 3, an autologous kol I-HYA chondrograft, 12 months after repair. Cross-section of the focus of defect repair showing the marginal zone of original chondrium

HE staining, 150× magnification (1 – original articular cartilage, 2 – chondrograft) (A). Staining with safranin O, 100× magnification (1 – original articular cartilage in the zone of weakened adhesion to the original chondrium, 2 – numerous cellular population and formation of HyLCa-resembling tissue (hyaline-like cartilage tissue), the arrow points to the weakened adhesion to the original chondrium) (B). Staining with anticollagen II antibody HE, 200× magnification under fluorescent light (population of transplanted chondrocytes is coloured light green) (C)

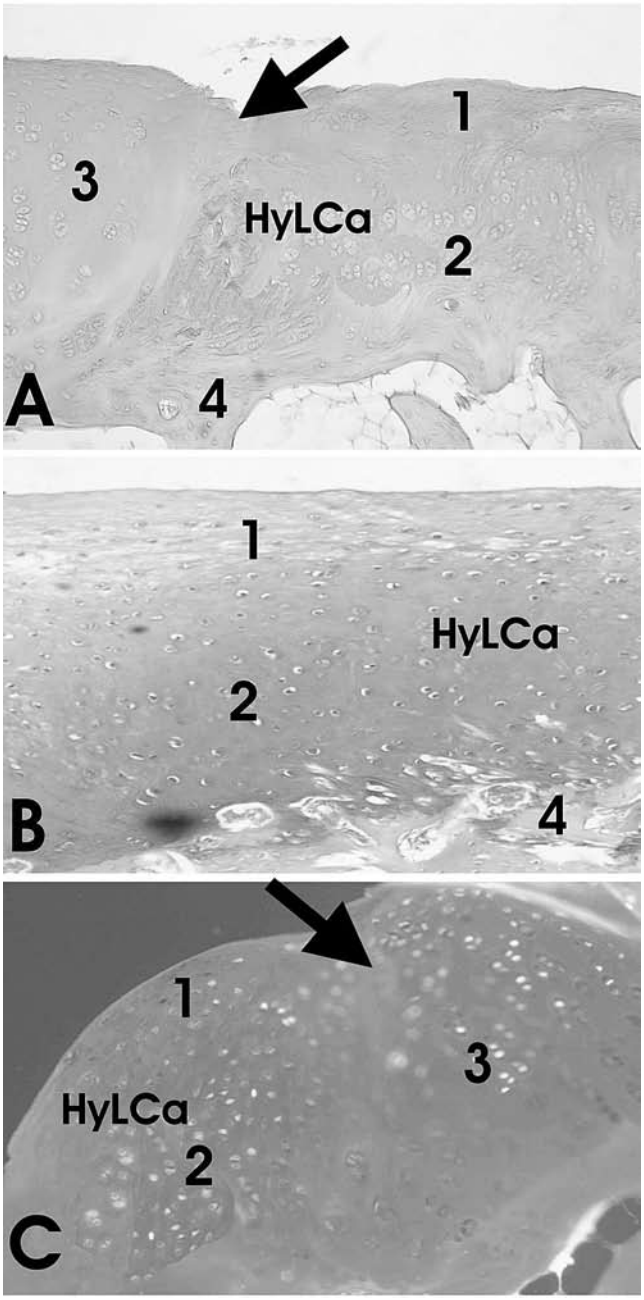


Fig. 2. Rabbit No. 6, cross-section in the place of defect repair by suspension of autologous chondrocytes in fibrin glue Beriplast® covered by a kol I-HYA membrane, 12 months after repair of the chondral defect

HE staining, magnification 100× (A). Safranin O stain, magnification 150× (central zone of repair focus with formation of HyLCa and high production of PG in surface zone and particularly in the deep layer (B). Staining with anticollagen II antibody HE, magnification 150× – picture under fluorescent light (numerous population of transplanted chondrocytes is coloured light green) (F)

1 – articular surface and surface zone of suspension chondrograft – covering kol. I-HYA membrane, 2 – deep zone of suspension chondrograft, 3 – deep layer zone of original chondrium, 4 – subchondral bone, the arrows indicate the location of continuous attachment to the original chondrium proceeding to the deeper layers

zones showed prevalence of fibres of the covering collagen I membrane the basis of which was infiltrated with population of smaller cells predominantly of ovoid shape. Production of proteoglycans was very high and in the deep repair zones reached the level of normal cartilage. The tissue in the repair focus corresponded almost completely to the hyaline cartilage.

The activity of staining for PG was somewhat lower in the surface zones, particularly at repair edges, but in the centre was very high. The surface of the repaired defect was smooth and continuously turned into surrounding chondrium. The attachment to the edges and base of the original chondrium was continuous and strong and during cutting of sections no detachment of chondrograft from the base was observed. Staining with anticollagen II antibody proved the presence of numerous cellular populations of chondrocyte phenotype.

In rabbit No. 4, evaluated 3 months after defect repair, we observed a detachment of the covering kol I-HYA membrane and the defect base was covered by a thin layer of whitish tissue. Histological sections proved that these were the residua of fibrinous suspension of chondrocytes. Preservation of chondrocyte phenotype of cells was confirmed by sections stained with anticollagen II antibodies. The defect base contained relatively large number of ovoid and spindle-shaped cells with high affinity to anticollagen II antibodies. Production of proteoglycans was confirmed in the defect focus although with low activity. The results obtained in Group 2 were evaluated as excellent in two rabbits (No. 5 and 6) and satisfactory in one rabbit (No. 4).

The deep chondral effect created in rabbits of the control Group No. 3 was allowed to heal spontaneously. The defect was not filled in neither of the two evaluated animals, even after 12 months. The rabbit No. 7, which died 3 weeks after the first operation, was not included in the evaluation (short interval). After 6 months, the deep chondral defect crated artificially in rabbit No. 8 still persisted without any signs of repair of defect base or edges. After 12 months, in rabbit No. 9 in which subchondrium had been exposed partially during creation of the defect, an inducement of a non-specific defect repair was observed in its central part, manifested by formation of a fibrinous tissue. Only a thin layer of tissue was produced so it could not reach the level of the surrounding chondrium. There were signs of only partial repair with irregular and defective articular surface. There were no indications of production of proteoglycans or presence of cells with affinity to anticollagen II antibodies in the fibrinous tissue. Metabolic activity of the original cartilage in the zone adjacent to artificial defect indicated low presence of PG and total regressive changes (acellular zones, irregularities and defibering of the surface layer).

On the whole, repair in the control group was evaluated as unambiguously insufficient in rabbit No. 8 and as satisfactory in rabbit No. 9.

DISCUSSION

In the recent years 3D implants came into limelight in the field of transplantation of chondrocytes (13, 16, 17). On the basis of our previous experience we decided to conduct a pre-clinical experiment to validate and modify subperiosteal application of culture-expanded autologous chondrocytes employing the 3 D system.

After re-evaluation of available information, advantages and disadvantages of individual types of matrices used for preparation of 3D chondrografts, and up-to date experience from clinical applications and animal models (12, 14, 15, 17), we selected two procedural modalities. The first involved the suspension form of fibrinous chondrograft which enabled direct application, sufficient adhesion and good possibility of formation of the implant. It also constituted a suitable degradable medium for implanted chondrocytes and the preserved fixation properties of fibrin glue ensured more or less sufficient fixation of the overlaying collagen membrane. The second approach was based on the use of solid, *in vitro* created collagen I-HYA chondrograft, fixed with fibrin glue. This involved a 3D implant allowing one to perform direct reconstruction in the place of deep chondral defect.

We used an available matrix consisting of collagen type I spatial network as the main component which was supplemented with hyaluronan. The collagen fibres provided a suitable spatial construction for proliferation and retention of chondrocytes. Hyaluronan served as a component of natural environment of chondrocytes and as a nutritive component.

The aim of the experiment was to verify *in vivo* the survival of culture-expanded chondrocytes without periosteal flap coverage, when used in the form of fibrinous suspension of chondrocytes or solid kol I-HYA chondrograft. Both approaches have their own advantages and stumbling blocks. The advantage of the solid kol I-HYA chondrograft consists in easier manipulation in the relatively small space of domestic rabbit knee joint. After formation of the implant into the required shape, its fixation by rapidly solidifying material Beriplast® was relatively simple. On the other hand it was necessary to manipulate with the implant very carefully during its formation to prevent its deformation or breakage. Damage to the chondrograft during its formation was most likely the reason for its partial detachment and incomplete filling of the defect in rabbit No. 2.

A disadvantage of the suspension form was the partial weakening of adhesive properties of the fibrin glue Beriplast® due to dilution of the fibrinogenous component by suspension of cultured chondrocytes despite the fact that the cellular component was concentrated in the smallest possible volume of culturing medium (50 µml). Because of the reduced adhesiveness of such a way diluted glue we strengthened fixation of the covering membrane from on surface side by a layer of pure, unmixed fibrin glue at the expense of slight prominence of the repair

site compared to the surrounding cartilage. Formation of the covering membrane was simple as well as overlaying of the defect after its filling with the chondrocyte suspension. Reduced adhesion and the mentioned discrete prominence were probably the reasons for detachment of the covering collagen membrane, observed in rabbit No. 4. Despite that a thin layer of fibrinous material with cells of chondrocyte phenotype was present at the defect base. In the place of application of chondrocyte suspension we observed formation of hyaline-like cartilage tissue with structure similar to that of the deep zone of normal chondrium. Distribution of cells was uniform and acellular zones were more or less an exception. The presence of chondrocytes in the covering membrane proved that the cellular suspension was capable of absorbing into the spongiform structure of the covering membrane before solidification of the fibrin material. The presence of chondrocytes in the covering membrane also indicated good ability of chondrocytes to adhere to the fibres of collagen I and proliferate further in this particular environment. Similar good experience with fibrografts was described also by other authors (8, 13, 17).

When using the kol I-HYA chondrografts, the surface layers of repair focus showed persistence of collagen network, with relatively thick fibres of collagen I running parallel with the articular joint surface. The fibres showed no signs of degradation or rebuilding even after 12 months. However, even after this time, the preservation of chondrocyte phenotype of cells was obvious. Histological sections showed presence of sporadic acellular zones but, on the whole, the distribution of chondrocytes was more uniform and total cellularity and metabolic activity were high. Formation of hyaline-like cartilage tissue was observed particularly in the deep layers.

Although rabbits were a suitable model with regard to manipulation and keeping, they were not optimal animals for evaluation of repair of the chondral defect. This was because of relatively small dimensions of rabbit knee joints and relatively thin layer of chondrium which was frequently not thicker than 1 mm. This was exactly the aspect that could contribute the most to the detachment of chondrografts in rabbit No. 2. Despite that, because of retaining the chondrografts in the other cases and formation of hyaline-like cartilage tissue, we concluded that both procedures appeared prospective for introduction into clinical practice.

CONCLUSION

The results of our experiment allowed us to conclude that both methods of repair of the deep chondral defect based on the autologous approach represented a very successful way of treatment. The differences between the groups with reparation by a solid kol I-HYA chondrograft and fibrinous suspension of chondrocytes applied below the kol I-HYA membrane were not unambiguous. With both treatment modalities, the chondrocytes were able to proliferate and secrete proteoglycans. In both cases the focus of repair of the chondral defect showed evident signs of repair and formation of hyaline-like cartilage tissue. The fibrin glue ensured sufficient fixation of chondrograft when it was applied undiluted. Dilution of glue with concentrated cellular suspension of chondrocytes reduced partially its fixative abilities. In clinical use, with regard to sufficient strength of the membrane used, its adhesion to surrounding chondrium could be strengthened by several fixation stitches.

We are aware of the fact that the small number of experimental animals in groups (altogether 9 rabbits, 1 excluded because of early death) was a weak point of this experiment which also prevented a more detailed statistical evaluation. Despite the limited sources we believe that we succeeded in confirming the suitability of both methods for therapy of knee joint defects.

Survival of cells at simultaneous preservation of chondrocyte phenotype and formation of hyaline-like cartilage tissue even after 12 months justifies our expectation of good clinical results in long-term horizons in the treatment of more extensive chondral defects on articular surfaces of animals.

REFERENCES

1. Adam, A., Pohunková, H., Klézl, Z., Pešáková, V., Čech, O., 1997: Use of bioimplants to replace cartilage. Part II: application of implants in animal experiments. *Acta Chir. Orthop. Traum. Čech.*, 64, 207–210.

2. Benya, P. D., 1982: Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels cell. *Cell*, 30, 215–224.

3. Brittberg, M., Lindahl, A.,1994: Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *New England J. Med.*, 331, 889–995.

4. Campbell, C. J., 1963: The transplantation of articular cartilage: an experimental study in dog. *J. Bone Jt. Surg.*, 45, 1579–1592.

5. Engvist, O., 1979: Reconstruction of patellar articular cartilage with freee autologous perichondral grafts: an experimental study in dogs. *Scand. J. Plast. Reconstr. Surg.*, 13, 361–369.

6. Grande, D. A., Breitbart, A., 1999: Cartilage tissue engineering: current limitations and solutions. *Clin. Orthop.*, 367, 176–185.

7. Grande, D. A., Pitman, M. I., 1989: The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. *J. Orthop. Res.*, 7, 208–218.

8. Grigolo, B., Roseti, L., Fiorini, M., Fini, M., 2001: Transplantation of chondrocytes seeded on a hyaluronan derivative (hyaff-11) into cartilage defects in rabbits. *Biomaterials*, 22, 2417–24.

9. Hangody, L., Kish, G., Kárpáti, Z., Szerb, I., Udvarhelyi, I., 1997: Arthroscopic autogenous osteochondral mosaicplasty for the treatment of femoral condylar articular defects. A

preliminary report. *Knee Surg. Sports Traumatol. Arthrosc.*, 5, 262–267.

10. Manning, W. J., Bonner, W. M., 1967: Isolation and culture of chondrocytes from human articular cartilage. *Arthritis Rheum.*, 10, 235–239.

11. Morgan, J. R., 1998: *Tissue Engineering Method and Protocols*. Humana Press, New Jersey, 173–215.

12. Pešáková, V., Adam, A., 1997: Use of bioimplants to replace cartilage. Part I: chondrocyte cultivation in three-dimensional gel. *Acta Chir. Orthop. Traum. Czech.*, 64, 201–206.

13. Plonczak, M., Czubak, J., Kawiak, J., 2006: Repair of articular cartilage defects with cultured chondrocytes on polysulphonic membrane: experimental studies in rabbits. *Transplant. Proc.*, 38, 320–322.

14 Rosocha, J., Vaško. G., Bačenková, D., Švihla, R., 2000: Three dimensional graft of autologous and allogenic chondrocytes for cartilage defect repair. *Book of Abstracts 3rd CEOC*, Portorož, Slovenia, 107.

15. Rotter, N., Aigner, J., Maumann, A., 1999: Behavior of tissue-engineered human cartilage after transplantation into nude mice. *J. Mat. in Med.*, 10, 689–693.

16. Vaško, G., Rosocha, J., Švihla, R., Bačenková, D., 2000: Combined method of cartilage defect reparation with osteochondral allografts and autologous chondrocytes in tissue glue. *Book of abstracts 3rd CEOC*, Portorož, Slovenia, 125.

17. Willers, C., Chen, J., Wood, D., Xu, J., Zheng, M. H., 2005: Autologous chondrocyte implantation with collagen bioscaffold for the treatment of osteochondral defects in rabbits. *Tissue Eng.*, 11, 1065–1076.

Received February 25, 2008

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 169–174, 2007

LABORATORY EVALUATION OF SANATIVE PAIR OF DIMINAZENE ACETURATE AND ISOMETAMIDIUM CHLORIDE AS COMBINATION THERAPY FOR ANIMAL TRYPANOSOMOSIS

Saba, A. B., Adedapo, A. A.
Oyagbemi, A. T., Odudu, Z. K.

Department of Veterinary Physiology, Biochemstry and Physiology
University of Ibadan, Ibadan
Oyo State – Nigeria

sabadee2000@yahoo.com

ABSTRACTS

This study investigated the efficacy of sanative pair of isometamidium chloride and diminazene aceturate in rabbits experimentally infected with *Trypanosoma brucei brucei*. Results show that the trypanosaemia is associated with low haematological parameters. There was significant difference between the means of the haematological parameters of the parasitaemic, non-treated rabbits of groups B, C and D and the control group. Generally, administration of the trypanocides in groups C and D reversed the depression of haematological values. Comparison between the parasitaemic, saline-treated and the trypanocides-treated rabbits shows that administration of trypanocides caused removal of trypanosomes from the blood and significantly improved the level of haematological parameters in both groups. More specifically, significant reversal of the mean haematological parameters was in rabbits administered with sanative pair of isometamidium chloride and diminazene aceturate (group D) which began by 7th day post-treatment while in rabbits administered with isometamidium only (group C) it was observed by 21st day post-treatment. The mean haematological parameters in group D were significantly higher than that of group C from 7th to 14th day post-treatment. This study shows that the concept of sanative pair in chemotherapy of trypanosomosis is still very much relevant and that synergy derivable from isometamidium and diminazene aceturate is still potent in spite of reported prevalence of resistance to currently available trypanocides.

Key words: combination therapy (laboratory); dimidazene acetusate; isometamidium chloride; rabbits; trypanosomosis

INTRODUCTION

Trypanosomosis is a protozoan disease of domestic animals and man characterized by dehydration, weight loss, stunting, drop in milk yield, abortion and death. It ranks as one of the most economically important disease of livestock in Africa. Among other measures such as vector control and genetic adaptation of trypanotolerant cattle, chemotherapy seems to be the only curative and prophylactic management of the disease (14, 2). Most of the drugs have been in use for fourty-five years and there is no indication for prospect of new drugs (12). Several measures have been taken over this period to reduce rate of resistance and improve the efficacy of the currently available trypanocides.

Slow release device were introduced in order to extend the period of protection provided by these drugs and to decrease their local toxicities. Different alternative delivery systems, such as suraminates, dextran complexes, liposomal formulations, carrier erythrocytes, and polymers, have been developed (19, 9). Except for the polymers, very few of these formulations have been successful (8, 10, 11).

The practice of sanative pairs was also introduced by Whiteside (23) and it involves pairing some of the trypanocides

known to have synergistic actions. The basis for the pairing was predicated on the fact that most trypanocides that were resistance to individual drugs were found to be susceptible to exposure to combination of two of them (2). Historically, drug combinations such as suramin/tartar emetic, isometamidium/ ethidium, isometamidium/diminazene or ethidium/diminazene have been employed (24, 5). Complexes of suramin/quinapyramine and suramin/diminazene aceturate were developed and found to be 80–100 % curative experimentally, whilst diminazene/ suramin complexes was found to protect for approximately five months (13).

However, recent reports on multiple drug resistance in some part of Africa (6, 7, 2) have created the need to re-evaluate the effectiveness of sanative pairs of trypanocides in the treatment of trypanosomosis in face of emerging cross-resistance to these drugs. Our study evaluates the efficacy of isometamidium chloride/diminazene aceturate pair as against the use of isometamidium chloride alone which was recently reported to be efficacious in experimentally infected laboratory rabbits (1).

MATERIAL AND METHODS

Experimental animals

Twenty New Zealand White adult male rabbits of 1.85 0.41 kg body weight were used in this study. The animals were housed individually in steel cages (60 × 60 × 45 cm), they were maintained on rabbit pellet *ad libitum* and allowed access to water without restriction. All the rabbits were randomly divided into four groups (A, B, C and D) of five rabbits each.

Experimental procedure

Infection of rabbits with trypanosomes

All the rabbits except those in group A (control group) were infected with strain of *Trypanosoma brucei brucei* obtained from Nigerian Institute of Trypanosomosis Research, Kaduna, Nigeria. The trypanosomes were first passaged in mice. At the peak of parasitaemia, the mice were bled. The 0.5 ml of the prepared diluted blood containing 8.53×10^4 trypanosomes was administered intraperitoneally into each of the fifteen rabbits. Blood smears were taken to confirm parasitaemia of the infected rabbits using the dark/ ground phase contrast buffy coat method (18) at three days interval until the trypanosomes were detected in the blood of all the rabbits of groups B, C and D.

Post-infection analysis

Parasitaemia was confirmed completely in all the fifteen rabbits in the test groups B, C and D by the 9th day post-infection. Blood samples were collected from each of the rabbits through the lateral ear vein into lithium-heparinized tubes on 14th and 21st days post-infection in all the four groups. The blood samples were analyzed to determine level of parasitaemia and haematological parameters of the animals. Total red blood cell count (RBC) was determined by the haemocytometer and total white blood cell counts (WBC) by Giemsa stained slides method. Haemoglobin concentration (Hb) was determined by cyanomethaemoglobin method, packed cell volume (PCV) by capillary tube method while the mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) were calculated from the data collected.

Treatment of parasitaemic rabbits

Immediately after collection of blood samples from the rabbits on the 21st days post-infection, the rabbits in groups A (control) and B were administered with 0.5 ml of normal saline intramuscularly. However, rabbits in group C were administered with isometamidium hydrochloride (Samorin®) at 1.0 $mg.kg^{-1}$ body weight while rabbits in group D were administered with simultaneous combination of diminazene aceturate and isometamidium hydrochloride at 3.5 $mg.kg^{-1}$ and 1.0 $mg.kg^{-1}$ body weight, respectively. All the drug administrations were done intramuscularly.

Post-treatment analysis

Blood samples were collected from all the rabbits in each of the four groups on the 7th, 14th and 21st days post-treatment. The total RBC and WBC counts, the Hb concentration, PCV, MCV, MCHC and MCH were determined on each day as previously described for the post-infection blood samples collected. Blood smears were taken to confirm parasitaemia from rabbits using the dark/ ground phase contrast buffy coat method.

Statistical analysis

Haematological parameters were expressed as mean standard deviation (S.D). Comparison of the mean values was done using Student's *t*-test and the differences of the means were considered significant at $P < 0.05$ (21).

RESULTS

Parasitology

Parasitological examination of blood samples revealed that *T. brucei brucei* were found in the blood of all the fifteen rabbits of groups B, C and D by 9th day post-infection and subsequently on the 14th and 21th days post-infection. Examination of the blood samples on the 7th, 14th and 21th days post-treatment did not reveal presence of the parasites in groups C, D and the control but the parasites were found in the rabbits of group B. Two of the rabbits in group B died in the course of the experiment.

Haematology

14 days post-infection – Table 1

There was no significant difference between the mean total RBC count of rabbits in the control group (3.60 ± 0.51) and each of the three infected groups: B (2.93 ± 0.12), C (3.01 ± 0.51) or D (2.71 ± 0.10). There

was no significant ($P > 0.05$) difference between the mean WBC values for groups A and B, C or D. However the mean PCV value obtained for the control group (35.2 ± 1.17) was significantly ($P < 0.001$) higher than the mean values obtained for group B ($23.3 \pm 1.03\%$), group C (22.45 ± 1.11) and group D (24.52 ± 1.15).

Similarly, the mean haemoglobin value of 19 ± 2.12 obtained for group A was significantly ($P < 0.001$) higher than 11.53 ± 0.50, 10.11 ± 0.52 and 12.11 ± 0.23 for groups B, C and D, respectively. There was no significant difference in the mean MCV value between group A (106.15 ± 4.17) and the parasitaemic groups B (116.79 ± 5.55), C (112.43 ± 4.54) and D (117.23 ± 0.77), respectively. The mean MCHC value for group A was higher that three parasitaemic groups but the difference of the means was only significant between that of groups A (50.87 ± 2.91) and B (36.49 ± 1.92). There was no significant difference between the mean values of MCH obtained for group A (54.29 ± 3.83) and groups B, C and D (41.79 ± 3.81, 45.71 ± 2.83 and 45.43 ± 4.30), respectively.

21 Days post-infection – Table 2

There was significant ($P < 0.05$) difference between the mean total RBC count of rabbits of the control group (3.65 ± 0.77) and group C (1.88 ± 0.62) or group D (1.24 ± 0.11). There was no significant ($P > 0.05$) difference between the mean WBC values for groups A and B, C or D. The mean PCV value obtained for the control group (35.79 ± 2.32) was significantly ($P < 0.001$) higher than the mean values obtained for group B ($23.01 \pm 1.52\%$), group C (9.72 ± 0.29) and for group D (9.33 ± 0.42). The mean Hb level of group A (20.01 ± 2.49) was also significantly ($P < 0.001$) higher than that of group B (10.52 ± 0.46), group C (9.72 ± 0.27) and group D (9.33 ± 0.42). The mean MCV values of the parasitaemic rabbits groups B (134.43 ± 3.27), C (122.29 ± 4.67) and D (131.81 ± 2.34) were higher than that of the control group (103.37 ± 3.65). The difference of the means is statistically significant ($P < 0.001$) groups A and B, and between group A and D. There was significant difference in the mean values of MCHC between group A (51.15 ± 2.44) and B (38.75 ± 2.48), and C (35.22 ± 2.14), and D (36.42 ± 1.33). However there was no significant difference exist for the mean MCH values between group A (53.42 ± 4.22) and the parasitaemic groups B (41.52 ± 2.60) and C (41.11 ± 2.15) but the difference between group A and D (40.11 ± 1.15) was significant at $P < 0.001$.

7 Days post-treatment – Table 3

The mean total RBC count for group A (3.66 ± 0.11) was significantly ($P < 0.001$) higher than those of groups B (1.95 ± 0.32), C (2.21 ± 0.10) and D (2.29 ± 0.15). There was no significant ($P > 0.05$) difference between the mean WBC values for groups A and B, C or D. However, the mean PCV value for group A (36.8 ± 11.04) was significantly ($P < 0.001$) higher than those of groups B (20.30 ± 1.14), C (19.45 ± 1.11) and D (26.00 ± 1.02). Likewise the mean Hb level for group A (19.19 ± 1.20) was significantly ($P < 0.001$) higher than those of groups B (8.42 ± 0.45), C (10.01 ± 0.12) and D (15.40 ± 0.62). There was also a significant difference between the mean value of MCHC of group A (52.03 ± 2.73) when compared with that of groups B (36.43 ± 2.83), C (36.31 ± 1.46) and D (53.22 ± 3.87). However there was no significant ($P > 0.05$) difference between the mean MCV value obtained for group A (106.33 ± 4.97) and B (111.98 ± 6.35), C (109.33 ± 3.21) and D (115.71 ± 4.83). There was also no significant ($P > 0.05$) difference between the mean MCH values obtained for group A (55.83 ± 5.21) and B (42.00 ± 2.73) and C (44.52 ± 2.05) and D (48.25 ± 2.69)

14 Days Post-treatment – Table 4

The mean total RBC count (3.68 ± 0.25) obtained for group A was significantly ($P < 0.001$) higher than 1.66 ± 0.21 obtained for group B and 2.25 ± 0.35 for group C ($P < 0.05$). There was no significant ($P > 0.05$) difference between the mean WBC values for groups A and B, C or D. The mean PCV value (36.89 ± 2.19) obtained for group A was significantly ($P < 0.001$) higher than 16.22 ± 0.74 for group B and 21.27 ± 2.81 for group C ($P < 0.05$) which also lower ($P < 0.01$) than 33.60 ± 1.00 obtained for group D. The mean Hb level obtained for group A (19.57 ± 3.05) was significantly ($P < 0.001$) higher than 8.02 ± 0.15 obtained for group B 12.13 ± 0.48 and also higher ($P < 0.001$) than 12.13 ± 0.48 obtained for group C which was lower ($P < 0.001$) than 19.69 ± 0.83 for group D. There was a significant ($P < 0.001$) difference between the mean MCHC values obtained for group A (53.0 ± 30.89) and B (30.13 ± 1.82), and C (39.10 ± 1.35) which is lower ($P < 0.001$) than 53.49 ± 2.95 obtained for group D. The mean MCH value of 56.13 ± 2.39 obtained for group A is higher ($P < 0.001$) than 39.00 ± 0.73 of group B and 45.69 ± 1.13 of group C ($P < 0.01$). There was no significant difference between the mean MCV value of group A and other groups.

21 Days post-treatment – Table 5

The mean RBC count of group A (3.69 ± 0.14) was only higher than ($P > 0.001$) than that of group B (1.05 ± 0.19) and the value obtained for that of group D (3.94 ± 0.17) was insignificantly ($P > 0.05$) higher than that of group C. There was no significant ($P > 0.05$) difference between the mean WBC values for groups A and B or C except with group D ($P < 0.001$). The mean PCV value for group A (37.01 ± 0.98) was only significantly higher ($P < 0.001$) than that of group B (14.29 ± 0.43) and the difference of means was not significant ($P > 0.05$) between that of group C and D. There was a significant ($P < 0.05$) difference between mean Hb values of group A (20.16 ± 1.15) and B (8.01 ± 0.29) and between C (20.12 ± 0.66) and D (23.02 ± 0.52). The difference of means for the mean

MCV values was not significant ($P > 0.05$) between any two of the groups studied.. The difference of the means were only significant between groups A and B for MCHC ($P < 0.001$) and MCH ($P < 0.01$) values.

Table 1. Mean haematological parameters obtained 14th day post-infection

Haematological parameters	Group A Control, n = 5	Group B Parasitaemic, Non-Treated n = 5	Group C. Parasitaemic, Non-Treated n = 5	Group D Parasitaemic, Non-Treated n = 5
RBC ($10^6.\mu l^{-1}$)	3.60 ± 0.51	2.93 ± 0.12	3.01 ± 0.15	2.71 ± 0.10
W.B.C ($10^3.mm^3$)	6.77 ± 0.52	5.98 ± 0.70	6.75 ± 0.57	6.67 ± 0.82
PCV (%)	35.2 ± 1.17[a]	23.30 ± 1.03 [b***]	22.45 ± 1.11[c***]	24.52± 1.15[d***]
Hb ($g.dl^{-1}$)	19.59 ± 2.12[a]	11.53 ± 0.50 [b**]	10.11 ± 0.52[c***]	12.11 ± 0.23[d**]
MCV (fl)	106.15 ± 4.17	116.79 ± 5.55	112.43 ± 4.54	117.23 ± 0.77
MCHC (%)	50.87 ± 2.91[a]	36.49 ± 1.92[b*]	42.53 ± 2.65	38.41 ± 7.30
MCH (pg)	54.29 ± 3.83	41.79 ± 3.81	45.71 ± 2.83	45.43 ± 4.20

Comparison between mean values with superscript a-b, a-c, a-d are significant (* – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$)

Table 2. Mean haematological parameters obtained 21st day post-infection

Haematological parameters	Group A Control n = 5	Group B Parasitaemic, Non-treated n = 4	Group C Parasitaemic, Non-treated n = 5	Group D Parasitaemic, Non-treated n = 5
RBC ($10^6.\mu l^{-1}$)	3.65 ± 0.77[a]	1.97 ± 0.33	1.88 ± 0.62[c*]	1.24 ± 0.11[d*]
W.B.C ($10^3.mm^3$)	6.52 ± 0.22	6.86 ± 0.55	6.73 ± 0.45	6.51 ± 0.48
PCV (%)	35.79 ± 2.32[a]	23.01 ± 1.52[b**]	16.52 ± 1.17[c***]	15.67 ± 1.59[d***]
Hb ($g.dl^{-1}$)	20.01 ± 2.49[a]	10.52 ± 0.46[b**]	9.72 ± 0.27[c***]	9.33 ± 0.42[d***]
MCV (fl)	103.37 ± 3.65[a]	134.43± 3.27[b***]	122.29 ± 4.67	131.81 ± 2.34[d***]
MCHC (%)	51.15 ± 2.44[a]	38.75 ± 2.48[b*]	35.22 ± 2.14[c***]	36.42 ± 1.33[d***]
MCH (pg)	53.42 ± 4.22[a]	41.52 ± 2.66	41.11 ± 2.15	40.11 ± 1.15[d*]

Comparison between mean values with superscript a-b, a-c, a-d are significant (* – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$)

Table 3. Mean haematological parameters obtained 7th day post-treatment

Haematological parameters	Group A Control, saline-treated n = 5	Group B Parasitaemic, saline-treated n = 3	Group C Parasitaemic, isometamidium-treated n = 5	Group D Parasitaemic, combination-treated n = 5
RBC ($10^6.\mu l^{-1}$)	3.66 ± 0.11 a	1.95 ± 0.32 b***	2.21 ± 0.10 c***	2.29 ± 0.15[d***]
W.B.C ($10^3.mm^3$)	6.57 ± 0.27	4.96 ± 0.57	6.75 ± 0.57	5.29 ± 0.46
PCV (%)	36.81 ± 1.04 a	20.30 ± 1.14 b***	19.45 ± 1.11c***,e	26.00 ± 1.02[d***,f**]
Hb ($g.dl^{-1}$)	19.19 ± 1.20 a	8.42 ± 0.45 b***	10.01 ± 0.12c*** e	15.40± 0.62[f***]
MCV (fl)	106.56 ± 4.97	111.98 ± 6.35	109.33 ± 3.21	115.71 ± 4.83
MCHC (%)	52.03 ± 2.73 a	36.43 ± 2.85 b*	36.31 ± 1.46[c*** e]	53.22 ± 3.87[f***]
MCH (pg)	55.83 ± 5.21	42.00 ± 2.73	44.52 ± 2.05	48.25 ± 2.69

Comparison between mean values with superscript a-b, a-c, a-d and e-f are significant (* – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$)

Table 4. Mean haematological parameters obtained 14th day post-treatment

Haematological parameters	Group A Control, saline-treated n = 5	Group B Parasitaemic, Ssline-treated n = 3	Group C Parasitaemic, isometamidium-treated n = 5	Group D Parasitaemic, combination-treated n = 5
RBC ($10^6.\mu l^{-1}$)	3.68 ± 0.25[a]	1.66 ± 0.21[b***]	2.25 ± 0.35[c*]	3.14 ± 0.14
W.B.C. ($10^3.mm^3$)	6.36 ± 0.24	4.55 ± 0.75	6.89 ± 1.02	6.25 ± 0.73
PCV (%)	36.89 ± 2.19[a]	16.22 ± 0.74[b***]	21.27 ± 2.81[c**, e]	33.60 ± 1.00[f**]
Hb ($g.dl^{-1}$)	19.57 ± 3.05[a]	8.02 ± 0.15[b***]	12.13 ± 0.48[e]	19.69 ± 0.83[f***]
MCV (fl)	104.32 ± 3.19	90.92 ± 3.22	100.17 ± 2.31	119.83 ± 6.80
MCHC (%)	53.03 ± 0.89[a]	30.13 ± 1.82[b***]	39.10 ± 1.35[c***, e]	53.49 ± 2.95[f***]
MCH (pg)	56.13 ± 2.39[a]	39.00 ± 0.73[b***]	45.69 ± 1.13[c**]	51.03 ± 1.47

Comparison between mean values with superscript a-b, a-c, a-d and e-f are significant (* – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$)

Table 5. Mean haematological parameters obtained 21st day post-treatment

Haematological parameters	Group A Control, saline-treated n = 5	Group B Parasitaemic, saline-treated n = 3	Group C Parasitaemic, isometamidium-treated n = 5	Group D Parasitaemic, combination-treated n = 5
RBC ($10^6.\mu l^{-1}$)	3.69 ± 0.14[a]	1.05 ± 0.19[b***]	3.90 ± 0.22	3.94 ± 0.17
W.B.C. ($10^3.mm^3$)	6.85 ± 0.27[a]	5.50 ± 0.58	4.89 ± 0.72	4.91 ± 0.17[b***]
PCV (%)	37.01 ± 0.98[a]	14.29 ± 0.43[b***]	34.11 ± 3.38	36.00 ± 2.83
Hb ($g.dl^{-1}$)	20.16 ± 1.15[a]	8.01 ± 0.29[b***]	20.12 ± 0.66[e]	23.02 ± 0.52[f*]
MCV (fl)	105.44 ± 3.56	90.01 ± 2.95	104.22 ± 3.26	91.22 ± 5.52
MCHC (%)	53.22 ± 2.68[a]	29.17 ± 0.89[b***]	53.14 ± 2.19	53.55 ± 3.35
MCH (pg)	56.03 ± 4.82[a]	37.13 ± 0.76[b**]	56.12 ± 1.72	52.11 ± 2.22

Comparison between mean values with superscripts a-b, a-c, a-d and e-f are significant (* = P < 0.05, ** = P < 0.01, *** = P < 0.001)

DISCUSSION

The findings from this study show a significant depression of haematological parameters by *T. brucei brucei* in the parasitaemic rabbits. Anaemia was established by the 14th day post-inoculation of trypanosomes into the rabbits in the test groups which became quite pronounced by the 21st day after infection. Depression of haematological parameters has been reported as consistent feature of animal trypanosomosis (4, 3).

Administration of trypanocides by the 21 days post infection cleared the parasite from the blood of infected rabbits. Conversely two rabbits had died out of the five rabbits in the parasitaemic, saline-treated rabbits (group B). Screening of blood smears of rabbits in groups C and D treated with trypanocides showed that they were negative for trypanosomes by the 7th day post-treatment.

Though *T. brucei brucei* is known to migrate from the blood into peripheral tissue (20), which sometimes creates false impression that there were no trypanosomes in the blood. This did not appear to be the case in this situation because of the accompanying improvement in the haematological parameters obtained following treatment with the trypanocides. Several workers have established a very strong correlation between trypanosomosis and PCV and erythrocyte indices such that persistent low PCV value is used as an index of the severity of trypanosomosis in the herd (17, 22).

Another notable finding from this study is the comparatively higher efficacy of the combination therapy of isometamidium chloride/diminazene aceturate over single therapy with isometamidium chloride only. The result obtained showed that though the rabbits treated with isometamidium chloride only in group C recovered their normal haematological parameters by day 21 post-treatment, the restoration of haematological parameters of rabbits in group D peaked faster and quite earlier than that of group C.

Comparison of the post-treatment haematological values of groups C and D showed that the parasitaemic rabbits treated with combination of isometamidium chloride and diminazene aceturate had significantly higher values than rabbits treated with isometamidium only from the 7th to 14th day post treatment. The practice of using sanative pairs of trypanocides was introduced to increase the efficacy of existing trypanocides in the absence of new drugs and emerging resistance to currently available drugs – Williamson (25), Kirby (16), Williamson and Scott-Finnigan (26). However as time goes on there were reports that the concept of sanative pair was no longer effective as a result of multidrug resistance (6, 7).

The findings in this study, however, show that the pair of isometamidium chloride and diminazene aceturate is still very much effective. The quickest explanation may be that the incidence of multidrug resistance to currently available trypanocides is still restricted and not widespread in agreement with findings by Afewok (2) or that the synergy of the combination of isometamidium chloride and diminazene aceturate has not yet been overwhelmed by prevalent resistance to trypanocides. In other words, the sanative pair of isometamidium chloride and diminazene aceturate could be relied upon to effectively treat or control animal trypanosomosis until the arrival of new trypanocides.

REFERENCES

1. Adedapo, A. A., Olayemi, F. O., Saba, A. B., Dina, O. A., Momoh, F. O., 2005: An assessment of a prophylactic dose of isometamidium chloride (Samorin®) on haematological and serum biochemical parameters of rabbits experimentally infected with *Trypanosoma brucei brucei* (Lafia strain). *Folia Vet.*, 49, 138–142.

2. Afewok, Y., Clausen, P. H., Abebe, G., Tilahun, G., Dieter, M., 2001: Appearance of multiple drug resistant trypanosome populations in village cattle of Metekel district, North-West Ethiopia. In **Livestock Community and Environment**. *Proceedings of the 10th Conference of the Association of Institutions for Tropical Veterinary Medicine*. Copenhagen, Denmark.

3. Akinbamijo, O. O., Bennison, J. J., Jaitner, J., Dempfle, L., 1998: Haematological changes in Ndama and Gobra Zebu bulls during *Trypanosoma congolense* maintained under controlled feeding regimen. *Act. Trop.*, 69, 181–192.

4. Anosa, V. O., 1988: Haematological and biochemical changes in human and animal trypanosomiasis. *Rev. d'Elev. Et de Med. Vet. Des. Pays*, 41, 151–164.

5. Boyt, W. P., 1986: *A Field Guide for Diagnosis, Treatment and Prevention of African Animal Trypanosomiasis*. FAO, Rome.

6. Clausen, P. H., Sidibe, I., Kabore. I., Bauer, B., 1992: Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse challenge in the pastoral zone of Samorogouan, Burkina Faso. *Act. Trop.*, 51, 229–236.

7. Codjia, V., Mulatu, W., Majiwa, P. A. O., Leak, S. G. A., Rowlands, G. J., Autjié, E., d'Ieteren, G. D. M., Peregrine, A. S., 1993: Epidemiology of cattle trypanosomiasis in Ghibe Valley, southwest Ethiopia. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. *Act. Trop.*, 53, 151–163.

8. De Deken, R. S., Geerts, P., Kageruka, F., Ceulemans, J., Brandt, E., Schacht, C., Pascucci, C., Lootens, C., 1989: Chemoprophylaxis of trypanosomiasis, due to *Trypanosoma* (*Nannomonas*) *congolense* in rabbits using a slow release device containing homidium bromide. *Ann. Soc. Belge Med. Trop.*, 69, 291–296.

9. Diarra, B., Diall, O., Geerts, S., Kageruka, P., Lemmouchi, Y., Schacht, E., Eisler, M. C., Holmes, P., 1998: Field evaluation of the prophylactic effect of an isometamidium eustained-release device against trypanosomiasis in cattle. *Antimicrob. Agents Chemotherap.*, 42, 1012–1014.

10. Geerts, S., De Deken, R., Kageruka, P., Lootens, C., Schacht, E., 1993: Evaluation of the efficacy of a slow release device containing homidium bromide in rabbits infected with *Trypanosoma congolense*. *Vet. Parasitol.*, 50, 15–21.

11. Geerts, S., Kageruka, P., De Deken, R., Brandt, J. R. A., Kazadi, J. M., Diarra, B., Eisler, M. C., Lemmouchi, Y., Schacht, E., Holmes, P. H., 1997: Prophylactic effects of isometamidium- and ethidium-sustained release devices against *Trypanosoma congolense* in cattle. *Act. Trop.*, 65, 23–31.

12. Geerts, S., Holmes, P. H., 1998: *Drug Management and Parasite Resistance in Bovine Trypanosomiasis in Africa*. FAO Corporate Document Repository.

13. Gill, B. S., Malhorta, M. N., 1971: Chemoprophylaxis of *Trypanosoma evansi* infection on ponies. *Trop. Anim. Health Prod.*, 3, 199–202.

14. Jordan, A. M., 1992: Degradation of the environment: an inevitable consequence of trypanosomiasis control? *World Animal Review*, 70/71, 2–7.

15. Kageruka, P., Kabore, H., Marcotty, T., Ibouesse, J. F., De Deken, R., Geerts, S., Lemmouchi, Y., Schacht, E., 1996: Comparative evaluation of the prophylactic effect of slow release devices containing homidium bromide and isometamidium on *Trypanosoma congolense* in rabbits. *Vet. Parasitol.*, 63, 179–185.

16. Kirby, W. W., 1960: Comparative prophylactic trial using prothidium antrycide prosalt and A and B4404. *Proceedings of I. S. C. T. R, Jos*, 8, 135–139.

17. Mdachi, R. E., Murilla, G. A., Omukuba, J. N., Cagnolati, V., 1995: Disposition of diminazene aceturate (Berenil) in trypanosome-infected pregnant and lactating cows. *Vet. Parasitol.*, 58, 215–225.

18. Paris, J., Murray, M., McDimba, F. A., 1982: Comparative evaluation of the parasitological techniques currently available for diagnosis of Africa Trypanosomiasis in cattle. *Act. Trop.*, 39, 307–316.

19. Peregrine, A. S., 1994: Chemotherapy and delivery systems: haemoparasites. *Vet. Parasitol.*, 54, 223–248.

20. Saba, A. B., Adedapo, A. A., Arowolo, R. O. A., Dina, O. A., 2002: *In vivo* assessment of the efficacy of Samorin and Berenil in nomadic cattle in Southwestern Nigeria. *Nig. J. Anim. Prod.*, 29, 81–87.

21. Steel, R. G. D., Torrie, J. I. I., 1996: P*rinciples and Procedure of Statistics*. A Biometrical Approach (2nd edn). McGraw Hill. New York, 6–15.

22. Van den Boische, P., Rowlands, G. J., 2001: The relationship between the parasitological prevalence of trypanosomal infections in cattle and herd average packed cell volume. *Act. Trop.*, 78, 163–170.

23. Whiteside, E. F., 1958: The maintenance of cattle in tsetse infested country. I. S. C. T. R. *7th Meeting International Scientific Committee for Trypanosomiasis Research, Brussels*. Commission for Technical Co-operation South of the Sahara, Lagos. Publication 41, 83–90.

24. Whiteside, E. F., 1960: *Proceedings of the 8th ISCTRC Meeting*, 141–154, Jos. Nigeria.

25. Willimason, J., 1962: Chemotherapy and chemoprophylaxis of African trypanosomiasis. *Exp. Parasit.*, 12, 274–322.

26. Williamson, J., Scott-Finnigon, T. J., 1976: Antitrypanocidal activity of antitumor antibiotic and other metabolic inhibitors. *Trans. R. S. Trop. Med. Hyg.*, 69, 1–2.

Received December 12, 2006

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 175–177, 2007

PREVALENCE OF *Rhipicephalus sanguineus* INFESTATION IN DOGS IN LAHORE, PAKISTAN

Ahmed, S. S., Abbas, T., Avais, M.

**University of Veterinary and Animal Sciences, Lahore 5400
Pakistan**

avaismerhammad@yahoo.com

ABSTRACT

A retrospective epidemiological study was conducted at Pet center, University of Veterinary and Animal Sciences, Lahore. A total 5490 pet dogs during year 2003–2004 were examined to determine the prevalence of *Rhipicephalus sanguineus* (brown dog ticks). The tick infestation rates were calculated on monthly basis and their distribution by age and sex. Chi-square test was used to compare the proportions of positive and negative tick infested dogs. The overall prevalence of *R. sanguineus* infestation was 12 % and there was significant difference ($P < 0.05$) in prevalence among the months. However, no association could be demonstrated between age and sex infestation rates. Domestic life habits and the environment were probably the main factors influencing the tick infestation patterns in dogs.

Key words: dogs; prevalence; *Rhipicephalus sanguineus*; tick

INTRODUCTION

The brown dog tick (*Rhipicephalus sanguineus*), is the most cosmopolitan tick species and is found too in all parts of south-east Asia. Its specific host is the dog, but it can also infest other domestic and wild species and even the human beings (7, 12). *R. sanguineus* is an efficient vector of *Ehrlichia canis* and *Babesia canis*, the causative agents of canine ehrlichiosis and canine babesiosis, respectively (4, 12). In humans, it is the confirmed vector of many diseases, such as Rocky Mountain Spotted Fever – H a r w o o d and J a m e s (7).

In Pakistan, *R. sanguineus* is present, widely distributed and in some places the dogs have heavy infestations of this tick and are a constant nuisance to the owners. Despite its frequency, little is known about its epidemiology in the dog in Pakistan. The objective of the present study was to describe the prevalence of the infestation with *R. sanguineus* in dogs in Lahore, Pakistan.

MATERIALS AND METHODS

Study site: The Lahore is the capital of the Punjab, located in the Pakistan; at an altitude of 300 feet above sea level.

Dogs: A total 5 490 dogs, during the year 2003–2004 were examined at pet center, University of Veterinary and Animal Sciences for the presence of brown dog tick (*Rhipicephalus sanguineus*). These dogs were presented to the clinic for a variety of reasons, including various infectious as well as non-infectious diseases, traumatic injuries, routine check-ups, grooming and health certification. All were the pet dogs with varying age and sex.

Information concerning the age (determined by questioning the owner and examining the dentition) and sex was recorded. Three categories were made regarding age, less than two year-old, two to four year-old and more than four year-old.

Specimen collection: Tick specimens were obtained from each positive dog. The dogs were examined beginning from the head, followed by the neck, dorsum, trunk, limbs and tail and all ticks stages were collected in a glass vial containing alcohol (70 %) for later identification. The tick specimens were taken to the laboratory and taxonomic identification was carried out (10).

Statistical analysis: The Chi-square test was used to compare proportions of positive and negative tick infested dogs according to age and sex in these variables. A P-value of less than 0.05 was considered as significant difference.

RESULTS AND DISCUSSION

Overall prevalence of infestation rate of *R. sanguineus* was 12 %. The monthly prevalence is shown in Table 1. There was statistical significant difference ($P < 0.05$) in the prevalence among months whereas no sex based difference was found. The prevalence rates were high throughout the year and the peak was found in October (16 %), the lowest prevalence was 3 % in August. Dogs with age over two years showed high prevalence. The prevalence was somewhat higher in spring, summer and autumn (may be due to high relative humidity; 20 % or more) then less in winter (low ambient temperature). Different stages of the tick were found and it was calculated that 2.5 generations could be completed each year.

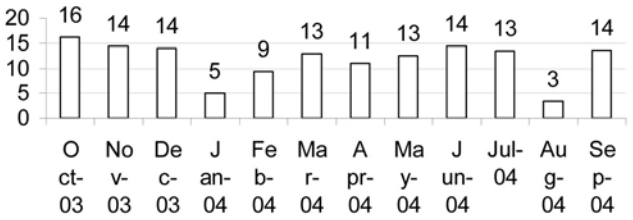
Dogs living outdoors, in rural areas and in close proximity to farm animals were infested with higher numbers of ticks than dogs living in confinement. The most heavily infested sites were the ear pinnae, neck, interdigital skin folds, trunk, head, ventrum, extremities and the tail. Cutaneous lesions at the attachment sites were noticed more severely infested animals. *R. sanguineus* is widely distributed in the world, and it is very important tick of the dogs. It can be peri- or intra-household because of the dog's domestic life habits (14, 13, 2).

In general the findings of present study are similar with other studies carried out by other workers in different climatic conditions. In those studies the peak population of ticks was always related with climatic conditions in the area studied (7, 5). The main factors influencing tick infestation in this study were probably the domestic life habits of the dogs studied, since most of them visit public gardens or live in private or common houses with gardens, which increased the possibility of contact with the ectoparasite (5). Adult dogs, more than two years old, showed higher tick infestations than young dogs (< 2 years old) which have less opportunity of visiting public gardens and the owners probably give more attention to them than to adult dogs. The high prevalence observed in all ages confirms previous report that dogs do not develop resistance to tick infestation by *R. sanguineus* even if they have continuous reinfestation (1). The environmental temperature and relative humidity in our area favours the development and maintenance of *R. sanguineus* ticks throughout the year (11, 8).

Table 1. Prevalence of *Rhipicephalus sanguineus* infestation during year 2003–2004

Month	No. of Dogs	Positive	Prevalence %
Oct-03	471	76	16
Nov-03	318	46	14
Dec-03	344	48	14
Jan-04	321	16	5
Feb-04	320	30	9
Mar-04	547	71	13
Apr-04	516	57	11
May-04	494	62	13
Jun-04	563	81	14
Jul-04	589	79	13
Aug-04	463	16	3
Sep-04	544	74	14

Prevalence % age of Ticks cases at Pet centre U.V.A.S. Lahore



REFERENCES

1. Bechara, G. H., Szabo, M. P. J., Mukai, L. S. Rosa, P. C. 1994: Immunization of dogs, hamster and guinea pigs against *Rhipicephalus sanguineus* using crude adult tick extracts. *Vet. Parasitol.*, 52, 79–90.

2. Carlos, C. V., Vazquez, Z. G., Soto, M. M., 1998: Prevalence of *Rhipicephalus sanguineus* infestation in dogs in Cuernavaca Morelos, Mexico. *Parasitol. Día.*, 22, 1–2. Santiago.

3. Cruz-Vazquez, C., Garcia-Vazquez, Z., 1999: Seasonal distribution of *Rhipicephalus sanguineus* ticks (Acari: Ixodidae) on dogs in an urban area of Morelos, Mexico. *Exp. Appl. Acarol.*, 23, 277–280.

4. Ewing, S. A., 1969: Canine ehrlichiosis. *Adv. Vet. Sci. Comp. Med.*, 13, 331–353.

5. Gilot, B., 1984: Biologie et écologie de *Rhipicephalus sanguineus* (Latreille, 1806) (Acariens, Ixodoidea) dans le sud-est de la France. Intéret dans l'épidémiologie de la fievre boutonneuse (premiere partie). *Sci. Vet. Med. Comp.*, 86, 25–33.

6. Gilot, B., Laforge, M. L., Cabassu, J. P., Romani, M., 1992: Elements pour la cartographie écologique des populations de *Rhipicephalus* du groupe sanguineus (Acariens, Ixodoidea) dans l'agglomération Marseillaise, en relation avec les diverses formes d'urbanisation. *Acarologia*, 33, 17–33.

7. Harwood, R. F., James, M. T., 1979: *Entomology in Human and Animal Health* (7th edn.). McMillan Publ. Co., USA. pp. 429–448.

8. Inokuma, H., Tamura, K., Onishi, T., 1996: Seasonal occurrence of *Rhipicephalus sanguineus* in Okayama Prefecture, Japan and effect of temperature on development of the tick. *J. Vet. Med. Sci.*, 58, 225–228.

9. Koch, G., 1982: Oviposition of the brown dog tick (Acari: Ixodidae) in the laboratory. *Ann. Entomol. Soc. Am.*, 75, 583–586.

10. Krantz, G. W., 1978: *A Manual of Acarology* (2nd edn.). Oregon State University Book Store, Inc., USA., pp. 218–223.

11. Papazahariadou, M. G., Saridomichelakis, M. N., Koutinas, A. F., Papadopoulos, E. G., Leontides, L., 2003: Tick infestation of dogs in Thessaloniki, northern Greece. *Med. Vet. Entomol.*, 17, 110–113.

12. Pergram, R. G., Clifford, C. M., Walker, J. B., Keirans, J. E., 1987: Clarification of the *Rhipicephalus sanguineus* group (Acari, Ixodoidea, Ixodidae). II. *R. sanguineus* (Latreille, 1806) and related species. *Syst. Parasitol.*, 10, 27–44.

13. Prinipato, M., Di-Giuseppe, L. Camerlengo, Liottier, B., 1989: Etude préliminaire sur la distribution saisonniere de certains Ixodidae (Acarina, Ixodoidea) d'intéret sanitaire dans la province de Perouse (Italie Centrale). *Bull. Soc. Franc. Parasitol.*, 7, 269–276.

14. Rivosecchi, L., Khoury, C. C., Lezzerini, G., Uomo, D., 1980: Osservazioni su *Rhipicephalus sanguineus* (Ixodidae) nella periferia di Roma. *Riv. Parasitol.*, 41, 273–276.

15. Shortt, H. E., 1973: *Babesia canis:* The life cycle and laboratory maintenance in its arthropod and mammalian hosts. *Int. J. Parasitol.*, 3, 119–148.

Received February 12, 2007

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 178–180, 2007

RENAL DYSPLASIA IN THE BERNESE MOUNTAIN DOG PUPPY

L. Novotný[1,2], K. Dembovská[3]

[1]Faculty of Military Health Sciences, University of Defence, Hradec Králové
[2]private veterinary pathologist, Hradec Králové
[3]HKvet – Veterinary Clinic for Small Animals, Hradec Králové
Czech Republic

novotnyl@pmfhk.cz

ABSTRACT

Renal dysplasia is usually a fatal disease described in many breeds of dogs. Histopathologic evaluation of the renal tissue proves a persistence of immature renal mesenchyma. A case of chronic renal failure with severe nephritis and azotaemia (creatinine 907 µmol.l^{-1} and urea 178 mmol.l^{-1}) in a ten-week-old Bernese mountain dog puppy has been described. Histopathology revealed a presence of primitive renal parenchyma and adenomatoid proliferation of primitive renal tubules and a diagnosis of renal dysplasia has been confirmed. There was only one published paper describing renal dysplasia in the Bernese mountain dog.

Key words: renal failure; juvenile nephropathy; uremia; immature renal mesenchyma; *pyelonephritis*

INTRODUCTION

Renal dysplasia has been documented in many breeds of dogs. This diagnosis is based on the post natal presence of poorly differentiated tissues (immature glomeruli and/or tubules, persistent mesenchyme) in the kidney (11). Renal dysplasia is commonly described together with inflammation of the kidney tissue (glomerulonephritis, intersititial nephritis and *pyelonephritis*) (7, 9, 1). Fibrous osteodystrophy of the skull has been also described in a six month old Rhodesian Ridgeback dog with renal dysplasia (6).

Clinical signs are represented with apathy, diarrhoea, poor body condition, polyuria and dehydration in the terminal phase. (13, 8). Laboratory tests reveal anaemia and uremia. *Post mortem* examination and histopathologic evaluation show shrunken, pale, and firm kidneys with microscopical finding of asynchronous differentiation of nephrons, persistent immature mesenchyme, persistent metanephric ducts, and adenomatoid proliferation of the tubular epithelium. Secondary degenerative and inflammatory changes consist of interstitial fibrosis and predominantly lymphocytic/plasmacytic inflammation (13, 11).

CASE REPORT

A ten-week-old female puppy of Bernese mountain dog was presented with a history of apathy, vomiting and diarrhoea in March 2007. Clinical examination revealed hypothermia (34 °C), tachycardia (140 pulses per minute) and tachypnoe (38 breathes per minute), pale mucous membranes, signs of dehydration and uremic foetor *ex ore*. An intravenous catheter was placed into cephalic vein, blood samples were obtained and the patient was placed into an incubator. An infusion of 0.9 % natrium chloride solution was started and amoxycilin-clavulanate was intravenously administrated on admis-

ADDRESS FOR CORRESPONDENCE:
MVDr. Ladislav Novotný, Ph.D., MRCVS
Mandysova 1298/22
500 12 Hradec Králové
Czech Republic
Phone +420 723 289 823
E-mail: novotnyl@pmfhk.cz

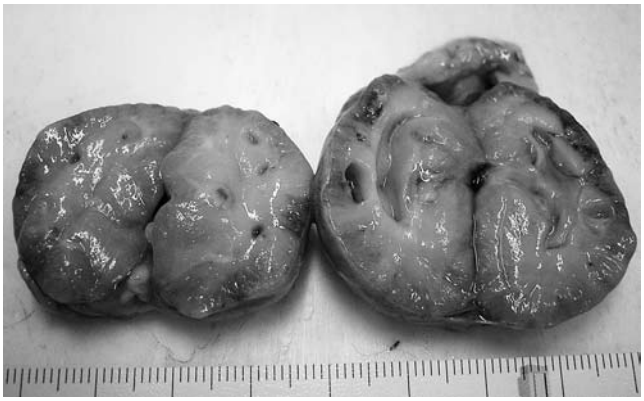


Fig. 1. Left and right kidney with severe fibrosis, mild cortical haemorrhage and cysts. Left kidney is markedly shrunken

Table 1. Haematological and biochemical values of the patient

Parameter (unit)	Value
Haemoglobin ($g.l^{-1}$)	99.00
Haematocrit ($l.l^{-1}$)	0.30
Erythrocytes ($10^{12}.l^{-1}$)	4.47
Leukocytes ($10^{9}.l^{-1}$)	28.74
Trombocytes ($10^{9}.l^{-1}$)	363
Total protein ($g.l^{-1}$)	61.6
Glucose ($mmol.l^{-1}$)	8.3
Creatinine ($\mu mol.l^{-1}$)	907.0
Urea ($mmol.l^{-1}$)	177.9
ALP ($IU.l^{-1}$)	2.75
ALT ($IU.l^{-1}$)	0.86
AST ($IU.l^{-1}$)	0.86

ALP – Alkaline phosphatase, ALT – Alanine aminotransferase
AST – Aspartate aminotransferase

sion. Ultrasonographic examination of the abdominal cavity showed irregular shape of the both kidneys and hyperechogenic areas in the renal cortex. Tonic/clonic convulsions of the legs and head appeared two hours later and was controlled with intravenous administrations of boluses of diazepam ($0.5\ mg.kg^{-1}$). Results of blood examination were delivered from the laboratory together with the onset of the convulsions. The results were consistent with azotaemia with serum creatinine at $907\ \mu mol.l^{-1}$ and urea at $178\ mmol.l^{-1}$. Other biochemical parameters were within normal limits and haematology showed leukocytosis and moderate anaemia (Tab. 1). Based on the above mentioned findings, a presumptive diagnosis of renal failure was rendered and euthanasia (thiopental 1 g i. v.) was performed after telephone consultation with the owner.

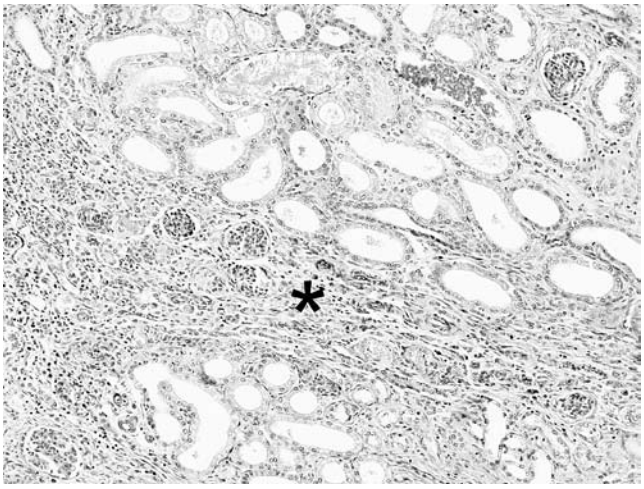


Fig. 2. Histological section of the renal cortex. There is presence of primitive renal parenchyma and adenomatoid proliferation of primitive tubules (asterisk). Haematoxylin-eosin, × 100

Post mortem examination

Autopsy revealed pale mucous membranes, oedematous subcutis, mild ascites and irregular shape of both kidneys with pale brown cortex, cortical cysts and focal haemorrhage (Fig. 1). Left kidney was shrunken with hardly recognizable pelvis. Histopathology revealed moderate to sever cortical fibrosis, dilatation of Bowman's capsule, dilated tubules, occasionaly with neutrophilic exsudation in the lumen, presence of primitive renal parenchyma and adenomatoid proliferation of primitive tubules and severe neutrophilic exsudation in the renal pelvis (Fig. 2).

DISCUSSION

The above described clinical and *post mortem* changes point at chronic renal failure with signs of uremia due the renal dysplasia and severe interstitial and tubular nephritis and suppurative pyelonephritis. The inflammation likely arises from abnormal renal excretion function related to the renal dysplasia and consecutive ascendant infection. There is only one published paper describing renal dysplasia in the Bernese mountain dog (10). A prognosis of renal dysplasia in dogs is in general very poor and euthanasia is made soon after diagnosis (4, 2). Treatment of dogs with chronic renal failure is usually supportive and a diet low in protein, phosphorus and lipids is administrated (3). The laboratory findings involved also mild anaemia which was possibly related to the decreased production of erythropoietin as it was described previously in patients with chronic renal failure (5) and leukocytosis related to the suppurative pyelonephritis.

The diagnosis of renal dysplasia should be put on the list of differential diagnoses in the puppies of Bernese mountain dog with signs of renal failure and concomitant nephritis.

REFERENCES

1. Abraham, L. A., Beck, C., Slocombe, R. F., 2003: Renal dysplasia and urinary tract infection in a Bull Mastiff puppy. *Australian Veterinary Journal*, 81, 336–339.

2. De Morais, H. S., Dibartola, S. P., Chew, D. J., 1996: Juvenile renal disease in golden retrievers: 12 cases (1984–1994). *J. Am. Vet. Med. Assoc*., 15, 209, 792–797.

3. Elliott, D. A., 2006: Nutritional management of chronic renal disease in dogs and cats. *Vet. Clin. North. Am. Small. Anim. Pract.*, 36, 1377–1384.

4. Kerlin, R. L., Van Winkle, T. J., 1995: Renal dysplasia in golden retrievers. *Vet. Pathol.*, 32, 327–329.

5. King, L. G., Giger, U., Diserens, D., Nagode, L. A., 1992: Anaemia of chronic renal failure in dogs. *J. Vet. Intern. Med.*, 6, 264–270.

6. Lobetti, R. G., Pearson, J., Jimenez, M., 1996: Renal dysplasia in a Rhodesian ridgeback dog. *J. Small. Anim. Pract.*, 37, 552–555.

7. Lucke, V. M., Kelly, D. F., Darke, P. G., Gaskell, C. J., 1980: Chronic renal failure in young dogs-possible renal dysplasia. *J. Small. Anim. Pract.*, 21, 169–181.

8. McKay, L. W., Seguin, M. A., Ritchey, J. W., Levy, J. K., 2004: Juvenile nephropathy in two related Pembroke Welsh corgi puppies. *J. Small. Anim. Pract.*, 45, 568–571.

9. Morton, L. D., Sanecki, R. K., Gordon, D. E., Sopiarz, R. L., Bell, J. S., Sakas, P. S., 1990: Juvenile renal disease in miniature schnauzer dogs. *Vet. Pathol.*, 27, 455–458.

10. Olenick, C. L., 1999: Congenital renal dysplasia and psychogenic polydipsia in a Bernese mountain dog. *Can. Vet. J.*, 40, 425–426.

11. Peeters, D., Clercx, C., Michiels, L., Desmecht, D., Snaps, F., Henroteaux, M., Day, M. J., 2000: Juvenile nephropathy in a boxer, a rottweiler, a collie and an Irish wolfhound. *Aust. Vet. J.*, 78, 162–165.

12. Raila, J., Aupperle, H., Raila, G., Schoon, H. A., Schweigert, F. J., 2007: Renal pathology and urinary protein excretion in a 14-month-old Bernese mountain dog with chronic renal failure. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.*, 54, 131–135.

13. Schulze, C., Meyer, H. P., Blok, A. L., Schipper, K., Van Den Ingh, T. S., 1998: Renal dysplasia in three young adult Dutch kooiker dogs. *Vet. Q.*, 20, 146–148.

Received January 28, 2008

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 181–184, 2007

PARACETAMOL IN SYMPTOMATIC TREATMENT OF RESPIRATORY DISEASES OF PIGS

Novotný, J., Kováč, G., Hromada, R.

University of Veterinary Medicine, Komenského 73, 041 81 Košice
The Slovak Republic

postgradual@pobox.sk

ABSTRACT

The work presented was aimed at evaluation of therapeutic using of paracetamol and its effects on haematological profile, as well as on body weight and concentration of total immunoglobulins in blood serum of pigs. From 240 weaned pigs (crossbreed large white × landrace) were chosen 15 animals (average body weight 12.27 kg) with clinically evident respiratory diseases symptoms. These stricken pigs were divided to 3 groups (1st experimental group – 5 animals, 2nd experimental group – 5 animals, and control group – 5 animals). The rations supplied to the 1st experimental groups were mixed with paracetamol in the form of a commercial preparation (Pracetam® 10 % premix, Vétoquinol) at a dose of 5 g preparation per 1 kg of the ration. The 2nd experimental group was treated with combination of paracetamol + marbofloxacin. Systemic antibiotic – marbofloxacin (Marbocyl® 10% inj. a. u. v., Vétoquinol) was administered intramuscularly in dosage 0.25 ml/12.5 kg of body weight, daily during 4 days. Control group was without treatment. A four days application of paracetamol resulted in a positive effect on concentration of leukocytes and body weight of treated pigs.

Key words: paracetamol; pigs; respiratory diseases

INTRODUCTION

In regard to health state of animals – the modern pig industry has disadvantage in being an extremely dense population of animals. High stocking density in a closed environment facilitates transmission of airborne pathogens within the herd (3) and between herds as well (4, 5). Consequently, respiratory disorders and systemic airborne diseases are today regarded as the most serious disease problem in modern swine production (2).

Paracetamol or acetaminophen is para amino-phenol derivatives with analgesic and antipyretic properties. In veterinary medicine it can be used mainly for symptomatic treatment – for reduction of pyrexia, in the context of acute infectious respiratory diseases, in combination with appropriate anti-infective therapy.

The aim of our experiment was to evaluate the influence of paracetamol on haematological parameters, concentration of total immunoglobulins and body weight of weaners with disease of respiratory system.

MATERIAL AND METHODS

The animals used: From 240 weaned pigs Crossbreed large white x Landrace) were chosen 15 animals (average body weight 12.27 kg) with disorders of respiratory system and clinically evident respiratory problems (change of upper respiratory tract – rhinitis, nasal discharge; or change of lower respiratory tract – sneezing, coughing, occurrence of pathological respiratory murmurs; dyspnoe). Experiment was made in practical conditions on pig farm PDP – Seleška. The pigs were housed in section for category pigs to 15 kg, in pens with identical, standard conditions. Animals were fed *ad libitum* dry mixed feed OŠ-02.

Experimental design:

1. experimental group: 5 weaners, treatment – only paracetamol

2. experimental group: 5 weaners, treatment – paracetamol + injection ATB (marbofloxacin)

3. control group: 5 weaners – no treatment

The rations supplied to experimental groups were mixed with paracetamol in the form of a commercial preparation (Paracetam® 10 % premix, Vétoquinol – France) at a dose of 5 g preparation per 1 kg of the ration (0.5 g paracetamol per 1kg of the ration). Systemic antibiotic – marbofloxacin (Marbocyl® 10 % inj. a. u. v., Vétoquinol – France) was administered intramuscularly in dosage 0.25 ml (25 mg)/12.5 kg of body weight, daily, during 4 days.

The blood was sampled from the ophthalmic sinus (6) in the beginning of experiment (sampling 0) and at the end of experiment (4th day of experiment). The body weight of pigs was also determined.

Determination of haematological profile: concentrations of Er, Lc, Hb, Htk, and MCV were measured by apparatus - animal blood counter (abc TM Vet.).

Differential leukocyte count (leukogram): the percentages of individual white cells were determined on Giemsa stained slides using a microscope at × 100 magnification by counting 100 white cells manually in laboratory of II Internal Clinic.

Determination of total immunoglobulins count: spectrofotometric on SPEKOL 211.

Cultivation of microorganisms: from respiratory tract of selected animals were taken swabs – sent to microbiological laboratory UVM and State Veterinary Institute in Košice.

Serological examination of blood: for intention of detection of respiratory viruses – State Veterinary and Food Institute Košice.

Monitoring of stable gas concentrations: concentration of CO_2 in pig stable – analyser ANAGAS CD 98, concentration of NH_3, H_2S, O_2, and CO -– TETRAGAS detector.

Coprological analyses: ovoscopic diagnosis – flotation and sedimentation method.

Statistical processing of results: The obtained results were processed by 1 way ANOVA.

RESULTS

Concentration of white blood cells ranged over the physiological limits in all tested groups (Table 1). Insignificant decrease of concentrations Lc was recorded on 4th day after beginning of therapy in 1st and 2nd experimental group. However Lc level of control group was insignificantly increased.

Table 1. Concentration of Lc in blood of pigs ($\bar{x} \pm sd$; physiological limit 11–18 G.l^{-1})

	1st day	4th day
1st group – paracetamol	23.16±6.77	20.96 ± 6.85
2nd group – paracetamol + marbocyl inj.	23.46 ± 6.07	20.98 ± 9.51
3rd group – control	22.40 ± 7.66	24.46 ± 4.93

Concentration of Er ranged within the reference range, in both samplings and in all 3 groups (Table 2). During the whole experiment, there were no significant differences between the groups.

Table 2. Concentration of Er in blood of pigs ($\bar{x} \pm sd$; physiological limit 5–8 T.l^{-1})

	1st day	4th day
1st group – paracetamol	6.52 ± 0.63	5.79 ± 0.63
2nd group – paracetamol + marbocyl inj.	6.54 ± 0.49	5.66 ± 0.18
3rd group – control	6.07 ± 0.59	5.93 ± 0.62

Similarly concentration of haemoglobin ranged within the normal limit. No significant differences were observed between experimental and control animals (Table 3).

Table 3. Concentration of Hb in pigs' blood ($\bar{x} \pm sd$; physiological limit 10–14 g.dl^{-1})

	1st day	4th day
1st group – paracetamol	10.80 ± 0.95	10.14 ± 0.61
2nd group – paracetamol + marbocyl inj.	11.54 ± 1.06	10.38 ± 0.47
3rd group – control	10.12 ± 0.94	10.12 ± 1.13

Except for the 2nd group (sampling 0) haematocrit was below physiological limit. The differences between groups were statistically insignificant (Table 4).

Table 4. Blood haematocrit of pigs ($\bar{x} \pm sd$; physiological limit 0.32–0.42 l.l^{-1})

	1st day	4th day
1st group – paracetamol	0.31 ± 0.03	0.28 ± 0.02
2nd group – paracetamol + marbocyl inj.	0.32 ± 0.04	0.28 ± 0.01
3rd group – control	0.28 ± 0.03	0.28 ± 0.03

The mean corpuscular volume (MCV), ranged below the normal limits. Statistical analysis showed insignificant differences.

Table 5. MCV blood of pigs
($\bar{x}$ ± sd; physiological limit 50–68 f.l^{-1})

	1st day	4th day
1st group – paracetamol	47.2 ± 2.17	48 ± 1.73
2nd group – paracetamol + marbocyl inj.	49.20 ± 1.79	49.8 ± 1.79
3rd group – control	46.80 ± 3.27	47.6 ± 3.13

Determination of leukogram showed no changes on 1st day of experiment (Table 6). On the other hand in 4th day was recorded an increased proportion of neutrophils in the blood (neutrophilia), and slightly lower level of lymphocytes (lymphopenia).

Table 6. Differential leukocyte count of blood pigs – mean value

	Day of experiment	Ly	Mo	Ba	Ne_{Band}	$Ne_{Seg.}$	Eo
		40–55	2–8	1–3	2–5	30–50	1–3
1st group	1st day	44	–	–	4	48.67	3
	4th day	39.33	–	–	2.67	56	3
2nd group	1st day	50.8	–	–	2	46.4	3
	4th day	39	–	–	2	59	2
3rd group	1st day	48.67	–	–	4	45.33	3
	4th day	39.20	–	–	4	56.60	2.60

Legend: Ly – lymphocytes; Mo – monocytes; Ba – bazophil granulocytes; Ne – neutrophil granulocytes (banded, segmented); Eo – eosinophil granulocytes

The comparison of levels of total immunoglobulins (T Ig; Table 7) in blood between the groups showed insignificant differences. These concentrations were physiological.

Table 7. Concentration T Ig in pigs' blood serum
($\bar{x}$ ± sd; normal limit 20–35 UZST)

1st day	4th day	
1st group – paracetamol	25.78 ± 2.38	24.88 ± 2.72
2nd group – paracetamol + marbocyl inj.	24.99 ± 2.76	25.18 ± 3.16
3rd group – control	25.58 ± 4.28	27.24 ± 5.39

During four days, the average live body weight increased by 1.06 kg in the 1st experimental group, 1.00 kg in 2nd experimental group and 0.90 kg in the control group. We did not observe any significant differences in the live body weight between the groups (Table 8).

Table 8. The average live body weight of the pigs
($\bar{x}$ ± sd; kg)

	1st day	4th day
1st group – paracetamol	13.60 ± 3.07	14.66 ± 3.12
2nd group – paracetamol + marbocyl inj.	11.70 ± 1.68	12.70 ± 2.22
3rd group - control	11.50 ± 3.00	12.40 ± 3.31

Cultivation of swabs from snouts of weaned pigs showed presence of: *Proteus mirabilis*, virid. *Streptococci*, *Enterobacter agglomerans*, *E. coli.* Serological examination focused on detection of antibodies against respiratory viruses was negative.

Coprological analysis oriented on identification of endoparasites ova was negative.

Analyse of stable microclimate showed defect of air circulation (no artificial ventilation system) and because of that – relative higher concentrations of NH_3 (0.0015 %) and CO_2 (0.25 %).

DISCUSSION

Analysis of haematological profile of pigs' blood showed differences from reference range (8). This is the consequence of intentionally selected sick animals – illness of respiratory tract. Elevated values were found in concentrations leukocytes (leukocytosis; Table 1) – indicating an infectious or inflammatory diseases with increased production and count of white blood cells. For this state we can consider also parasitary infestation. However, this cause was excluded by coprological examination and by leukogram. In treated groups of pigs (1st a 2nd group) was achieved lower concentration of Lc – what suggest effective therapy. On the contrary, in control group was reached higher concentration of Lc – it apparently results from development of pathological processes in respiratory tract. Lower values of haematocrit (Table 4) in all groups of animals is related to lower mean corpuscular volume – MCV (microcytosis – abnormally small red blood cells; Table 5), because haematocrit mainly depends on number and size of red blood cells (1). Concentrations of erythrocytes and haemoglobin (Tables 2, 3) were in physiological range – there was not anaemia (relate to parenteral application of Fe in perinatal period of piglets life).

As far as differential leukocyte count differences were recorded only in 4th day of experiment (Table 6), in all groups of animals:

– neutrophilia – indicated bacterial infections (acute inflammatory processes, acute infections), because neutrophils are the first line of defence against bacteria that invade tissues and blood. Neutrophils – kill bacteria in blood or interstitial fluid by phagocytosing them (7).

– mild lymphopenia – similar level in all three groups – it excludes some immunosuppressive effect of used drugs (as have e. g. corticosteroids)

By detection of infectious germs were not recorded any primary respiratory pathogens (such as e. g. viruses – PRRS, pseudorabies, pig influenza; or bacteria – such as e. g. *Actinobacillus pleuropneumoniae, Haemophilus parasuis* etc.). In regard to insufficient stable ventilation (natural ventilation by windows – did not manage to provide sufficient circulation of air) and dustiness connected with dry feeding system – we suppose that animals suffered by intensive irritation of respiratory mucose membranes, suppression of local mucosal immunity, and by reason of that pathological influence determined bacteria (*Proteus mirabilis*, virid. *Streptococci*, *Enterobacter agglomerans*, *E. coli*).

Supplementation of paracetamol did not influence appetite of animals (higher average body weight of experimental groups), it had positive effect on reduction clinical symptoms indicating respiratory disease (positive change or decrease of nasal discharges, coughing, fever reduction – rectal temperature below 39.6 °C). No side effect, no interactions, no adverse effect was observed after oral administration of paracetamol and intramuscular application of marbofloxacin.

We may conclude that administration of paracetamol favourable influenced Lc concentrations in blood of weaned pigs with respiratory problems. The mean daily weight gain of treated piglets achieved higher value than in control group. Therapeutic using of paracetamol can by appropriate addition to anti-infective therapy of respiratory diseases pigs.

ACKOWLEDGEMENT

This investigation was supported by Grant agency VEGA 1/3486/06.

REFERENCES

1. Bartko, P., Hofírek, B., 1993: Blood and blood forming organs. In **Slanina, Ľ., Dvořák, R. *et al.*:** *Veterinary Clinical Diagnostics of Internal Diseases* (In Slovak), 291–325.

2. Christensen, G., Sorensen, V., Mousing, J., 2003: Diseases of respiratory system. In *Diseases of Pigs*. (**Straw E. B., D'Allaire, Mengeling W. L., Taylor D. J.**), 659–678.

3. Donham, K. J., Scallon, L. J., Popendorph, W., Treuhaft, M. W., Roberts, R. C., 1986: Characterization of dust collected from swine confinement buildings. *Am. Ind. Hyg. Assoc. J.*, 47, 404–410.

4. Flori, J., Mousing, J., Gardner, I. A., Willeberg, P., Have, P., 1995: Risk factors associated with seropositivuty to porcine respiratory coronavirus in Danish swine herds. *Prev. Vet. Med.*, 25, 51–62.

5. Jorsa, S., Thomsen, B. L., 1988: A Cox regression analyses of risk factors related to *Mycoplasma suipneumoniae* reinfection in Danish SPF-herds. *Acta Vet. Scand. (Suppl.)*, 84, 436–437.

6. Kováč, G., Martinček, M., Mudroň, P., Cilk, D., Demonovič, T., Paštek, J., Stanislavský, V., Ružička, S., 1990: Collection of blood samples from pigs. Comparison all used methods and description of recent method (In Slovak), *Veterinářsti*, 40.

7. Li, Y., Karlin, A., Loike, J. D., Silverstein, S. C., 2002: A critical concentration of neutrophils is required for effective bacterial killing in suspension. *Proc. Natl. Acad. Sci*., 99, 8289–8294.

8. Vrzgula, L., Sokol, J., 1990: Interpretation of haematological profile. In **Vrzgula** *et al.*: *Metabolic Disorders and Their Prevention in Farm Animals* (2nd edn.), 470–473.

Received May 7, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 185–188, 2007

HISTOPATHOLOGICAL AND GENETIC ASPECTS OF STRESS SYNDROME OF THE SKELETON MUSCULATURE OF PIGS WITH RESULTING PSE MEAT

Makovický, P. E.[1], Makovický, P. A.[2]
Levkut, M.[3], Chrenková, M.[4], Kulíšek V.[2], Jílek, F.[1]

[1]Department of Veterinary Science, Czech University of Life Sciences Prague
The Czech Republic
[2]Department of Animal Husbandry, Slovak University of Agriculture Nitra
[3]Department of Pathology, University of Veterinary Medicine Košice
[4]Institute of Nutrition, Research Institute for Animal Production Nitra
The Slovak Republic

pmakovicky@email.cz

ABSTRACT

The aim of this study was to analyse the samples of skeleton muscles of pigs (n = 10) weighing from 90 to 120 kg. The animals were detected as stress sensitive animals at the slaughterhouse before the slaughter. One of the criteria was pH of the meat with the values below 5.8 taken 45 minutes *post mortem*. The samples were taken from blood for DNA isolation and *MYF-4* and RYR1 gene detection. All pigs were detected as *MYF-4* AB with *RYR1* gene. The samples were taken also from the muscles *m. triceps brachii* (MTB); *m. longissimus dorsi* (MLD), and *m. rectus femoris* (MRF) and then they were fixed in liquid nitrogen. After cutting the samples on the cryocut microtome the sections were stained by haematoxylin-eosin for the evidence of neutral lipids: oil red "O" for detection of dehydrogenases for distinguishing the individual types of muscular fibres and for the evidence of glycogen. All other types of muscular fibres are sharply limited with scattered giant muscular fibres with a tendency of their localisation on the periphery of muscular fascicles. There is also an individual, but also mass shifting of the muscular nuclei from the periphery to the centre of muscular fibres. Muscular fibres are condensed with some vacuolisation. The fat cells were localised in the peripheral clusters in the interstitial tissue among the tertiary fascicles, while more centrally they occurred only occasionally. There was a deficit of glycogen determined histochemically in A (white) muscular fibres.

Key words: muscular fibres; MYF-4; pigs; RYR-1; skeletal muscles; stress syndrome

INTRODUCTION

Long-term trends of intensive pig breeding with the aim of high meat productivity at the present limiting of the portion of deposit and thermo-regulating fat layers caused higher perception of such selected animals to the environmental stress factors. Bickhardt (3) already mentioned the existence of negative correlation between the levels of meatiness and the stress susceptibility, and on the other hand between the quantity and quality of meat, which leads to the conclusion that all the breeding procedures with the aim to increase the meat production represent the risk of deterioration of meat quality. Bonelli and Schifferli (4) describe the stress syndrome of pigs as follows: the degeneration of tail muscles, breathing difficulties, high increase in the body temperature with following collapse of the animal, and animal dies in shock. As mentioned by Warnants *et al.* (28) there is an occurrence of PSE meat after the death already in 70 % of pigs with a stress syndrome, or those susceptible to stress. PSE (pale, soft, exsudative) meat was already mentioned more than one hundred years ago. During the development of this disorder, there is an extremely fast glycolysis inside the muscles and the formation of lactate, and as a result of this pH is decreasing while the muscular temperature is still high. Maksimov (15) reported that a rate decrease in pH is approximately twice higher than that in normal muscles.

While the pH in normal meat decreases from 7.2 to 5.5 during 24 hours, in PSE meat the pH decreases below 5.8 already

after 45 minutes, whereas the lactate concentration achieves the maximum values in one hour after *post mortem*. The levels of creatinine phosphate and ATP are lower at the time of death according to S e e w a l d *et al.* (22) and they deplete in about one hour. There is an increased concentration of ions Ca^{2+} in the muscles, which cause the increased activity of enzyme of ATP-ase and due to the surplus of ADP, IMP and non-organic phosphate a more rapid process of glycogenesis occurs (1).

Due to fast glycogenolysis and ATP a considerable amount of heat releases and the muscular temperature rises up to 43 °C. Due to the effect of lactic acid rise a rapid acidification of muscles occurs, and combination of the increased temperature and low pH value manifests by a partial denaturalisation of muscular proteins (18). On the basis of this fact, the PSE meat water retention ability is restricted and there is a loss of meat juice. For the purpose to analyse the samples of skeleton muscles of pigs, stress sensitive animals were detected before their slaughterring.

MATERIAL AND METHODS

For reaching the goals, the samples from several collections (n = 10) from the striated skeletal musculature of pigs, at the slaughter weight 90–120 kg were used. The samples were collected from pigs over a longer period of time. In this study only the pigs showing higher susceptibility to changed environmental conditions were included. The animals due to the stress syndrome sustained shock, or died in a collapse, and the pH of meat 45 minutes *post mortem* was below the value of 5.8. After the slaughter, the blood was collected for isolation of DNA according to the methodological procedure of S a m b r o o k *et al.* (21). Gene MYF-4 was detected by the PCR-RFLP method according to M e n d e z *et al.* (16).

After bleeding and the removals of internal organs the samples from three muscles were collected: *m. triceps brachii* (MTB), *m. longissimus dorsi* (MLD) and *m. rectus femoris* (MRF). Selection of analysed muscular samples corresponds with the most important muscular groups from the point of view of meat productivity. The muscular samples were taken according to the methodology of K u l í š e k *et al.* (12) and they were collected at the very latest to 30 minutes *post mortem*. The samples were labelled and the respective protocol documentation filled in and then they were preserved in liquid nitrogen. Such fixed samples in a container with liquid nitrogen were sent to a histological laboratory and they were stored in a freezing box at the temperature below 30 °C, or practically immediately sliced by means of freezing microtome (cryocut).

The serial slices were cut at the temperature below 18 °C with thickness of 10 μm and afterwards exposed to histological and histochemical dyeing processes. The sections were stained by haematoxylin-eosin for the evidence of neutral lipids, the oily red "0" for detection of dehydrogenisation for distinguishing of the individual muscular fibres according to the methodological processes recommended by S t e i n and P a d y k u l a (24). For glycogen evidence the sections were stained by the PAS reaction. The microscopic samples were evaluated subjectively using the light microscope Olympus Provis.

RESULTS

1. Genetic results

All pigs were detected as stress sensitive before slaughtering and with DNA analysis revealed that all the pigs were MYF-4 AB with *RYR1* gene. Results are in the Table 1.

2. Histopathological results

The light microscopic picture documents in all samples the transverse cross section of the studied muscles. It is a case of dystrophic picture with fluctuating of the diameter of muscular fibres, focal necrosis in the initial stadium, which are accompanied with regeneration and reparation processes with proliferation of interstitial tissue.

Table 1. Genetic results

Pig	Weight of pigs (kg)	MYF-4 gene	RYR1 gene	pH of meat *post mortem* 45 min
1	95	AB	detected	below 5.8
2	99	AB	detected	below 5.8
3	102	AB	detected	below 5.8
4	96	AB	detected	below 5.8
5	112	AB	detected	below 5.8
6	113	AB	detected	below 5.8
7	96	AB	detected	below 5.8
8	90	AB	detected	below 5.8
9	103	AB	detected	below 5.8
10	120	AB	detected	below 5.8

The muscular fibres loose their characteristic oval shape and acquire irregular one. Sporadically the muscular fibres granularly break up with irregular homogenous clots of oxyphilic sarcoplasm with partial loss of stainability of nuclei. In the samples there are also solitary giant muscular fibres localised on the periphery of individual muscular bundles. The centre of almost all muscular fibres is non homogenous and created by condensing formations represented by myofibrils.

There are also mouldy-granular muscular fibres with homogenous dollops of condensed *oxyfil sarcoplasm* with necrosis of muscular fibres. The muscular nuclei are localised beneath the sarcolemma, but it is possible to observe the nucleus with spirally multiplied cores from the peripheral edges towards the centre of the muscular fibres. *Perymisium externum* is only partially determined in the samples at the edges of muscles. *Perymisium internum* is very well detected in all samples when it is anastomosed thickly on the whole muscle surface, while it is thicker on the peripheral area in comparison to the centre where it forms only narrow septums. Endomysium is enveloping the individual muscular fibres but it is detectable only faintly.

The vascular system is only very faintly detected but the nerve fibres are represented numerously and in the framework of the whole tissue there are clearly detectable blood capillaries. It is possible to detect more or less numerous clusters of fat cells in the samples coloured in order to detect the fat in *perymisium internum* among the tertiary bundles of muscular fibres. It is possible to observe the individual small multivacuolar and univacuolar fat cells even in the primary bundles.

All the three types of muscular fibres A (white), B (intermediary) and C (red) are detectable in SDH reaction. There is an intra-muscular fat dispersed peripherally beneath the sarcolemma in C (red) muscular fibres. The muscular fibres have no reaction to the neutral fat. Only a small response in glycogen reaction with localisation among the individual myofibrils can be observed.

DISCUSSION

Selection of pigs for their lean slaughter body together with a high daily gain specified the ability of animals to retain high levels of nitrogen, the abilities to synthesise the albumens and high activity of growth hormone (STH) which is in contradiction with the productivity of adrenocortictrophic hormone and corticosteroid hormones of the adrenal cortex. The preferential treatment of the individuals with these abilities caused the reduced ability to react effectively to stress situations (13).

PSE meat is being created as a result of malignant hypethermic syndrome (MHS), or porcine stress syndrome (PSS). The pigs susceptible to PSS react to changed environmental conditions so that this would lead to the syndrome which could be characterised by hypermetabolism, acidosis, increased body temperature, muscle stiffening leading to the animal exit. At stress syndrome the weakening of tail muscles, increased breathing and heart rate, paleness with a following cyanosis are described. At the same time the body temperature increases rapidly, the muscles are tightening and causing the collapse and the animal is dying in shock (4).

As it was already mentioned by L e n g e r k e n *et al.* (14) there is a relation among the individual syndromes and at the same time there is a relationship in the direction of PSS *via* SMH and PSE. Some authors quantify the influence of psychogenic stresses on the usage of animals and especially meat, while the animal behaviour is being described under the normal and changed conditions. They observed that in a group of animals during their development and growth there are social and psychological relations. Transport, mixing up of pens, waiting time at the slaughterhouse, or the unsuitable handling of animals represent stress for animals (19).

The pigs not resistant and tolerant to the influence of stresses are affected by various disorders in the metabolic, constitutional and functional state of their organisms. It is necessary to consider the increase of stress sensitivity according to W e n d t *et al.* (29) as a symptom of physiological unstability of sensitive animal. The pigs with anincreased stress sensitivity react to the tension by an increase of the lactic acid formation in the muscles. They have lower oxidised phosphorylysis and the anaerobic glycolysis is higher. According to C h e a n *et al.* (10) the PAS reaction in the muscular fibres is decreasing to minimum *post mortem* and this means that there is a rapid degradation of glycogen.

Another explanation was already provided by S y b e s m a and E i k e l e n b o o n (25) according to which those processes are based on the insufficient oxygen supply of mitochondria, then inadequate systems of intracellular enzymes or the penetration of hormonal systems outside the cell. PSE meat is related with the increase of Ca^{2+} concentration and because the mitochondria bind approximately 10 % of Ca^{2+} of muscular fibres M i t c h e l and H e f f r o n (17) admitted the possibility of its insufficient feedback at mitochondria.

The histological and histochemical changes in stress sensitive pigs are from the point of view of pathological anatomy variable and only small. D u t s o n *et al.* (6) mention the muscular fibre necrosis. We can compare the results with those gained by H e n d r i c k s *et al.* (9), and they point at the hyaline degeneration and loss of striation of myofibrils, disorganisation and condensation of muscular fibres. J e a n n e t *et al.* (11) stated that the multiplied muscular nuclei appear in the middle of muscular fibres, while they form the chains of centrally located muscular nuclei. G a y a t h r i *et al.* (7) stated that the presence of central or centrally migrating muscular nuclei in more than 3 % of muscular fibres is frequent in myogenic lesions. When their frequency exceed 30 %, it is necessary to think at the chronic muscular dystrophy and at the values of more than 50 or more % the centronuclear or myotubular myopathy is probable.

Regarding the blood capillaries C o o p e r *et al.* (5) did not observe the difference between the stress resistant and stress sensitive animals but they found that the muscles of stress sensitive pigs have a higher proportion of B (intermediary) muscular fibres with a higher activity of amylphosphorylysis and ATP, and thus they are responsible for PSE meat. It can be concluded from the work of R e i n e r *et al.* (20) that the muscular fibres of skeletal muscles determine the quality of meat while the thickness and representation of A (white) muscular fibres point to the increase of R-value (degree of dissemination of adenosine) and the production of lactic acid, which indicates to the cases of PSE in European and American pigs.

The changes in PSE meat appear more or less immediately *post mortem*. As Š k a r d a *et al.* (27) stated the muscles are pale, of soft consistency, watery with small water retention ability and thus a considerable losses during the heat preparation. According to S e e w a l d *et al.* (22) the paleness is caused by precipitation of soluble sarcoplasmatic proteins that mask the red colour of sarcoplasmatic proteins. In this sense B e e (2) stated that the growth of glycolytical potential is negatively influences

the meat colour with regard to the fact that there is faster glycogen degradation, and thus the pH changes in meat occur. Meat with pH lower than 5.6 is paler.

The increase in the enzyme activity in blood plasma is according to H a m m (8) caused by the disorder of muscular fibre permeability with a high degree of destruction of giant protein molecules, which leads to myopathic changes. Š i m e k *et al.* (26) mention the meat quality abnormalities detection, as it is in the case of PSE meat after using electric conductivity. Similarly, S c h w a g e l e *et al.* (23) have this opinion, but as Š i m e k *et al.* (26) state the pH value of meat is one of the best verified methods for PSE meat detection.

REFERENCES

1. Batlle, N., Aristoy, M. C., Toldra, F., 2000: Early post-mortem detection of exsudative pork meat based on nucleotide content. *J. Food Sci*., 65, 413–216.

2. Bee, G., 2002: Effect of available dietary carbohydrate on glycolyte potential and meat quality of swine musles. *Can. J. Anim. Sci.*, 82, 311–320.

3. Bickhardt, K., 1998: Exertional myopathy and osteochondrosis of pigs as a result of breeding for growth rate. *Tier. Umschau.*, 53, 129–134.

4. Bonelli, A. M., Schifferli, C., 2001: Porcine stress syndrome. *Arch. Med. Vet.*, 33, 125–135.

5. Cooper, C. C., Cassens, R. G., Briskey, E. J., 1969: Capillary distribution and fiber characteristics in skeletal muscle of stress-susceptibile animals. *J. Food. Sci.*, 34, 299–302.

6. Dutson, T. R., Pearson, A. M., Merkel, R. A., Koch, D. E., Weatherspooh, J. B., 1971: Histochemical activity of some lysosomal enzymes in normal and pale soft exsudative pig muscle. *J. Anim. Sci.*, 32, 233–240.

7. Gayathri, N., Das, S., Vasanth, A., Devi, M. G., Ramamohan, Y., Santosh, V., 2000: Centronuclear myopathy – morphological relation to developing human skeletal muscle: a clinicopathological evaluation. *Neurol. India*, 48, 19–28.

8. Hamm, R., 1991: Lactate-dehydrogenase and its importance in meat research. 4. Effects of muscle disease and PSE conditions on LDH in serum and tissue-influence of treatment of meat. *Fleischwirtschaft*, 71, 345–349.

9. Hendricks, H. B., Lafferly, D. T., Aberle, E. D., Forrest, J. C., 1971: Relation of Porcine muscle fiber type and size to post-mortem shortening. *J. Anim. Sci.*, 32, 57–60.

10. Chean, K. S., Chean, A. M., Ľahučký, R., Mojto, J., Kováč, Ľ., 1993: Prediction of meat quality in live pigs using stress susceptibile and stress resistant animals. *Meat Sci.*, 34, 179–189.

11. Jeannet, P. Y., Basez, G., Eymard, B., Laforel, P., Urtizberea, J. A., Rouche, A., Guicheney, P., Fardeau, M., Romero, N. B., 2004: Clinical and histological findings in autosomal centronuclear myopathy. *Neurol.*, 62, 1484–1490.

12. Kulíšek, V., Dvořák, J., Makovický, P. E., Vykoukalová, Z., Civáňová, K., 2003: Methodical steps samples taking from pigs embryos for genetically and histological analysis (In Slovak). *Infovet*, 10, 39–41.

13. Lee, Y. B., Choi, Y. I., 1999: PSE (pale, soft, exsudative) pork. The causes and solutions – Review. *Asian Aust. J. Anim. Sci.*, 12, 244–252.

14. Lengerken, G., Maak, S., Wicke, M., Von-Lengerken, G., 2002: Muscle metabolism and meat quality of pigs and poultry. *Vet. Zoo.*, 20, 82–86.

15. Maksimov, G. V., 1995: Meat quality and sterss resistance of pigs in relation to selection for meatiness (In Russian). *Selskokhozyaistvennaya Biologiya* (In Russian), 16, 13–36.

16. Mendez, E. A., Ernst, C. W., Rothschild, M. F., 1997: Rapid communication: A novel DNA polymorphism of the porcine myogenin (MYOD) gene. *J. Anim. Sci.*, 75, 1984–1984.

17. Mitchel, G., Heffron, J., 1980: Porcine stress syndrome: A mitochondrial defect? *S. Afr. J. Sci.*, 76, 546–551.

18. Monin, G., Przybylski, W., Kocwin-Podisadla, M., Kuril, J., Klosowska, D., Pospiech, E., Lyczynski, A., 2003: Glycolytic potential as meat quality determinant. *The IV Conference on Effect of Genetic and Non-genetic Factors on Carcass and Meat Quality of Pigs*. 109–120.

19. Provazník, L., Valenta, J., 1994: The effect of transport and resting time of pigs before slaughter on meat quality. *Cze. J. Anim. Sci.*, 39, 1063–1072.

20. Reiner, G., Heinricy, L., Muller, E., Geldermann, H., Dzapo, V., 2001: Indications of associations of the porcine FOS proto-oncogene with skeletal muscle fibre traits. *Anim. Genet.*, 33, 49–55.

21. Sambrook, J., 1989: *Molecular Cloning: a Laboratory Manual* (2nd edn.). Cold spring Harp. Lab. Press, USA 122–123.

22. Seewald, M. J., Iaizoo, P. A., Heisswolf, E., Eichinger, H. M., 1993: Effects of meat quality and storage on the breakdown of adenosine-triphosphate in muscle from swine. *Meat Sci.*, 35, 47–61.

23. Schwagele, F., Lopez, P., Haschke, C., Honikel, K. O., 1994: Rapid pH drop in PSE muscles – enzymological investigations into the causes. *Fleischwirtschaft*, 74, 95–98.

24. Stein, Y. M., Padykula, A. H., 1962: Histochemical classifications of individual skeletal fibres of the rat. *Amer. J. Anat.*, 72, 103–110.

25. Sybesma, W., Eikelenboon, G., 1969: Malignant hyperthermia syndrome in pigs. *Nether. J. Vet. Sci.*, 28, 155–159.

26. Šimek, J., Vorlová, L., Malota, L., Steinhauserová, I., Steinhauser, L., 2003: Postmortal changes of privalue and lacticacid content in the muscles of pigs and bulls. *Cze. J. Anim. Sci.*, 48, 295–299.

27. Škarda, R., 1991: *Pathological Anatomy* (In Slovak). Príroda, Bratislava, 208.

28. Warnants, N., Eckhout, W., Boucque, C. V., 1992: The porcine stress syndrome – detection and repercussions – Review. *Landbouwtijdschrift – Revue de l'Agriculture*, 45, 1129–1175.

29. Wendt, M., Bickhardt, K., Herzog, A., Fischer, A., Martens, H., Richter, T., 2000: Porcine stress syndrome and PSE meat clinical symptoms, pathogenesis, aetiology and aspects of animal welfare. *Berliner Münch. Tierärztl. Wochensehrft.*, 113, 173–190.

Received October 23, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 189–196, 2007

THE ROLE OF CYCLOOXYGENASES IN TUMORIGENESIS AND ITS POSSIBILITY IN ANTICANCER THERAPY (A review)

Ledecká, K., Hajurka, J., Ledecký, V.

University of Veterinary Medicine, Komenského 73, 041 81 Košice
The Slovak Republic

katarinaledecka@yahoo.com

ABSTRACT

We summarised the role of inflammatory reaction and chronic irritation in cancer development and described the mechanism of action of cyclooxygenase (COX) in tumorigenesis. Subsequently we discuss about therapeutic possibilities using nonsteroidal antiinflammatory drugs. Although the precise mechanisms of the present results are still obscure, more detailed pharmacological profiling of COX inhibitors may be required for their rational clinical application.

Key words: anticancer therapy; cyclooxygenases; non-steroidal antiinflammatory drugs

INTRODUCTION

Cyclooxygenases (COX_s), also known as prostaglandin H synthases or prostaglandin endoperoxide synthases, are fatty-acid oxygenases of the myeloperoxidase superfamily. They are most closely related to the pathogen inducible oxidases and linoleate dial synthases of plants and fungi (D a i y a s u and T o h: 12). The purification of COX-l (then called simply COX) from sheep (H e m l e r *et al.*: 25) and bovine (M i y a m o t o *et al.*: 44) seminal vesicles led to the cloning of the COX-l gene in 1988 (Y o k o y a m a and T a n a b e: 66, M e r l i e *et al.*: 39, De W i t t and S m i t h: 14). For many years, it was thought that the constitutively active COX-l protein was the only cyclooxygenase in eukaryotic cells, but in 1991 a second, inducible enzyme was identified, which is now called COX-2 (X i e *et al.*: 65, K u j u b u *et al.*: 31).

COX-l is ubiquitously and constitutively expressed in mammalian tissues and cells, where as COX-2 is highly inducible and is generally present in mammalian tissues at very low levels, unless increased by one of many types of stimuli such as cytokines and growth factors (C h a n d r a s e k h a r a n and S i m m o n s: 10). Both COX_s are largely located on the lumenal side of the endoplasmatic reticulum (ER) membrane and the nuclear envelope although they have also been detected in some situations in lipid bodies, mitochondria filamentous structures, vesicles and in the nucleus (B o z z a *et al.*: 9, L i o u *et al.*: 34, L i o u *et al.*: 35). The lumen of the ER is important for both the structure and function of COX_s: its oxidative potental allows formation of the disulfide bonds of the enzymes and N-linked glycosylation (which occurs in the ER) appears to be necessary for proper protein folding (O t t o *et al.*: 46). More over, the final product of COX_s, prostaglandin H_2 is sufficiently non-polar to difuse through the membrane of the ER to isomerases located on the cytosolic surface of the ER or in the cytosol.

Both classes of COX_s are bifunctional enzymes with two distinct catalytic activities: cyclooxygenase (or bis-dioxygenase) activity and peroxidase activity. The primary products of COX_s were first detected in human seminal fluid by clinicians studying uterine contractions (G o l d b l a t t: 19). Thought to be the product of the prostate gland were given the name prostaglandins. They are synthesized in virtually all tissues in vertebrates that lack prostate glands. Thus, in many respects the term prostaglandin is a misnomer. Initially, the enzym activity that synthetized prostaglandins was frequently called prostaglandin synthase, but because it does not require ATP it is now called prostaglandin G/H synthase to fit the nomenclature

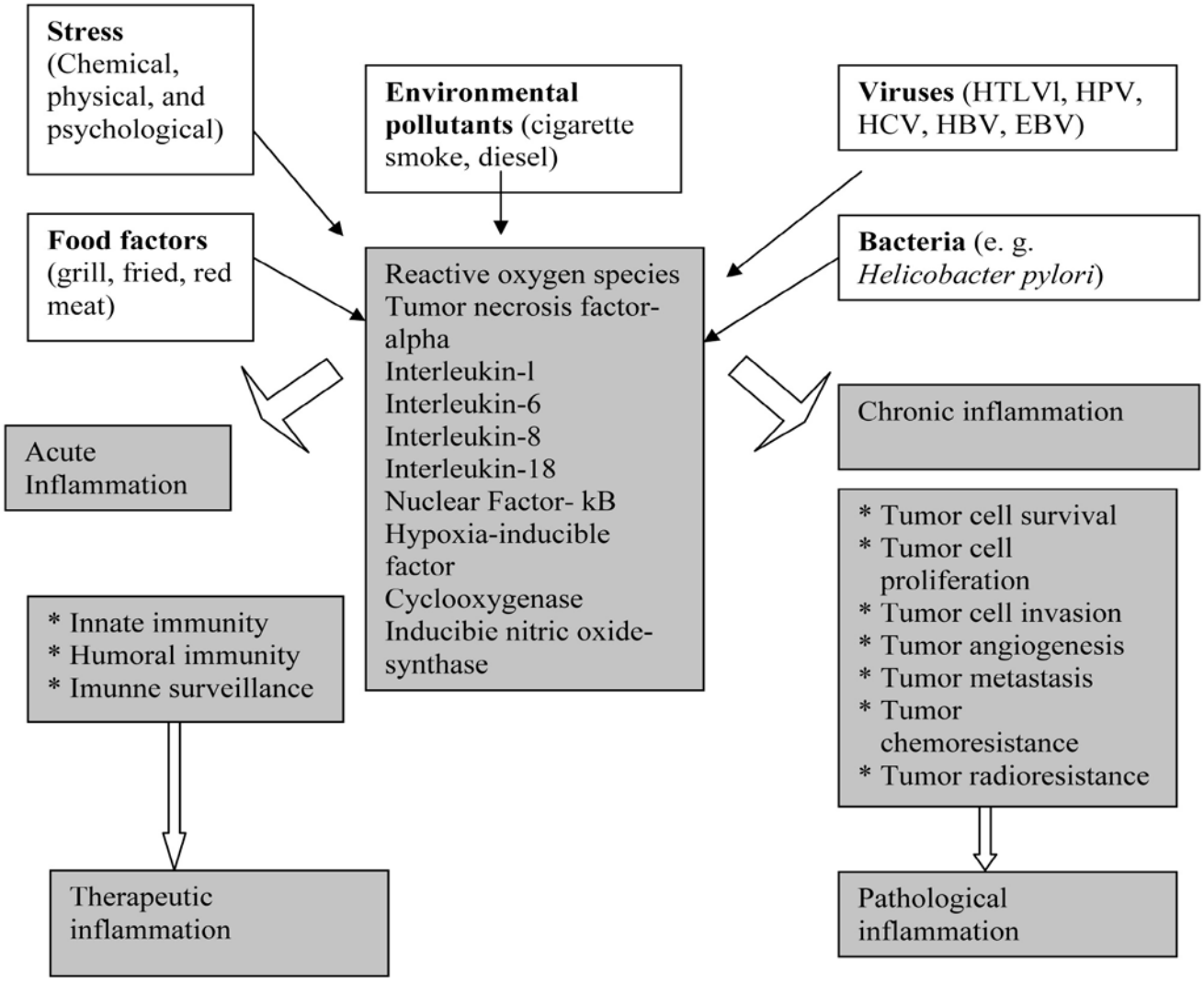


Fig. l. Different faces of inflammation and its role in tumorigenesis (A g g a r w a l *et al.*, 2006)

convention. It is more popularly known as cyclooxygenase, a name that only partially describes the enzyme since it refers to only one of its two enzymatic activities (C h a n d r a s e k h a r a n and S i m m o n s: 10).

The cyclooxygenase activity of COX_s oxygenates arachidonic acid to produce prostaglandin G_2. The perioxidase activity of COX_s then reduces this to prostaglandin H_2. This is the root of prostaglandin from which prostaglandin isomers such as tromboxane and prostaglin are made by dowstream synthese, *via* isomerisation and oxidation or reduction reactions. Cyclooxygenases have short catalytic life span (frequently 1–2 minutes) because the enzyme is autoinactivated (C h a n d r a s e k h a r a n and S i m m o n s: 10).

The exact distinct functions of COX-l and COX-2 are still being unraveled. There is increasing evidence for the involvement of COX_s in the development and progression of cancer, Alzheimers diseases and other pathophysiological conditions. Development of therapeutic and other diagnostic tools to treat these diseases is being actively investigated.

INFLAMMATION AND CANCER

Although inflammation has been known as localised protective reaction of tissue to irritation, injury or infection, characterized by pain, redness, swelling, and sometimes loss of function, there has been a new realization about its role in a wide variety of diseases, including cancer (A g g a r w a l *et al.*: 3).

When acute inflamation or fever is manifested for a short period of time, it has a therapeutic consequence. However, when inflammation becomes chronic or last too long, it might be harmful and may lead to disease. The role of proinflammatory cytokines, chemokines, adhesion molecules and inflammatory enzymes have been linked with chronic inflammation (Figs. l, 2). Chronic inflammation has been found to mediate a wide variety of pathological conditiones, including cardiovascular, pulmonary, neurogical, autoimmune disorders, also in cancer diabetes and arthritis (A g g a r w a l: 2).

Chronic inflammation has been linked for various steps

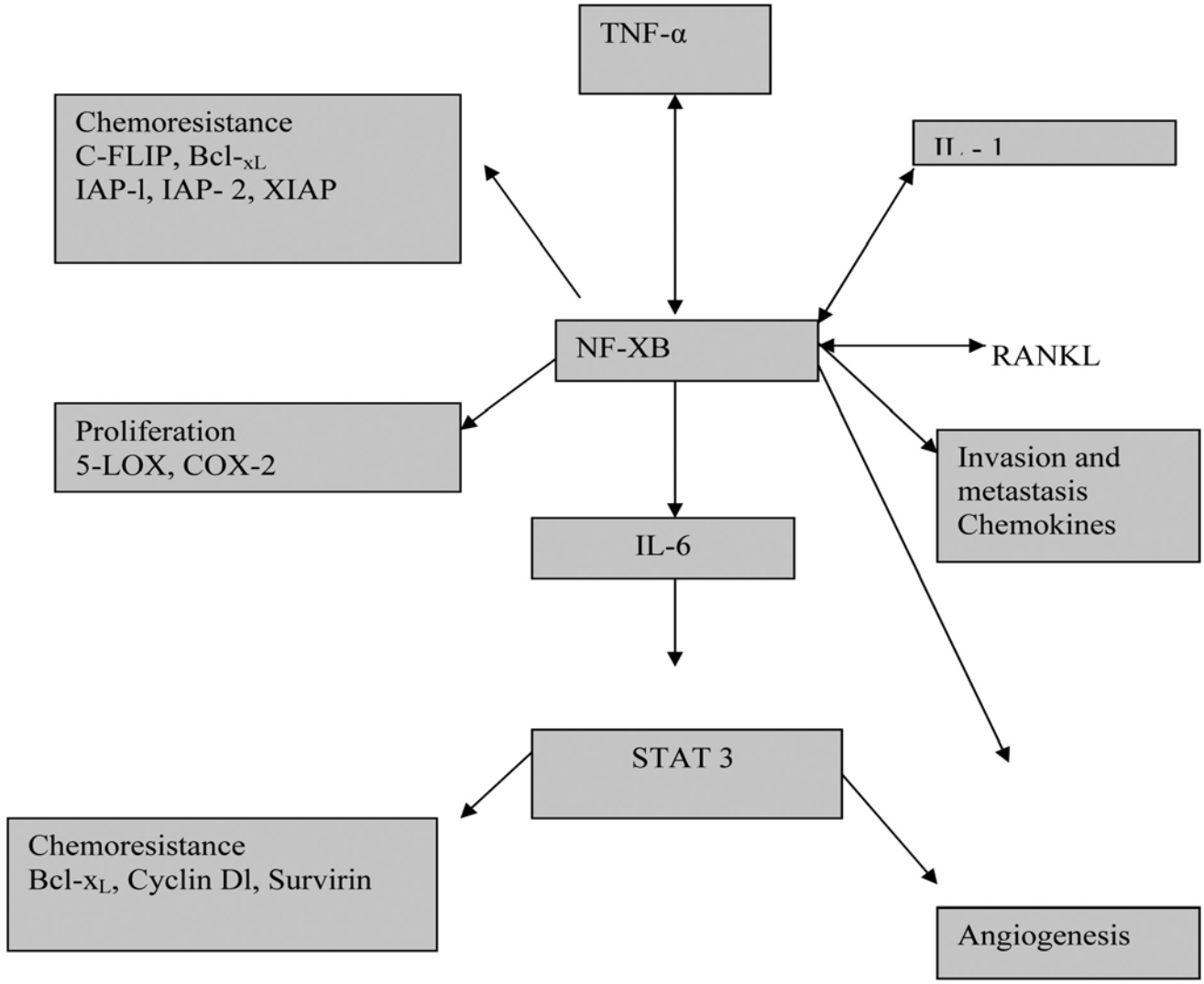


Fig. 2. Inflammatory networking in cancer (Aggarwal *et al.,* 2006)

involved in tumorigenesis, including cellular transformation promotion, survival, proliferation, invasion, angiogenesis and metastasis (Mantovani: 38, Coussens and Werb: 11). The inflammation is a risk factor for most types of cancer and include various inflammatory intermediates responsible for the steps leading to formation of tumors their growth and metastasis.

Role of tumor necrosis factor

Tumor necrosis factor (TNF-α) was first isolated as an anticancer cytokine more than two decades ago (Aggarwal: 1). When expressed locally by cells of the immune system, TNF-α has a therapeutic role. However, when dysregulated and secreted in the circulation, TNF-α can mediate a wide variety of diseases including cancer (Aggarwal: 1). TNF-α has been shown to be one of major mediators of inflammation induced by a wide range of pathogenic stimuli, TNF-α induced other inflammatory mediators and proteases that orchestrate inflammatory responses (Balkwill: 5). TNF-α is also produced by tumors and can act as an endogenous tumor promoter (Balkwill: 5). The role of TNF-α has been linked to all steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Fig. 2: Aggarwal *et al*: 3).

Role of cyclooxygenases in tumorigenesis

Cyclooxygenase-2 an inducible enzyme with expression regulated by NF-κB, mediates tumorigenesis. COX-2, the inducible isoform of prostaglandin H synthase, has been implicated in the growth and progression of variety of cancers. Enhanced COX-2 expression has been found in colon cancer tissues in human with clinically diagnosed colorectal cancer (Sano *et al.*: 49, Tsuji *et al.*: 60, Tsuji *et al.*: 61, Eisinger *et al.*: 16). Cyclooxy-

genase regulates colon carcinoma-induced angiogenesis by two mechanisms: COX-2 can modulate production of angiogenic factors by colon cancer cells, while COX-l regulates angiogenesis in endothelial cells (E i n s p a h r *et al.*: 15).

COX-2 expression in human tumors can be induced by various growth factors, cytokines, oncogenes, and other factors. IL-lβ has been reported to upregulate COX-2 expression in human colorectal cancer cells *via* multiple signaling pathways (L i u *et al.*: 36). COX-2 over expression reduces apoptotic susceptibility by inhibiting the cytochrome C-dependent apoptotic pathway in human colon cancer cells (S u n *et al.*: 58). Paradoxically, COX-2 overexpression can also inhibit death receptor 5 expression and confers resistance to TRAIL-induced apoptosis in human colon cancer cells (T a n g *et al.*: 59).

COX-2 is expressed at an intermediate or high level epithelial cells of invasive breast cancers cell (H a l f *et al.*: 22, L u *et al.*: 37). Expression of COX-2 in breast cancer correlates with poor prognosis and COX-2 enzyme inhibitors reduce breast cancer incidence in human. COX-2 overexpression has been also found in the mammary gland of transgenic mice induced mammary cancer (K u n d u and F a l t o n: 32). COX-2 also plays an important role in the progression of human lung adenocarcinoma (W o l f f *et al.*: 68). Both survivin and COX-2 are overexpressed and they seem to be early events in the occurence of endometrial adenocarcinoma (E r k a n l i *et al.*: 17). COX-2 and iNOS expression has been observed in human ovarian tumors and in tumor-associated macrophages (K l i m p *et al.*: 36). COX-2 expression levels in tumor specimens from patients with low-and high-grade astrocytomas indicated a correlation between the percentage of COX-2 expression and patient survival (S h o n o *et al.*: 50). These findings indicate that high COX-2 expression in tumor cells is associated with clinically more aggressive gliomas and it is strong predictor of poor survival.

S u b b a r a y a n *et al.* (57) compared and contrasted the expression levels and subcellular distribution paterns of COX-l and COX-2 in normal prostate (prostate epithelial cell (PrEC), prostate smooth muscle – (PrSm), and prostate stromal (PrSt) primary cell cultures and prostatic carcinoma cell lines (PC-3, LNCaP, and Du 145). The basal COX-2 mRNA and protein levels were high in normal PrEC and low in tumor cells, unlike many other normal cells and tumor cells. They concluded that COX-2 expression may be important to PrEC cell function although it is low in stromal and tumor cells, COX-2 expression is induced by TNF-α in these cells, and this responsiveness may play on important role in prostate cancer progression.

COX-2 is also expressed in 93% of melanomas, with a moderate to strong expression in 68% (D e n k e r t *et al.*: 13). Increased expression of COX-2 plays a functional role in the development and progression of malignant epithelial cancers (G o u l e t *et al.*: 20). COX-2 appears to play an important role as well as in gastrointestinal carcinogenesis, and COX-2 overexpression has been demonstrated both in oesophageal adenocarcinomas and in the metaplastic epithelium of Barretts oesophagus. It has been reported that inhibition of COX-2 supressed growth and induced apoptosis in human oesophageal adenocarcinoma cells (S o u z a *et al.*: 56). COX-2 expression has been reported in 91% of the squamous cell, carcinomas and in 78% of the oesophageal adenocarcinomas (Z i m m e r m a n n *et al.*: 70).

It has also been found that both cox isoforms may be involved in the pathogenesis of oesophageal adenocarcinoma, as they are linked to the expression of important modulators of angiogenesis (VEGF-A) and lymphangiogenesis (VEGF-C) (von R a h d e n *et al.*: 64). COX-2 in RNA and protein expression has been found in 9 of 10 cases of adenocarcinoma of the pancreas, but not in nontumorous pancreatic tissue (T u c k e r *et al.*: 62). Potential involvement of COX-2 pathway also contribute to tumor angiogenesis in hepatocellular carcinoma (Z a o *et al.*: 69). Human gastric adenocarcinoma tissues also contain significantly higher levels of COX-2 mRNA as compared with paired gastric mucosal specimens devoid of cancer cells (R i s t i m a k i *et al.*: 47). COX-2 expression was not found in normal. Urinary bladder samples but was detected in invasive transitional cell carcinomas of the urinary bladder (M o h a m m e d *et al.*: 40).

Role of NSAIDs in cancer prevention and therapy

Inflammation is the way of the body which is dealing with infections and tissue damage, but there is a fine balance between the beneficial effects of inflammation cascades and their potential for long-term tissue destruction. If they are not controlled or resolved, inflammation cascades can lead to development of disease or tumor (Fig.l). (A g g a r w a l *et al.*: 3, Fig.l). Within many inflammation cascades or pathways there are often pivotal molecular targets that, when antagonized or mentralized, block the output of the pathway. A relatively small number of pivotal targets have been identified that have yielded any successful antiinflammatory drugs. Most of these are antagonists of endogenous proinflammatory mediators such as prostaglandins, leukotrienes and histamine. These targets include the H1 receptor for histamine, the enzymes cyclooxygenase 1 and 2, the cytokine tumor necrosis factors-α, and receptor for cysteinyl leukotrienes C4 and D4.

This table lists all major anti-inflammatory targets organised according to the class of target (enzymes, G-protein-coupled receptors, nuclear hormone receptors, cytokines, and cell adhesion molecules and co-stimulatory molecules with specific targets).

Abbreviations: COX, cyclooxygenase, IMPDH, inosine monophosphate dehydrogenase; NSAIDS, nonsteroidal anti-inflamatory drugs, CysLT1, cysteinyl leukotriene l; histamine l; TNF-α tumor necrosis factor-α, TNF-R II, tumor necrosis factor α receptor II, IL, interleukin. IL-2R, interleukin 2 receptor; IL-lRA, interleukin l receptor

Table l. Major anti-inflammatory targets (by class) (S i m o n s, 2006)

Target class	Specific targets
Enzymes	COX-2
	COX-l and COX-2
	IMPDH
G-protein-coupled receptors	CysLTl
	H1
Nuclear hormone receptors	Corticosteroids
Cytokines and cytokine receptors	TNF-α and TNF-RII
	IL-lβ and IL-lRA
	IL-2 and IL-2R
	Interferon α2
	Interferon β1
	Interferon γ
Cell interaction molecules (cell adhesion molecules and co-stimulatory molecules)	LFA-1 and CD11a
	CD2 and LFA-3
	VLA-4 and CD49d
	CTLA-4-ig

antagonist. LFA-l, leukocyte function-associated antigen-l; VLA-4, very late activation antigen 4; LFA-3, leukocyte function-associated antigen 3; CTLA4-Ig; cytotoxic T-lymfocyte antigen 4 immunoglobin chimera.

Prostaglandin research underwent a dramatic paradigm shift in early 90's in that regulation of COX enzyme levels was recognized as a major control point in the biosynthesis of prostanoids (B a i l e y *et al.*: 6, A l-b r i g h t s o n *et al.*: 4). Cyclooxygenases (also known as prostaglandin the synthases or prostaglandin endoperoxide synthases) are all close to 600 amino acids in size and have a similar primary structure (S i m m o n s *et al.*: 53). COX is a bifunctional enzyme catalysing cyclo-oxygenation and peroxidation of arachidonic acid derived from cellular membranes. The COX and peroxidase activities occur at distinct but structurally and functionally related sites. COX-l is expressed constitutively, fulfilling a housekeeping role in most cells, and contributes to cellular responses that require instanteneous or continual regulation.

In 1991, a second enzyme with similar activity was discovered (K u j u b u *et al.*: 31). This second enzyme, COX-2, was dubbed in the inducible form. Although COX-2 is undetectable in most mammalian tissues, it is found constitutively in certain specific tissues such as brain, kidney, testicles and tracheal epithelium. Both COX isoforms can be expressed simultaneously in the same cell, although not all cells in each tissue will show expression of either COX-1 or COX-2 (S m i t h *et al.*: 52).

Prostaglandins synthetized by the COX-l pathway are responsible for cytoprotection in the stomach, vasodilation, in the kidney and production of proagregatory prostanoid tromboxane by platelets. Generally, COX-2 plays a role in inflammation, ovulation, growth and differentiation of cells and carcinogenesis. There have been developed some specific to inhibit COX-2 which are commonly used today (H a y e s: 23).

In 1800, the pain relieving efects of willow bark, which was chewed to obtain its medical benefits by the ancient Greeks, was well known. The active ingredient salicin, the precursor of acetylsalicylic acid (aspirin), was later identified as the active substance and is still used as nonsteroidal anti-inflammatory drug (NSAID). The discovery of the action of NSAIDs showed their ability to inhibit COX, was found by J. Vane in 1970s, who's work was later awarded the Nobel Prize (V a n e: 63). The link with cancer prevention and NSAIDs is based on evidence in human gastrointestinal tumours. In 1983, a case report documented the disappearance of colorectal polyps in one patient treated by common NSAID combination for pain relief (W a d d e l l and L o u g h r y: 67). Studies have shown that NSAID sulindac (a non-specific COX-2 inhibitor) can reduce both the size and the number of polyps, in patients, and trend that was reversed when treatment was with drawn (L a b a y l e *et al.*: 33, G i a r d i e l l o *et. al.*: 18).

COX inhibition in cancer treatment and prevention in animals

Studies documenting both COX-2 overexpression and response to NSAID treatment mainly comprise small number of animals are nonrandomized and collate date which have been generated retrospectively. These are valid criticisms that authors readily acknowledge, and randomized, prospective, controled trials with ultimately be required to uncontrolled trials will ultimately be required to answer many of questions surrouding COX-2 and cancer development and progression (H a y e s: 23).

Several lines of evidence should be explored. The first step is to determine whether a particular animal cancer expresses COX-2 over and above that expressed by normal, noncancerous tissue. Then we can seek how the inhibition of COX-2 slows cancer development or progression or increases survival time. There is a question of whether it is actually COX-2 that matters or other yet to be identified molecular events or alteration occuring downstream. Does COX-2 over expression result in increased enzyme activity and increased downstream PG overproduction and is the mechanism by which we detect COX-2 actually an accurate in demonstrating this step?

Few studies address all these problems, but some answers are beginning to emerge (H a y e s: 3). COX-2 expression or/and levels of PGE2 have been examined in various canine malignities. The effect of the COX-inhibiting NSAID piroxicam, have been evaluated in the treatment of canine transitional cell carcinoma of the urinary bladder (K n a p p *et al.*: 27, K n a p p *et al.*: 28, M o h a m m e d *et al.*: 41, M o h a m m e d *et al.*:

42). In this spontaneously occuring tumor in dogs, there was a reduction in tumor volume in 12/18 dogs treated with piroxicam (K n a p p *et al.*: 27).

In a randomized study, when piroxicam was combined with cisplatin, there was a significant increase in remision rates (K n a p p *et al.*: 28). Piroxicam alone (S c h m i d t *et al.*: 51) and in combination with cisplatin (B o r i a *et al.*: 8) has also been evaluated as therapy for canine oral squamous cell carcinoma. Remission was induced in 17.6 % (3/17) dogs given piroxicam alone and in 55.6 % (5/9) dogs receiving piroxicam and cisplatin.

In dogs treated with piroxicam and cisplatin for oral malignant melanoma, remission was induced in 18.2 % (2/11) dogs (B o r i a *et al.*: 8). Sixteen dogs with prostatic carcinoma that received either piroxicam or carprofen were shown to have a survival advantage of 6.9 *versus* 0.7 months (PL 0.0001) in a similar number of untreated dogs (S o r e n m o *et al.*: 55). Subjective response were seen in a group of 15 dogs treated with piroxicam suppositories for colorectal adenocarcinoma (K n o t t e n b e l t *et al.*: 30). In dogs who underwent treatment of lymphoma, a piroxicam/doxorubicin combination resulted in no survival advantage over dogs receiving doxorubicin alone (M u t s a e r s *et al.*: 43).

Evidence of COX-2 expression in feline tumors is sparse. COX-2 immunohistochemical expression was not shown in feline cutaneous squamous cell carcinoma, mammary, pulmonary and intestinal adenocarcinoma, lymphoma or vaccine-associated sarcoma, but has been reported in oral squamous cell carcinoma and transitional cell carcinoma (B e a m *et al.*: 7, H a y e s *et al.*: 24).

Predicting which tumour is likely to respond to which NSAID therapy and the drug dose required to achieve a measurable cell kill is not straight forward. Similarly the amount of drug required to inhibit cell growth *in vitro* may not relate directly to the dose which the patient requires or may tolerate. Knowing the relative inhibition of COX-l *versus* COX-2 can be perhaps helpful in anticiparting of tumor response and side-effects, but on accurate comparison between drugs is difficult to establish with currently availabe data. Selectivity (anti-COX-l *versus* anti-COX-2 activity) of the drug is dose dependant and interspecies variations in pharmacodynamics and pharmacokinetics exist such as an oral dose of one drug in one species may have different effects than in other species. Compared with humans, dogs and cats seem to be more sensitive to effects of NSAIDs, which may occure because of enterohepatic recirculation and relative inability to metabolize NSAIDs owing to poor glucoronyl transferase activity (J o n e s and B u d s b e r g: 26).

Definitive tissue diagnosis must be obtained in all cases, the full extent of disease should be determined and conventional surgical and medical options such as chemotherapy and radiation therapy should be considered. In some cases, it may be logical to use a COX-2 inhibiting drug along side conventional anticancer treatment, however, there are dangers in assuming that an NSAID can be choosen strategically to offer anticancer effects.

Tumors that have been to shown overexpress COX-2, respond to COX-2 inhibiting therapy or have a survival advantage when treated with COX-2 inhibitors (H a y e s: 23). However, routine COX-2 testing of tumors is not commercially available. Additionally, inherent tumour heterogenity. Both within a group of tumour histologies an even within an individual tumor exist for COX-2 expression. Lastly, recent work also indicates that cancer inhibition may in fact take place even in the absence of COX-2 expression (S a n c h e z - A l c a z a r *et al.*: 48, K n o t t e n b e l t *et al.*: 30). The picture becomes even less clear with the speculation that in some cancers, COX-l rather than COX-2 plays a role in tumorigenesis and thus cancer treatment (G u p t a *et al.*: 21) and also that COX-1 and COX-2 derived prostanoids may play differential or sometimes antagonistic roles in the late phase of acute inflammation (N a k a n o *et al.*: 45).

REFERENCES

1. Aggarwal, B. B., 2003: Signalling pathways of the TNF superfamily: a double-edged sword. *Nat. Rev. Immunol.*, 3, 745–750.

2. Agggarwal, B. B., 2004: Nuclear factor-Kappa B: the enemy within. *Cancer Cell*, 6, 203–208.

3. Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K., Sethi, G., 2006: Inflammation and cancer: How hot is the link? *Biochem. Pharmacology*, 72, 1605–1621.

4. Albrightson, C. R., Baenziger, N. L., Needleman, P. A., 1985: Mononuclear cell factor is potent agonist of prostaglandin biosynthesis in cultured human fibroblasts and smooth muscle and endothelial cells. *Adv. Prostaglandin Thromboxam Leukot. Res.*, 15, 213–216.

5. Balkwill, F., 2002: Tumor necrosis factor or tumor promoting factor? *Cytokine Growth Factor Rev.*, 13, 135–141.

6. Bailey, J. M., Muza, B., Hla, T., Salata, K., 1985: Restoration of prostacyclin synthase in vascular smooth muscle cells after aspirin treatment: regulation by epidermal growth factor. *J. Lipid. Res.*, 26, 54–61.

7. Beam, S. L., Rassnick, K. M., Moore, A. S., McDonough, S. P., 2003: An immunohistochemical study of cyclooxygenase-2 expression in various feline neoplasms. *Vet. Pathol.*, 40, 496–500.

8. Boria, P. A., Murry, D. J., Bennet, P. F., Glickman, N. W., Snyder, P. W., Merkel, B. L., Schittler, D. L., Mutsaers, A. J., Thomas, R. M., Knapp, D. W., 2004: Evaluation of cisplatin combined with piroxicam for the treatment of oral malignant melanoma and oral squamous cell carcinoma in dogs. *J. A. V. M. A.*, 224, 388–394.

9. Bozza, P. T., Yu, W., Penrose, J. F., Morgan, E. S., Dvorak, A. M., Weller, P., 1997: Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation. *J. Exp. Med.*, 186, 909–920.

10. Chandrasekharan, N. V., Simmons, D. L., 2004: The cyclooxygenases. *Genome Biology*, 5, article 241.

11. Coussens, L. M., Werb, Z., 2002: Inflammation and cancer. *Nature*, 420, 860–867.

12. Daiyasu, H., Toh, H., 2000: Molecular evolution of the meyloperoxidase family. *J. Mol. Evol.*, 51, 433–445.

13. Denkert, C., Kobel, M., Berger, S., Siegert, A., Leclere, A., Trefzer, U., Hauptmann, S., 2001: Expression of cyclooxygenase-2 in human malignant melanome. *Cancer Res.*, 61, 303–308.

14. De Witt, D. L., Smith, W. L., 1988: Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Nath. Acad. Sci USA*, 85, 1412–1416.

15. Einspahr, J. G., Krause, R. S., Yochim, J. M., Danenberg, P. V., Danenberg, P. V., Bhattacharyya, A. K., Martinez, M. E., Alberts, D. S., 2003: Association between cyclooxygenase expression and colorectal adenoma characteristics. *Cancer Res.*, 63, 3891–3893.

16. Eisinger, A. L., Prescott, S. M., Jones, D. A., Stafforini, D. M., 2007: The role of cyclooxygenase-2 and prostaglandins in colon cancer. *Prostaglandin-other lipid mediators*, 82, 147–154.

17. Erkanli, S., Bolat, F., Kayaselcuk, F., Demihran, B., Kuscu, E., 2007: Cox-2 and survivin are overexpressed and positively correlated in endometrial carcinoma. *Gyn. Oncology*, 104, 320–325.

18. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R., Offerhaus, G. J., 1993: Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *New England J. Med.*, 328, 1313–1316.

19. Goldblatt, M. M., 1993: A depresor substance in seminal fluid. *J. Soc. Chem. Ind.*, 52, 1056–1057.

20. Goulet, A. C., Einsphar, J. G., Alberts, D. S., Beas, A., Burk, C., Bhattacharyya, A., Bangert, J., Harmon, J. M., Fujiwara, H., Kaki, A., Nelson, M. A., 2003: Analysis of cyclooxygenase-2 (COX-2) expression during malignant melanoma progression. *Cancer Biol. Ther.*, 2, 713–718.

21. Gupta, S., Srivastava, M., Ahmad, N., Boostwick, D. G., Munktar, H., 2000: Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma. *Prostate*, 42, 73–78.

22. Half, E., Tang, X. M., Gwyn, K., Sahin, A., Wathen, K., Sinicrope, F. A., 2002: Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma *in situ*. *Cancer Res.*, 62, 1676–1681.

23. Hayes, A., 2007: Cancer, cyclo-oxygenase and nonsteroidal anti-inflamatory drug can we combine all three? *Veterinary Comparative Oncology*, 5, 1–13.

24. Hayes, A., Scase, T., Miller, J., Murphy, S., Sparkes, A., Adams, V., 2006: Cox-l and Cox-2 expression in feline oral squamous cell carcinoma. *J. Comparative Pathol.*, 135, 93–99.

25. Hemler, M., Lands, W. E. M., Smith, W. L., 1976: Purification of the cyclooxygenase that form prostaglandins: demonstration of two form of iron in the holoenzyme. *J. Biol. Chem.*, 251, 5575–5579.

26. Jones, C. J., Budsberg, S. C., 2000: Physiologic characteristics and clinical importance of the cyclooxygenase isoforms in dogs and cats. *J. A. V. M. A.*, 217, 721–729.

27. Knapp, D. W., Richardson, R. C., Chan, T. C., Bottoms, G. D., Widmer, W. R., DeNicola, D. B., Teclaw, R., Bonney, P. L., Kuczek, T., 1994: Piroxicam therapy in 34 dogs with transitional cell carcinoma of the urinary bladder. *J. Vet. Int. Med.*, 8, 273–278.

28. Knapp, D. W., Glickman, N. W., Widmer, W. R., DeNicola, D. B., Adams, L. G., Kuczek, T., Bonney, P. L., De Gortari, A. E., Han, C., Glickman, L. T., 2000: Cisplatin *versus* cisplatin combined with piroxicam in a canine model of human invasive urinary bladder cancer. *Cancer Chemotherapy Pharmacol.*, 46, 221–226.

29. Klimp, A. H., Hollema, H., Kempinga, C., Van der Zee, A. G., de Vries, E. G., Daemen, T., 2001: Expression of cyclooxygenase-2 and inducible nitric oxide synthase in humans ovarian tumors and tumor-associated macrophages. *Cancer Res.*, 61, 7305–7309.

30. Knottenbelt, C., Chambers, G., Gault, E., Argyle, D. J., 2006: The *in vitro* effects of piroxicam and melocixam on canine cell lines. *J. Small Anim. Practice*, 47, 14–20.

31. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., Herschman, H. R., 1991: TIS 10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.*, 266, 12866–12872.

32. Kundu, N., Fulton, A. M., 2002: Selective cyclooxygenase (COX)-l or COX-2 inhibitors control metastatic disease in murine model of breast cancer. *Cancer Res.*, 62, 2343–2346.

33. Labayele, D., Fischer, D., Vielh, P., Drouhin, F., Pariente, A., Bories, C., Duhamel, O., Trousset, M., Attali, P., 1991: Sulivac causes regression of rectal polyps in familial adenomatous polyposis. *Gastroenterology*, 101, 635–639.

34. Liu, J. Y., Shyue, S. K., Tsai, M. J., Chung, C. L., Chu, K. Y., Wu, K. K., 2000: Colocalisation of prostacyclin synthase with prostaglandin H synthase-l (PGHS-l) but not phorbol ester-induced PGHS-2 in cultured endothelial cells. *J. Biol. Chem.*, 275, 15314–15320.

35. Liou, J. Y., Deng, W. G., Gilroy, D. W., Shyne, S. K., Wu, K. K., 2001: Cocolonisation and interaction of cyclooxygenase-l with caveolin-l in human fibroblasts. *J. Biol. Chem.*, 276, 34975–34982.

36. Liu, W., Reinmuth, N., Stoettzing, O., Parikh, A. A., Tellez, C., Williams, S., 2003: Cyclooxygenase-2 is up regulated by interleukin-l beta in human colorectal cancer cells *via* multiple signaling pathways. *Cancer Res.*, 63, 3632–3636.

37. Lu, S., Yu, G., Zhu, Y., Archer, M. C., 2005: Cyclooxygenase-2 overexpression in MCF-10F human breast epithelial cells inhibits proliferation, apoptosis and differentiation, and causes partial transformation. *Int. J. Cancer*, 116, 847–852.

38. Mantovani, A., 2005: Cancer: Inflammation by remote control. *Nature*, 435, 752–753.

39. Merlie, J. P., Fagan, D., Mudd, J., Needleman, P., 1988: Isolation and characterisation of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). *J. Biol. Chem.*, 263, 3550–3553.

40. Mohammed, S. I., Knapp, D. W., Bostwick, D. G., Foster, R. S., Khan, K. N., Masferrer, J. L., Woerner, B. M., Snyder, P. W., Koki, A. T., 1999: Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. *Cancer Res.*, 59, 5647–5650.

41. Mohammed, S. I., Bennett, P. F., Craig, B. A., Glickamn, N. W., Mutsaers, A. J., Snyder, P. W., Widmer, W. R., De Gortari, A. E., Bonney, P. L., Knapp, D. W., 2002: Effects of the cyclooxygenase inhibitor piroxicam on tumor response

apoptosis and angiogenesis in a canine model of human invasive urinary bladder cancer. *Cancer Res.*, 62, 356–358.

42. Mohammed, S. I., Craig, B. A., Mutsaers, A. J., Glickman, N. W., Snyder, P. W., DeGortari, A. E., Schlittler, D. L., Cofman, K. T., Bonney, P. L., Knapp, D. W., 2003: Effects of the cyclooxygenase inhibitor piroxicam in combination with chemotherapy on tumor response, apoptosis and angiogenesis in a canine model of human invasive urinary bladder cancer. *Molecular Cancer Therapeutics*, 2, 183–188.

43. Mutsaers, A. J., Glickman, N. W., DeNicola, D. B., Widmer, W. R., Bonney, P. L., Hahn, K. A., Knapp, D. W., 2002: Evaluation of treatment with doxorubicin and piroxicam or doxorubicin alone for multicentric lymphoma in dogs. *J. A. V. M. A.*, 220, 1813–1817.

44. Miyamoto, T., Ogino, N., Yamamoto, S., Hayaishi, O., 1976: Purification prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.*, 251, 2629–2636.

45. Nakano, M., Denda, N., Matsumoto, M., Kawamura, M., Kawakubo, Y., Hatanaka, Ko., Hiramoto, Y., Sato, Y., Noshiro, M., Harada, Y., 2007: Interaction between cyclooxygenase (COX)-l and COX-2 products modulates COX-2 expression in the late phase of acute inflammation. *European J. Pharmacology*, 559, 210–218.

46. Otto, J. C., De Witt, D. L., Smith, W. L., 1993: N-Glycosylation of prostaglandin endoperoxide synthase-l and -2 and their orientations in the endoplastic reticulum. *J. Biol. Chem.*, 268, 18230–18242.

47. Ristimaki, A., Honkanen, N., Jankala, H., Siponen, P., Karkonen, M., 1997: Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res.*, 57, 1276–1280.

48. Sanchez-Alcazar, J. A., Bradbury, D. A., Pang, L., Knox, A. J., 2003: Cyclooxygenase (COX) inhibitors induce apoptosis in non-small lung cancer through cyclooxygenase indepent pathways. *Lung Cancer*, 40, 33–44.

49. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., Hla, T., 1995: Expression of cyclooxygenase-l and -2 in human colorectal cancer. *Cancer Res.*, 55, 3785–3789.

50. Shono, T., Tofilon, P. J., Braner, J. M., Owolabi, O., Lang, F. F., 2001: Cyclooxygenase-2 expression in human gliomas: prognostic significance and molecular correlations. *Cancer Res.*, 61, 4375–4381.

51. Schmidt, B. R., Glickman, N. W., DeNicola, D. B., DeGortari, A. E., Knapp, D. W., 2001: Evaluation of piroxicam for the treatment of oral squamous cell carcinoma in dogs. *J. A. V. M. A.*, 218, 1783–1786.

52. Smith, W. L., Garavito, R. M., De Witt, D. L.,1996: Prostaglandin endoperoxide H synthases (cyclooxygenases)-1- 2. *J. Biol. Chem.*, 271, 33157–33160.

53. Simmons, D. L., 2006: What makes a good anti-inflammatory drug targets? *Drug Discovery Today*, 11, 210–219.

54. Simmons, D. L., Botting, R. M., Hla, T., 2004: Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol. Rev.*, 56, 387–437.

55. Sorenmo, K. U., Goldschmidt, M. H., Shofer, F. S., Goldkamp, C., Ferracone, J., 2004: Evaluation of cyclooxygenase-l and cyclooxygenase-2 and the effect of cyclooxygenase inhibitors in canine prostatic carcinoma. *Vet. Comparat. Oncology*, 2, 13–23.

56. Souza, R. F., Shewmake, K., Beer, D. G., Cryer, B., Spechler, S. J., 2000: Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human oesophageal adenocarcinoma cells. *Cancer Res.*, 60, 5767–5772.

57. Subbarayan, V., Sabichi, A. L., Llansa, N., Lippman, S. M., Menter, D. G., 2001: Differential expression of cyclooxygenase-2 and its regulation by tumor necrosis factor-alpha in normal and malignant prostate cells. *Cancer Res.*, 61, 2720–2726.

58. Sun, Y., Tang, X. M., Half, E., Kuo, M. T., Sinicrope, F. A., 2002: Cyclooxygenase-2 overexpression reduces apoptopic susceptibility by inhibiting the cytochrome C-dependent apoptopic pathway in human colon cancer cells. *Cancer Res.*, 62, 6323–6328.

59. Tang, X., Sun, Y. F., Half, E., Kuo, M. T., Sinicrope, F. A., 2002: Cyclooxygenase-2 overexpression inhibits death receptor 5 expression and confers resistence to tumor necrosis factor-related apoptosis-inducing ligandinduced apoptosis in human colon cancer cells. *Cancer Res.*, 62, 4903–4908.

60. Tsuji, M., Kawano, S., DuBois, R. N., 1997: Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potencial. *Proc. Natl. Acad. Ser.*, 94, 3336–3340.

61. Tsuji, M., Kawano, S., Tsujii, S., Sawaoka, H., Hori, M., Du Bois, R. N., 1998: Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*, 93, 705–716.

62. Tucker, O. N., Dannenberg, A. J., Yang, E. K., Zhang, F., Teng, L., Daly, J. M., Soslow, R. A., Masferrer, J. L., Woerner, B. M., Koki, A. T., Fahey, T. J., 1999: Cyclooxygenase-2 expression in up-regulated in human pancreatic cancer. *Cancer Res.*, 59, 987–990.

63. Vane, J. R., 1971: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs. *Nature: New Biology*, 231, 232–235.

64. Von Rahden, B. H., Stein, H. J., Puhringer, F., Koch, I., Langer, R., Piontek, G., 2005: Coexpression of cyclooxygenases (COX-l, COX-2) and vascular endothelial growth factors (VEGF-A, VEGF-C) in esophaegeal adenocarcinoma. *Cancer Res.*, 65, 5038–5044.

65. Xie, W., Chipman, J. G., Robertson, D. L., Erikson, R. L., Simmons, D. L., 1991: Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by in RNA splicing. *Proc. Natl. Acad. Sci. USA*, 88, 2692–2696.

66. Yokoyama, C., Tanabe, T., 1989: Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. *Biochem. Biophys. Res., Commun.*, 165, 888–894.

67. Waddell, W. R., Loughry, R. W., 1983: Sulindac for polyposis of the colon. *J. Surg. Oncology*, 24, 83–87.

68. Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H., Ristimaki, A., 1998: Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res.*, 58, 4997–5001.

69. Zao, Q. T., Yue, S. Q., Cui, Z., Wang, Q., Cui, X., Zhai, H. H., Zhang, L. H., Dou, K. F., 2007: Potential involvement of the cyclooxygenase-2 pathway in hepatocellular carcinoma-associated angiogenesis. *Life Sci.*, 80, 484–492.

70. Zimmermann, K. C., Sarbia, M., Weber, A. A., Borchard, F., Gabbert, H. E., Schror, K., 1999: Cyclooxygenase-2 expression in human oesophageal carcinoma. *Cancer Res.*, 59, 198–204.

Received September 6, 2007

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 197–201, 2007

SOME ASPECTS OF MORPHOGENESIS (A Review)

Petrovová, E.[1], Lešník, F.[1, 2], Luptáková L.[1]
Flešárová, S.[1], Bálent P.[1], Valenčáková A.[1]

[1]University of Veterinary Medicine, Komenského 73, 041 81 Košice
The Slovak Republic
[2]Institute of Animal Physiology and Genetics AS CR, Rumburská 89, 277 21 Liběchov
The Czech Republic

uvl13ster@gmail.com

ABSTRACT

The biological process of morphogenesis is a process in which living systems produce forms and structures through mechanical and biological factors including morphogens. Various negative regulating factors can come into these processes. Teratogenesis and consequently malformation, eventually death of embryo or foetus can be a result. One of the morphogenetic systems is the patterning along the future head to tail (anteroposterior) axis of the fruit fly *Drosophila melanogaster*. A group of genes known as homeobox genes has emerged as important master regulators of development. Apoptosis is also known as "programmed cell death" because in many cases the patches of cells die in a particular location of the embryo at a specific time in development and plays an important role in morphogenesis. Morphogens can be neurotransmitters when they act as dose-dependent morphogenic signals in neural and non-neural tissues. In animal organisms morphogenetic responses may be induced by hormones, or by environmental chemicals ranging from substances produced by other organisms to toxic chemicals or radionuclides released as pollutants.

Key words: apoptosis; embryo; morphogenesis; morphogens

INTRODUCTION

Morphogenesis of animal eukaryotic multicellular organism is a complicated multistage process at the molecular, physiological and morphological levels. Various negative regulating factors can come into these processes. Teratogenesis and consequently malformation, eventually death of embryo or foetus can be a result (13).

Morphogenesis is a one of the three aspects of developmental biology along with the control of cell growth and cellular differentiation. Morphogenesis is concerned with the shapes of tissues, organs and entire organisms and the positions of the various specialized cell types (18). Cell growth and differentiation can take place in cell culture or inside of tumor cell masses without the normal morphogenesis that is seen in an intact organism.

The study of morphogenesis involves an attempt to understand the processes that control the organized spatial distribution of cells that arises during the embryonic development of an organism and which give rise to the characteristic forms of tissues, organs and overall body anatomy. Another class of molecules involved in morphogenesis are molecules that control cell adhesion (16). For example, during gastrulation clumps of stem cells switch off their cell-to-cell adhesion, become migratory, and take up new positions with an embryo where they again activate specific cell adhesion proteins and form new tissues and organs (7).

GENES AND MORPHOGENESIS

Homeobox genes

It is a fascinatig thought that the single cell zygote contains all the information required for the development of the adult organism. Understandig how this information is encoded and deciphered is a major uncompleted scientific challenge. A group of genes known as homeo-

box genes has emerged as important master regulators of development. Homeobox is about 180 base pairs and encodes a protein domain (homeodomain) which can binds DNA in a specific manner. Homeobox genes encode transcription factors which typically switch on cascades of other genes (15). These genes have been highly conserved throughout evolution. They are expressed during embryonic development in highly co-ordinated manner and continue to be expressed in virtually all tissues and organs throughout adult life (10).

One of the best understood morphogenetic systems is the patterning along the future head to tail (antero-posterior) axis of the fruit fly *Drosophila melanogaster*. The development of *Drosophila* is particurlarly well studied, and it is representative of one major class of insects. Other multicellular organisms sometimes use similar mechanisms for axis formation, although the relative importance of signal transfer between the earliest cells of many developing organisms is greater than in *Drosophila* (11).

In the *antennapedia* mutation the antennae are changed into legs, whereas in the *bithorax* mutation is transformed into part of a wing. These changes were described as homeotic transformations from the Greek word homeosis, signifying a change of a complete body structure into another. Subsequently *Drosophila* was found to contain a cluster of genes consisting of the *bithorax* complex with three homeobox genes: *Ultrabithorax* (Ubx), *Abdominal-A (Abd-A)*, and *Abdominal-B (Abd-B)* and the *antennapedia* complex with five homeobox genes: *Labial (Lab), Proboscipaedia (Pb), Deformed (Dfd), Sex comb reduced (Scr),* and *Antennapaedia (Antp)*. The relationship between the chromosomal arrangement of HOX genes and localisation of their expression was established by Lewis in 1978 (14). In effect, these genes specify positional identity of the body segments of the fly along the antero-posterior axis.

Evolution of HOX genes

Homeobox genes are present in the genomes of all animals which have so far been mapped as well as in the genomes of plants and fungi, indicating that the origins are ancient and precede the divergence of these kingdoms. Plants, fungi and unicellular animals do not, however, have clustered homeobox genes. Shortly after the origins of animals the primordial homeobox gene duplicated to form a protohox cluster of two genes which are still present in cnidara such as hydra. Sponges do not have clustered homeobox genes, suggesting that this duplication occurred before the divergence of the parazoa. This is also reflective of the very simple body structure of sponges compared to other multicellular animals.

The nematode *Caenorhabditis elegans* has a single cluster of at least five homeobox genes (21). *Amphioxus* is a vertebrate-like chordate which has a notochord and segmental muscles derived from somites but does not develop a true vertebral column. It has only one HOX cluster which contains ten HOX genes and this cluster is regarded as being homologous to the ancestral cluster from which all vertebrate HOX clusters were derived. Two duplication events, early in vertebrate evolution, resulted in the four clusters seen in mammals and birds. Loss of some of the HOX genes in each cluster has also occurred with the result that not every primordial vertebral HOX gene is represented in each of the four clusters. Interestingly in some fish, such as zebrafish, a further duplication has occurred resulting in seven clusters (19).

HOX genes in vertebrates

The vertebrate counterparts of the *bithorax*/*antennapedia* cluster are the HOX genes, usually found in four clusters. In man the four HOX gene clusters (A–D) are located on different chromosomes, at 7p15, 7q21.2, 12q13, and 2q31. Each cluster consists of 13 paralog groups with nine to eleven members assigned on the basis of sequence similarity and relative position within the cluster. A high degree of homology is evident between the human HOX genes and the HOM-C genes of *Drosophila*.

Mice and human have on 4 HOX clusters (a total 39 genes in humans) located on four different chromosomes:

– In mice: HOX1, HOX2, HOX3, HOX4
– In humans: HOXA, HOXB, HOXC, HOXD

As in *Drosophila*, they act along the developing embryo in the same sequence that they occupy in the chromosome. In the mammalian HOX clusters all the genes show some sequence homology to each other but very strong sequence homology to the equivalent genes in *Drosophila* (10).

On the basis of these evidence homeodomain proteins as the products of homeobox genes, regulated embryonic development (6).

HOX genes and development

The order of expression of HOX genes within a cluster is co-ordinated during development, so that the low number, 3' genes, are expressed more anteriorly and earlier than high number, 5' genes. During embryogenesis, cells require positional information to ensure that uncommitted location within the developing embryo. Thus groups of cells, known as functional domains, become committed to form body structures such as limbs and organs. There is growing evidence that it is the combination of HOX genes expressed within the functional domains along the antero-posterior axis which results in specifying the development of structures within these domains. In both *Drosophila* and man the spatial patterning corresponds to the relative position on the chromosome, thereby conforming to the "principle of colinearity" (9).

In the developing vertebrate HOX genes are first expressed during early gastrulation at a stage when the embryo generates its major body axis (4). In a pattern

which correlates with the spatial expression of HOX genes, 3' genes are expressed earlier than 5' and as the embryo develops more progressively 5' genes are expressed. This pattern is termed "temporal colinearity" and is evident in other models of development such as haematopoiesis (10).

APOPTOSIS AND MORPHOGENESIS

As well as regions of high proliferation there are also areas that have a high rate of cell death. Probably in all embryonic tissues there is a continuous loss of cells through death, but in certain parts of the developing body the death rate outstrips the proliferative rate. This means that just as some regions become enlarged by rapid cell division, others become eroded away. The dying cells undergo a process of breakdown and gradually become phagocytosed by other cells. Apoptosis is also known as "programmed cell death" because in many cases the patches of cells die in a particular location of the embryo at a specific time in development and play an important role in morphogenesis.

Two classical examples in the chick occur in the shaping of the wing bud and of the toes. Apoptotic cell are found in normal embryos even as early as gastrulation (1) and after that in many well-defined sites in the differentiating tissues (mesonephros, heart, nervous system, sclerotome, neural crest, tail bud, branchial arches, lateral body wall). In most of these examples cell death is focused on a highly localized region and occurs within a restricted period of time, e. g. cell death within the endocardial cushions of the developing heart occurs only between about embryonic days 5.5–7.5 in the chick embryo (2).

MORPHOGENS

Several types of molecules are particularly important during morphogenesis. Morphogens are soluble molecules that can diffuse and carry signals that control cell differentiation decisions in a concentration-dependent fashion. Morphogens typically act through binding to specific protein receptors. An important class of molecules involved in morphogenesis are transcription factor proteins that determine the fate of cells by interacting with DNA. These can be coded for be master regulatory genes and either activate or deactivate the transcription of other genes and, in turn, these secondary gene products can regulate the expression of still other genes in a regulatory cascade (20).

Morphogens can be neurotransmitters when they act as dose-dependent morphoghenic signals in neural and non-neural tissues (2).

Several neurotransmitters may coexist together inside individual neurons, where usually one neurotransmitter is accompanied by one or more neuropeptides. "Prenervous" neurotransmitter present in the same embryonic cells may act as multifunctional regulators, which is the additional important property of neurotransmitters. Neurotransmitters can affect in the same way as the morphogens that control the positive regulation of embryo development (12).

Neurotransmitter molecules are present in a wide variety of animal species. Throughout development, thus giving support to their role as signal molecules controlling various basic cellular processes. Neurotransmitters take up new functions, ending up in the nervous system as mediators of synaptic communication. It is well known that neurotransmitter synthesis as well as neurotransmitter receptor expression are activated in an early phase of neurogenesis, before the formation of synaptic contacts, in several regions of the nervous system (3).

XENOBIOTICS AND MORPHOGENESIS

In animal organisms morphogenetic responses may be induced by hormones, or by environmental chemicals ranging from substances produced by other organisms to toxic chemicals or radionuclides released as pollutants.

Cholinesterase inhibitors

A well-known neurotransmitter is acetylcholine (ACh), a concentional synaptic neurotransmitter, chemical substance, which neurons in the brain use to communicate with one another. Acetylcholinesterase (AChE) enzyme normally degrades unused ACh, by breaking it into its components – acetate and choline. Cholinesterase inhibitors (ChEI) is a group of chemical compounds, which inhibits ACh hydrolysis by AchE, thereby ACh accumulates in the reactive locations of a living organism.

Great numbers of known pesticides on the basis of carbamate or organophosphates belong to group of ChEI (12).

Bendiocarbamate

Bendiocarbamate is a carbamate insecticide, which belongs among the ChEIs. Moreover reproductive, teratogenic, mutagenic and carcinogenic effects of this insecticide have been studied. Every carcinogenesis and teratogenesis is connected with an initiation and promotion step and with a common accumulated mutation. Therefore bendiocarbamate is a suitable model for study from multiple views, including its influence in animal morphogenesis (12).

2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)

Angiogenesis and neovascularization are a specific example of morphogenesis and describe the recruitment and proliferation of vascular endothelial cells from existing vascular system in order to develop a new vascular

network. Angiogenesis occurs during the natural course of foetal development, wound healing as well as in tissue regeneration. A pathological 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) is known to be the most toxic congener among the dioxin and related compounds found in the environment.

Exposure to TCDD causes a diverse spectrum of toxicities in humans and laboratory animals. The foetus is one of the most sensitive targets of TCDD and exhibits a wide range of biological responses at low TCDD levels that have no detectable effects on maternal side. The incidence has been shown in all species studied to date, including the monkey, hamster, rat and mouse. One of the most severe adverse effects of TCDD is intrauterine foetal death. The increased incidence of the foetal death is due to reduced blood flow into the placenta because blood vessel formation in the placenta was severely impaired by the TCDD. Interferon gene re-expression is strongly up-regulated by the TCDD whereby interferon is known to be involved in the regulation of angiogenesis. Interferon alpha- and -gamma inhibit endothelial cell proliferation (17).

Toxicants during lung development

Exposure to a variety of toxicants during lung development has the potential to significantly affect the overall growth and function of the respiratory system. The target of a toxic insult to the lung during development is likely to involve the disruption and alteration of a specific molecular signal or transcription factor, but to date, little information is available as to the precise effect of such exposures. Timing of exposure during development appears to be critical in the subsequent effects observed (8).

Cellular differentiation, branching morphogenesis and overall lung growth encompass several different phases of lung development. A growing body of evidence suggests that these processes can be affected by exposure to chemicals. However, the effects of exposure are likely to be different during each phase of development. For example, during embryogenesis and progressive stages of foetal development, cell number, cell type and cell function of the airways and alveoli continue to change, and may be differentially affected based on the timing of the exposure (5).

Since cells continue to differentiate and divide during the postnatal period, chemical exposure during the postnatal period is also likely to impact on the respiratory system in a different fashion, based on further changes in the cellular differentiation and anatomical growth. Although less is known about the effects of toxicants in the developing lung, a number of toxicants are known to affect the developing lungs. These include environmental tobacco smoke (5, 8), bioactivated compounds, and oxidant gases. The target for a number of these compounds is largely airway epithelial cells undergoing maturation and rapid proliferation (8).

ACKNOWLEDGEMENT

The present work was carried out within the framework of the grant of VEGA MŠ SR No. 1/4430/07 of the Slovak Ministry of Education.

REFERENCES

1. Bellairs, R., 1961: Cell death in the chick embryo as studied by electron microscopy. *J. Anat.*, 95, 54–60.

2. Bellairs, R., Osmond, M., 2005: In *The Atlas of Chick Development*. Elsevier Academic Press edn., California, 42–43.

3. Buznikov, G. A., Shmukler, Y. B., Lauder, J. M., 1996: From oocyte to neuron: do neurotransmitters function in the same way throughout development? *Cell. Molec. Neurobiol.*, 16, 532–559.

4. Duboule, D., 1992: The vertebrate limb: a model system to study the Hox/HOM gene network during development and evolution. *Bioassays*, 14, 375–384.

5. Gebremichael, A., Chang, A., Buckpitt, A. R., Plopper, C. G., Pinkerton, K. E., 1995: Postnatal development of cytochrome P 450 1A1 and 2B1 in rat lung and liver: effect of aged and diluted sidestream cigarette smoke. *Toxicol. Appl. Pharmacol.*, 135, 246–253.

6. Gehring, W. J., Affolter, M., Burglin, T. R., 1994: Homeodomain proteins. *Ann. Rev. Biochem.*, 63, 487–526.

7. Ingber, D. E., 2006: Mechanical control of tissue morphogenesis during embryological development. *Int. J. Dev. Biol.*, 50, 255–266.

8. Joad, J. P., Bric, J. M., Peake, J. L., Pinkerton, K. E., 1999: Perinatal exposure to aged and diluted sidestream cigarette smoke produces airway hyperresponsiveness in older rats. *Toxicol. Appl. Pharmacol.*, 155, 253–260.

9. Kmita, M., Duboule, D., 2003: Organizing axes in time and space; 25 years of colinear tinkering. *Science*, 301, 331–333.

10. Lappin, R. J., Grier, D. G., Thompson, A., Hallidey, H. L., 2006: Hox genes: seductive science, mysterious mechanisms. *Ulster. Med. J.*, 75, 23–31.

11. Lawrence, P. A., Morata, G., 1994: Homeobox genes: their function in *Drosophila* segmentation and pattern formation. *Cell*, 78, 181–189.

12. Lešník, F., 2005: A medical view of agrochemicals. *Folia Veter.*, 49, 4–6.

13. Lešník, F., Danko, J., Legáth, J., Ucekaj, N., 2004: *Agrochemicals and morphogenesis* (In Slovak) Slov. vet. čas., 29, 24–25.

14. Lewis, E. B., 1978: A gene complex controlling segmentation in *Drosophila*. *Nature*, 276, 565–570.

15. Lodish, H. F., 2003: In *Molecular Cell Biology*. W. H. Freeman and Company edn., New York.

16. Lukáš, Z., Dvořák, K., 2003: Adhesion molecules in biology and oncology. *Acta Vet. Brno*, 73, 93–104.

17. Mizutani, T., Yoshino, M., Satake, T., Nakagawa, M., Ishimura, R., Tohyama, C. *et al.*, 2004: Identification of 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD)-inducible and-suppressive genes in the rat placenta: Induction of interferon-regulated

genes with possible inhibitory roles for angiogenesis in the placenta. *Endoc. J.*, 51, 569–577.

18. Olovnikov, A. M., 1996: Molecular mechanism of morphogenesis: a theory of locational DNA (In Russian). *Biokhimiya*, 61, 1948–1970.

19. Schilling, T. F., Knight, R. D., 2001: Origins of anteroposterior patterning and Hox gene regulation during chordate evolution. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 356, 1599–1613.

20. Tabata, T., Takei, Y., 2004: Morphogens, their identification and regulation. *Development*, 131, 703–712.

2**1. Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J.** *et al.*, **1994:** 2,2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans. *Nature*, 368, 32–38.

Received November 17, 2007

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 202–207, 2007

THE IONOPHOROUS ANTICOCCIDIALS USED IN POULTRY (A Review)

Váczi, P., Neuschl, J., Čonková, E.

University of Veterinary Medicine, Komenského 73, 041 81 Košice, The Slovak Republic

vaczi@uvm.sk

ABSTRACT

In this report we would like explain some attributes of ionophorous anticoccidials used in poultry production. The certain substances from this group became already the tradition in broiler poultry breeding; the others are adversely novel and less known. In several sections we present the history, general mode of action, toxicity and the benefits and disadvantages of polyether antibiotics. Beyond this, the concise characteristics of individual drugs are introduced. These are for example the name, spectrum of activity and dosage.

Key words: anticoccidials; ionophores; poultry

INTRODUCTION

Significant economic losses caused by coccidiosis in poultry industry come forward above all countries with intensive production of poultry meat, where large number of poultry is concentrated in a relatively small area. The objective of majority of farmers is to produce as many broiler chickens per 1 m^2 as possible, of the largest weight and with minimal costs. It has been proven, that the most advantageous procedure is life-long administration of anticoccidials from early age. The prevention of coccidiosis by long-term administration of anticoccidials has lots of disadvantages associated with high financial expenses for anticoccidials, quick development of resistance – development of resistant coccidia, leaving residues in meat and eggs, environmental impacts. Increased concentrations of some anticoccidials (also in case of uneven distribution of anticoccidials in feed mixtures) can have undesirable effects showed by retarded growth, as well as death of whole flocks, etc.

In spite of these disadvantages, actually safer anticoccidials are used and in chicken farms they occupy important position. Ionophores meet higher criteria for anticoccidials, also the very high ones, regarding the range of their efficiency, residues, resistance development, price, and the important fact being their toxic consequences on animals and humans. Moreover, ionophores show positive impact on increasing body weight gain, which is welcome by the breeders.

IONOPHORES AND THE MODE OF THEIR ACTION

These substances have been discovered in 1950s and their anticoccidial efficacy was proven in the late 1960s (52). Very soon after that discovery, due to its wide range of efficiency and growing resistance development in other substances, they started to be heavily used (43).

They are categorized as carboxylic polyether antibiotics (5, 15). Most ionophorous anticoccidials are fermentation products of *Streptomyces* spp. and *Actinomadura* spp. Ionophores have been successfully used in production of broilers for 30 years, due to their excellent effect against coccidia infecting poultry (37, 38). They are the main group of feed additives and at present they are considered the most efficient and widely used coccidiostats.

The mode of their action consists in the fact that they create lipophylic complexes with various alkali metal cations, above all with natrium, potassium and

calcium and transfer them in biological membranes as well as across them, which explains their activity (46, 53). Simply said, irreversible damage of the natrium pump occurs, followed by energetic exhaustion of the cells damaged, as a result of its increased activity. They affect prokaryotic and eukaryotic cells. Ionophores affect extracellular stages, because sporozoites and merozoites, which penetrate the host cells, escape from the effect of these compounds. Intracellular stages of parasitic protozoa significantly lose their capability to produce oocysts. Their main activity shows during early asexual stages of developing coccidia (8, 30).

RESISTANCE TO IONOPHORES

Speed of resistance development differs in the individual ionophores. The onset of resistance in poultry industry can be limited by using two methods of anticoccidial administration, which are equal from the practical aspect. The first one is termed the "shuttle program", which recommends continual administration of two or several drugs during the life of a flock. It is commonly practiced in many countries in the effort to reduce the selection on resistance. Another method, termed the "rotation program" consists in administration of one substance during one period, and in the next one this is altered for another drug (12, 30).

In general it is known that by using ionophorous antibiotics, the natural immunity or resistance does not develop, however, development of resistance is just a question of time (39). Monensin, narasin and salinomycin are considered products of the same group (monovalent polyether antibiotics), that is why resistance development is assumed. At lower frequency, also crossed resistance with other groups of ionophores occurs (33).

Each ionophorous anticoccidial drug has strong points and weak points regarding its range and potential. Monensin, salinomycin and narasin are heavily active ones against *E. acervulina* and they are less appropriate for severe infection caused by *E. tenella* and *E. maxima*. Lasalocid and maduramicin are more efficient against *E. tenella* and show weaker activity against *E. acervulina* (33).

TOXICITY OF IONOPHORES

In recent years, polyether antibiotics are used successfully. Their therapeutic range is not large; however, some of them have also strict toxicity in some species and age categories of poultry. The most susceptible ones are considered turkeys older than 9 weeks. For that reason, the requirement of high safety in administration and keeping preventive concentration of those drugs in feed mixtures is justified (19).

On one side, there is development of resistant coccidia species and onset of coccidiosis at insufficient dosage, on the other side there is a chance of acute or chronic intoxications in feeding excessive concentrations.

In relation to that fact, it is necessary to consider the fact that the efficiency and toxicity of certain drug as well as other drugs, depends not only of its dosage, pharmacological profile, functional state of biological systems, but of any other endogenous and exogenous factors, including drug form (19, 34, 35). The same quantity of the efficient substance contained in the drugs of the same type produced by different producers does not yet guarantee their same effect and tolerability (49).

Toxicity of any ionophorous antibiotics is known worldwide for decades. Some cases of overdosing mammals with ionophorous anticoccidials have been published, most often it was acute intoxication, and however, some describe also chronic intoxication (31, 40). Different authors describe anamnesis and clinical symptoms of acute intoxication by ionophores, which are most frequently related to significant degeneration of myocardium (20). Horses and rabbits are extremely sensitive to acute intoxications.

Well known are intoxications by ionophorous anticoccidials in poultry (1, 3, 4). Mild exceeding of the recommended preventive doses causes growth retardation. Administration of even higher doses can end up by acute poisoning. Characteristic symptom of overdose by ionophorous anticoccidials in poultry is lying in the sternal position with extremities stretched backwards and lowered wings (50). It is caused by high affinity of ionophores to muscle cells of the skeletal muscles, as well as myocardium (32).

Besides the concentration used, toxicity of ionophores is affected also by other factors, overall health condition, immunological status, breed, age, sex. From exogenous factors above all zoohygienic and microclimatic situation, drug form and the way of binding the efficient substance to the carriers, contents of some elements in feed (presence of natrium, and potassium increase toxicity), interaction with other drugs (furasolidone, virginiamycin, tiamulin, erythromycin, oleandomycin, sulphonamides), as well as the presence of another ionophore simultaneously (above all with salinomycin, monensin and semduramicin) and also some antioxidants (48).

IONOPHORES AND OTHER FARM ANIMALS

Ionophores were used in cattle and pigs as substances supporting growth (28). Although they were originally developed as coccidiostatics exclusively for poultry (16), later they showed as advantageous feed additives for cattle increasing conversion of feed and total revenues of meat production. It is explained by the ability of ionophores to reduce microbial degradation of proteins in the rumen, and this way to arrange their digestion in other parts of digestive tract – the so called bypass-protein (16). They decrease production of methane and also they prevent acidosis in animals that are rapidly changed

from roughage diets to high carbohydrate diets. In monogastric animals they considerably decrease microbial colonisation in gastrointestinal tract (36).

IMPACT ON WEIGHT GAINING

Ionophores are a part of feed mixtures used above all in the categories of broiler poultry. They improve feed conversion, resulting in increased live weight accompanied by larger musculature of the parts most valuable from consumers' aspect (breasts and thigh muscles). E. g. in chicken they start to be formed approximately from the 21st day of age, immediately after stopping administration of chemical anticoccidials, it means in the phase, when the largest growth of physical weight is expected (49).

Watkins and Bafundo (51) compared narasin with monensin and salinomycin. During 40 days these anticoccidials were continually administered to broiler chickens. The concentration of the individual drugs was administered in the recommended concentrations and they were the following: narasin 63 $mg.kg^{-1}$ and 72 $mg.kg^{-1}$, monensin 100 $mg.kg^{-1}$ and salinomycin 60 $mg.kg^{-1}$ in the feed. The results showed almost the same effect of all the anticoccidials used for increasing the weight of chicken, conversion of feed and mortality. No significant differences were observed in affecting the parameters observed.

In general, in all the ionophores the withdrawal period of 5 days is observed, maximum residue limit in meat have been so far determined only for lasalocid (22).

CLASSIFICATION OF IONOPHORES

So far 6 polyether antibiotics are known, as effective in prevention of coccidiosis: monensin, lasalocid, salinomycin, narasin, maduramicin and semduramicin. According to cation preference and chemical structure they are divided in 3 groups:

1. monovalent polyethers: *monensin, narasin, salinomycin*
2. monovalent glycoside polyethers: *maduramicin, semduramicin*
3. divalent polyethers: *lasalocid* (23)

REVIEW OF IONOPHOROUS ANTICOCCIDIALS

Lasalocid

Chemical name of lasalocid is: (Na-6-3R, 4S, 5S, 7R)-7-[2S, 3S, 5S)-5-ethyl-5-[(2R, 5R, 6S)-5-ethyl-5-hydroxy-6-methyltetrahydro-2H-pyran-2-yl]-tetrahydro-3-methyl-2-furyl]-4-hydroxy-3,5-dimethyl-6-oxononyl]-2,3-cresoate.

Lasalocid is used in the form of natrium salt as feed additive. It is a product of fermentation of *Streptomyces lasaliensis* subsp. *lasaliensis*. It is used at the concentration of 75–125 $mg.kg^{-1}$ in the feed.

Lasalocid sodium, as well as other ionophorous antibiotics, has the ability to bind to different divalent cations as well as monovalent ions, affecting their transport through biological membranes and this way it causes osmotic unbalance. This causes disturbance of normal physiological processed in the cell of the parasite. Sporozoites of coccidia exposed to the effect of lasalocid, in the lumen of the intestine, show significant swelling of their surface and creation of different pathological changes encouraging development of potentially lethal damages. After attacking the cells of intestinal mucosa, these disruptive changes occur only on the surface of sporozoites, because lasalocid natrium in the concentration recommended acts selectively and it remains relatively harmless to the lost cells.

Lasalocid is available as 15 % premix under the name Avatec150G and it affects sporozoites, early non sexual and later sexual stages *E. tenella, E. necatrix, E. acervulina, E. brunetti, E. mivati* and *E. maxima* (14). Withdrawal period of lasalocid is 5 days (23, 24). It is transmitted in eggs of hens that have been fed lasalocid for 1 week. European Union permits the maximum residue limit of lasalocid residues in poultry meat and eatable organs. In muscle the maximum residue limit is 20 $\mu g.kg^{-1}$, in skin and fat 100 $\mu g.kg^{-1}$, liver 100 $\mu g.kg^{-1}$ and kidneys 50 $\mu g.kg^{-1}$ (10).

Monensin

Chemical name of monensin is: 2-(5-ethyl-tetrahydro-5-(tetrahydro-3-methyl-5-tetrahydro-6-hydroxy-6-(hydroxy-methyl)-3,5-dimethyl-2H-pyran-2-yl-2-furyl)-2-furyl)-9-hydroxy-β-methoxy-α, γ-2,8-tetramethyl-1, 6-dioxaspiro (4, 5) decane-7-butyrate.

Monensin is used as feed additive to prevent and control coccidiosis in poultry. This polyether ionophore is monovalent and it started to be used in 1970s. Originally marked as monensin acid, later it was assigned the name monensin (52). It has wide range anticoccidal effect, which is mostly coccidiocidic.

Monensin sodium is very efficient, produced by *Streptomyces cinnamonensis*. Besides coccidia it acts against some Gram-positive bacteria. It is used for coccidioses caused by *E. acervulina, E. brunetti, E. maxima, E. necatrix, E. tenella* and *E. mivati* in broilers as feed additive at the concentrations of 99–121 $mg.kg^{-1}$. Monensin is contained in the preparation Elancoban G 100 and Elancoban G 200.

Mode of action is not different from other ionophores. Significant being high affinity to natrium ions, which is ten times higher that to potassium ions (23, 25). This contributes to the fact, that monensin has strong positive inotropic effect on myocardium of the host (3, 41, 44, 47).

In 1980s was developed the resistance of *E. meleagrimitis* parasiting on turkey at American farms, where monensin was administered in the dose 100 $mg.kg^{-1}$ during 20 months, in another case in the dose 60 $mg.kg^{-1}$ during 8 months (17).

Monensin is apparently not transmitted to eggs of hens fed monensin for one week (26), nor does it adversely affect egg production or quality (18). Monensin is also efficient to control coccidiosis in lambs and calves (6, 11, 29). Withdrawal period of monensin is 5 days (21, 23).

Salinomycin

Chemical name of salinomycin is: nitric salt of the ethyl-6-[5-{2-(5-ethyltetrahydro-5-hydroxy-6-methyl-2H-pyrano-2-yl)-15-hydroxy-2, 10, 12-trimethyl-1, 6, 8-trioxadispiro [4, 1, 5, 3] pentadec-13-en-9-yl}2-hydroxy-1,3-dimethyl-4-oxoheptyl] tetrahydroxy-5-methyl-2H-pyran-2-acetoacetic acid.

Salinomycin is monovalent monokarboxyl ionophore, which is produced by the fermentation of *Streptomyces albus* (derived from the type ATCC 21838). It is available as 6.6 % premix used for preventing coccidiosis of broiler chicken, above all against *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti* and *E. mivati* (43). In early phases of its implementing in practice it was found that low concentration of salinomycin highly positively affect weight gain. It is used in concentration 60—66 $mg.kg^{-1}$. It is sold as Sacox 120 microGranulate or Salinomax 120 G.

Mechanism of action and biological activity of salinomycin is based on the capability of this ionophore to create in lipids solvable complexes with monovalent and divalent cations (above all with ions K^+, Na^+ and Rb^+). Salinomycin also acts against sporozoites and early and later non-sexual forms of coccidia in the intestines of chicken (9). Withdrawal period of salinomycin is 5 days (23).

Narasin

Chemical name of narasin is: (αβ, 2β, 3α, 5α, 6α)-α-ethyl-6-[5-[5-(5α-ethyltetrahydro-5β-hydroxy-6α-methyl-2H-pyrane-2β-yl)-3´´α4, 4´´, 5, 5´´α, 6-hexahydro-3´β-hydroxy-3´´β, 5α5´´β-trimethylspiro]furan-2(3H),2´-[2H] pyrane-6´(3´H),2´´-[2H]pyran]6´´α-yl]2α-hydroxy-1α,3β-dimethyl-4-oxoheptyl]-tetrahydro-3,5-dimethyl-2H-pyran-2-acetic acid.

It is monovalent ionophore, which is fermentation product of *Streptomyces aureofaciens*. It was discovered in 1986. Its structure is similar to the one of salinomycin, it differs only by the presence of the methyl group, which is absent in the molecule of salinomycin, so it is methyl-salinomycin (43).

Mechanism of action consists in creating liposoluble complexes with cations of potassium, natrium and rubidium. Resulting changes in transmembrane ion gradient and in electric potential unfavourably affect metabolic functions in the cell of the parasite. Experimentally it was proven, that narasin acts rather coccidocidic effect. It affects early and later non sexual stages of coccidia.

It is used for preventing infection of broilers caused by *E. acervulina, E. brunetti, E. maxima, E. necatrix, E. tenella a E. mivati*. It can be administered only to broilers; it is not permitted for other categories of chicken. Narasin should not be fed to adult turkeys. Preventive concentration of narasin in feed was determined 70 mg.kg-1 (60—80 $mg.kg^{-1}$) (18). It is contained in the preparation Maxiban G 160. Withdrawal period of narasin is 5 days (23).

Study targeted at comparing administration of narasin in the way called "shuttle program" and continual administration was taking place in the following countries: Columbia, Brasilia, Venezuela, Thailand, Malaysia, Taiwan and South Africa, in total in 17 384 specimens of one day old broiler chicken. Preventive application of narasin at concentrations of 60 $mg.kg^{-1}$, as well as 70 $mg.kg^{-1}$ decreased the incidence of damaging intestinal mucosa in comparison to the untreated chickens. In both concentrations narasin showed positive impact on body weight gain and feed conversion (12).

Maduramicin

Chemical name of maduramicin is: ammonium salt of (3R, 4S, 5S, 6R, 7S, 22S)-23, 27-didemethoxy-2, 6, 22-tridemethyl-11-O-demethyl-22-[(2,6-dideoxy-3,4-di-O-methyl-β-l-arabino-hexopyranosyl) oxyl]-6-methyloxy-lonomycine A. It is monovalent monoglykoside ionophore, which is fermentation product of *Actinomadura yumaensis*. It was discovered in 1989. Maduramicin is antibiotic with significant commercial value in fighting coccidiosis in the whole poultry processing industry. It is more efficient than other polyether antibiotics used to control coccidiosis of poultry (13), it has stimulating effect for muscle growth and it is coccidostat determined exclusively for feeding broiler turkeys and chickens (45).

It shows extensive anticoccidal activity in prevention and therapy of poultry and it is 10—12 × more efficient that other ionophore antibiotics. As other ionophores, also maduramicin is efficient above all in the stage of sporozoites. This is based on the fact, that in no animals treated by maduramicin the presence of early development stages was not found.

The killing of early stages results in much weaker invasion and also less damage of intestinal epithelium. Further development of sporozoites, which escaped the effect of maduramicin is also slowed and retarded maturation of schizonts, gamonts and oocysts (7). *In vitro* tests showed significant anticryptosporidial activity (2).

It is available as 1 % immixture for preventing coccidiosis of broilers caused by *E. acervulina, E. brunetti, E. maxima, E. necatrix, E. tenella* and *E. mivati*. The advantage is the option to use low concentration in fed, at the concentration of 5 $mg.kg^{-1}$ (18). It is known as Cygro 1 %. Withdrawal period of maduramicin is 5 days (23).

Semduramicin

Chemical name of semduramicin is: (2R, 3S, 4S, 5R, 6S)-tetrahydro-2,4-dihydroxy-6-[(1R)-1-[(2S, 5R, 7S, 8R, 9S)-9-hydroxy-2,8-dimethyl-2-[(2R, 6S)-tetrahydro-5-methyl-5

-[(2S, 3S, 5R, 6S)-tetrahydro-6-hydroxy-3,5, 6-trimethyl-2H-pyran-2-yl]-3-[[(2S, 5S, 6R)-tetrahydro-5-methoxy-6-methyl-2H-pyran-2-yl]-2-furyl]-2-furyl]-1,6-dioxaspirol[4,5]dec-7-yl] ethyl]-5-methoxy-3-methyl-2H-pyran-2-acetic acid.

Semduramicin is monovalent monoglykoside polyether antibiotic produced by *Actinomadura roseorufa* (42). Microorganisms produce semduramicin in the form of diglycoside, this is then semisynthetically modified. However, both forms have the same effect (27).

It is efficient against sporozoites and early and later non sexual stages of coccidia (9). So far it is used in broiler chickens only. It was published, that 25 $mg.kg^{-1}$ semduramicin is efficient equally to 60 $mg.kg^{-1}$ salinomycin. It acts well against *E. acervulina, E. maxima, E. brunetti* and *E. tenella*. It is sold under the name Aviax 5 %. Withdrawal period of semduramicin is 5 days (22, 23).

CONCLUSION

Ionophorous anticoccidials are generally administered to the poultry in feed, to avoid acute diseases and economic losses often related to subacute infections. The advantage of all the ionophores is their positive impact in weight gain of poultry. Anyway, they are ionophores, that belong to substances administered to meat categories of poultry during growth phase and they fill in the longest part of their life. Commercially produced products are prepared by such technologies, that guarantee their perfect capability to be mixed with feed mixtures and this way they guarantee high safety of their application.

ACKNOWLEDGEMENT

This study has been supported by the grant Kega No. 3/3202/05 and by the grant Vega No. 1/349/06.

REFERENCES

1. Andreasen, J. R., Schleifer, J. H., 1994: Salinomycin toxicosis in male turkey breeders. *Avian Dis.*, 39, 638–642.

2. Arrowood, M. J., Xie, L. T., Hurd, M. R., 1994: *In vitro* assays of maduramicin activity against *Cryptosporidium parvum*. *J. Eukar. Microbiol.*, 41, 23.

3. Baird, G. J., Caldow, G. L., Peek, I. S., Grant, D. A., 1997: Monensin toxicity in a flock of ostriches. *Vet. Rec.*, 140, 624–626.

4. Bartov, I., 1994: Effect of growth promoters on monensin toxicity in broiler chicks. *Brit. Poult. Sci*., 35, 123 133.

5. Bergen, W. G., Bates, D. B., 1994: Ionophores: Their effects on production efficiency and mode of action. *J. Anim. Sci.*, 58, 1465–4483.

6. Bergstrom, R. C., Maki, L. R., 1978: Effect of monensin in young crossbred lambs with naturally occurring coccidiosis. *J. Am. Vet. Med. Assoc.*, 165, 288.

7. Cerruti Sola, S., Leoni, A., Agostini, A., Castagnaro, M., 1996: Efficacy of maduramicin against turkey coccidiosis in battery: a clinical and pathological study. *Schweiz. Arch. Tierheilkde*, 138, 201–206.

8. Chapman, H. D., 1993: 21 years of monensin for control of coccidiosis – a review. *Proceeding of 6th International Coccidial Conference*. Gueph; Canada, 37–44.

9. Conway, D. P., Johnson, J. K., Guyonnet, V., Long, P. L., Smothers, C. D., 1993: Efficacy of semduramicin and salinomycin against different stages of *Eimeria tenella* and *E. acervulina* in the chicken. *Vet. Parasitol*., 45, 215–229.

10. Council regulation (EEC) No. 2377/1990, new consolidated version 02/2007.

11. Fitzgerald, P. R., 1973: Efficacy of monensin against bovine coccidiosis in young Holstein-Friesian calves. *J. Protozool.*, 20, 121.

12. Gunerate, J. R. M., Gard, D. L., 1991: A comparisom of three continuous and four shuttle anticoccidial programs. *Poult. Sci.*, 70, 1888–1894.

13. Gutierrez-Lugo, M. T., Lotina-Hennsen, B., Farres, A., Sanchez, S., Mata, R., 1999: Phytotoxic and photosynthetic activities of maduramicin and maduramicin methyl ester. *Zeitschrift für Naturforschung*, 54, 325–332.

14. Guyonnet, V., Johnson, J. K., Long, P. L., 1990: Studies on the stage of action of lasalocid against *Eimeria tenella* and *Eimeria acervulina* in the chicken. *Vet. Parasitol.*, 37, 93–100.

15. Hirohiko, A., Hideaki, M., Ken-Ichi, H., Makoto, S., Hisao, O., Yoshitomo, I., 1994: Improvement of chemical analysis of antibiotics 21. Simultaneous determination of three polyether antibiotics using high performance liguid chromatography with fluorescence detection. *J. Agricult. Food Chem.*, 42, 112–117.

16. Horton, G. M. J., Mislevy, P., Pitman, W. D., 1992: Ionophore effects on performance of heifers grazing subtropical grass pastures. *J. Prod. Agricult.*, 5, 77–80.

17. Jeffers, J. K., Bentley, E. J., 1980: Monensin sensitivity in recent isolates of turkey coccidian. *Poult. Sci.*, 59, 1722–1730.

18. Jones, J. E., Solis, J., Hughes, B. L., Castaldo, D. J., Toler J. E., 1990: Production and egg quality responses of white leghorn layers to anticoccidial agents. *Poult. Sci.*, 69, 378–387.

19. Julian, R. J., 1991: Poisons and toxins. In *Diseases of Poultry* (9th edn.). **Calnek, B. W., Barnes, H. J., Beard, C. W., Reid, W. M., Yoder, H. W., Jr.** (eds.). Iowa State University Press. Ames, Iowa., 963–984.

20. Kamphues, J., Meyer, H., Lieber, E. M., Johannsen, A., 1990: Animal nutrition for veterinarians – recent cases of clinical disorders in horses after intake of ionofore containing feed. *Dtsch. Tierärzt. Wschr.*, 97, 537–539.

21. Kožárová, I., 2006: Anticoccidial drug residues and safety of animal products derived from poultry. In *Proceedings of Lectures and Posters of the International Scientific Conference "Hygiena Alimentorum XXVII"*, Štrbské Pleso, High Tatras, 18–22 May, 2006, 109–112.

22. Kožárová, I., Máté, D., Sopková, D., 2002: The prevention and control of coccidiosis in poultry rearing. *Folia Veterinaria*, 46, 203–206.

23. Kožárová, I., Máté, D., Pipová, M., Laciaková, A., Jevinová, P., 2003: Legislative treatment of anticoccidial drugs and their residues in poultry. *Slov. Vet. Čas.*, 28, 29–32.

24. Kožárová, I., Máté, D., 2003: Anticoccidial drugs and their residues. In *Proceedings of International Scientific Conference "Food Safety and Quality in Transition Countries and Internet-Portal on Food Safety – Communication systems to ensure food safety and build consumers confidence"*, Nitra, SUA, 24–27 March, 2003, 62–65.

25. Kožárová, I., 2006: Alternatives in control of coccidiosis in poultry rearing – Elimination of the risk of the presence of anticoccidial residues in animal products derived from poultry. In *Proceedings of Lectures and Posters of the 2nd International Scientific Conference "Infectious and Parasitic Diseases of Animals"* (In Slovak), Košice, UVM, 18–20 September, 2006, 299–301.

26. Long, P. L., Sheridan, K., Mc Dougald, L. R., 1981: Maternal transfer of some anticoccidial drugs in the chicken. *Poult. Sci.*, 60, 2342–2345.

27. Mc Dougald, L. R., 1993: Chemotherapy of coccidiosis. *Proceeding of 6th International Coccidial Conference*. Guelph, Canada, 45–47.

28. McKellar, Q., Lawrence, K., 1996: Ionophores. *In practice*, 18, 385–386.

29. Meral, I., 1998: Effects of different concentrations of monensin in the contractility changes of guinea-pig papillary muscle. *Turk. J. Vet. Anim. Sci.*, 22, 145–149.

30. Merck Veterinary Manual. Eighth edition, 2001: Merck and Co., Inc., Whitehaus station, N.Y.; USA, 1826–1831.

31. Miller, R. E., Boewer, W. J., Junge, R. E., Thornburg, L. P., Raisbeck, M. F., 1990: Acute monensin toxicosis in stone sheep (*Ovis dalli stonei*), blesbok (*Damaliscus dorcus*), and a bactrian camel (*Camelus bactrienus*). *J. Am. Vet. Med. Assoc.*, 196, 131–34.

32. Mollenhauer, H. H., More, J. D., Rowe, D. L., 1990: Alteration of intracellular traffic by monensin: Mechanism, specifity and relationship to toxicity. *Biochim. Biophys. Acta*, 1031, 225–246.

33. Mudríková, R., 2002: The rational anticoccidial planing (In Slovak). *Slov. Vet. Čas.*, 27, 47–49.

34. Neuschl, J., Šály, J., Šimko, Š., Magic, D., Tučková, M., Lenhardt, Ľ., Nagy, J., Kremeň, J., 1998: Subacute toxicity of sodium salinomycin in the anticoccidial preparation Synvertas *plv. ad us. vet.* preparation in chickens. *Bull. Vet. Inst. Pulawy*, 45, 323–331.

35. Neuschl, J., Šály, J., Šimko, Š., Magic, D., Ševčiková, Z., Kremeň, J., Nagy, J., Korim, P., 2001: Acute toxicity of sodium salinomycin in Synvertas *plv. ad us. vet.* preparation in chickens. *Bull. Vet. Inst. Pulawy*, 45, 315–321.

36. Oehme, F. W., Pickrell, J., 1999: An analysis of the chronic oral toxicity of polyether ionophore antibiotics in animals. *Vet. Hum. Toxicol.*, 41, 251–257.

37. Pesti, G. M., Bakalli, R. I., Cervantes, H. M., Bafundo, K. W.,1999: Studies on semduramicin and nutritional responses – 1. Level and source of protein. *Poult. Sci.*, 78, 102–106.

38. Pesti, G. M., Bakalli, R. I., Cervantes, H. M., Bafundo, K. W., 1999: Studies on semduramicin and nutritional responses – 2. Methionine levels. *Poult. Sci.*, 78, 1170–1176.

39. Plíšek, K., 1987: Current and perspective anticoccidials in poultry. *Research Institute for Biofactors and Veterinary Drugs*, Merkur, 98–174.

40. Plumlee, K. H., Johnson, B., Galey, F. D., 1995: Acute salinomycin toxicosis of pigs. *J. Vet. Diagn. Inv.*, 7, 419–420.

41. Pressman, B. C., 1976: Biological applications of ionophores. *Ann. Rev. Biochem*., 45, 501–529.

42. Ricketts, A. P., Glazer, E. A., Migaki, T. T., Olson, J. A., 1992: Anticoccidial efficacy of semduramicin in battery studies with laboratory isolates of coccidia. *Poult. Sci.*, 71, 98–103.

43. Ruff, M. D., 1982: Veterinary applications In **Westly, J. W.:** *Polyether Antibiotics. Naturally Occuring Acid Ionophores 1, Biology*. Marcel Dekker; NY, 303–332.

44. Shlafer, M., Kane, P., 1980: Subcellular actions and potential adverse cardiac effects on the cardiotonic ionophore monensin. *J. Pharmacol. Exper. Ther.*, 214, 567–573.

45. Shlosberg, A., Perl, S., Harmelin, A., Hanji, V., Bellaiche, M., Bogin, E., Cohen, R., Markusfeld-Nir, O., Shpigel, N., Eisenberg, Z., Brosh, A., Holzer, Z., Aharoni, Y., 1997: Acute maduramicin toxicity in calves. *Vet. Rec.*, 140, 643–646.

46. Smith, C. K., Galloway, R. B., 1983: Influence of monensin on cation influx and glycolysis of *Eimeria tenella* sporozoites *in vitro*. *J. Parasitol.*, 67, 511–516.

47. Sutko, J. L., Besch, H. R., Bailey, J. C., Zimmerman, G., Watanabe, A. M., 1997: Direct effect of the monovalent cation ionophores monensin and nigericin on myocardium. *J. Pharmacol. Exper. Ther.*, 203, 685–700.

48. Šály, J., Magic, D., Kalus, J., Šnirc, J., Sovík, L., Bíreš, I., Jantošovič, J., Neuschl, J., 1997: Salinomycin poisoning in turkey rearing. *11th Symposium on Diseases of Poultry and Exotic Birds*, Košice, 27–28.

49. Švec, P., 1998: On dynamic development of drug forms and development of galenic pharmacy. *Slovakofarma Revue*, 3, 82–83.

50. Váczi, P., Neuschl, J., Šály, J., Lenhardt, Ľ., 2006: Comparison of the acute toxicity of sodium salinomycin in Synvertas and Sacox preparations in chickens. *Bull. Vet. Inst. Pulawy*, 50, 379–382.

51. Watkins, K. L., Bafundo, K. W., 1993: Effect of anticoccidial programs on broiler performance. *J. Appl. Poult. Res.*, 1993, 55–60.

52. Westly, J. W., 1982: Notation and clasification. In **Westly, J. W.** (ed.)**:** *Polyether Antibiotics: Naturally Occurring Acid Ionophores. Vol. 1: Biolog*. NY, Marcel Dekker, 1–20.

53. Zhu, G., Mc Dougald, L. R., 1992: Characterization *in vitro* and *in vivo* of resistance to ionophores in the strain of *Eimeria tenella*. *J. Parasitol.*, 78, 1067–1073.

Received February, 15, 2008

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 208–216, 2007

LIMITED DRUG RESPONSE OF CANCER CELLS CAUSED BY MULTIPLE RESISTANCE MECHANISMS* (A Review)

Rybárová , S.

Department of Anatomy, Faculty of Medicine
P. J. Šafárik University, Šrobárova 2, 040 01 Košice
The Slovak Republic

silvia.rybarova@upjs.sk

ABSTRACT

Unresponsiveness to anticancer drugs is very often caused by drug resistance. Cancer cells exhibit a wide variety of mechanisms of drug resistance. Studies on drug resistance in cancer have been dominated for decades by the notion that mechanisms are primarily localized inside the cell. Recent findings regarding the tumor microenvironment will significantly reduces penetration and drug toxicity and finaly may be equally important in leading to clinical resistance. The broad spectrum of drug resistance mechanisms can be divided according the localization into the extracellular (e. g. cell surface and tumor microenvironment) and intracellular mechanisms (cytoplasmic membrane, cytoplasm, nuclear membrane and nucleus). Nevertheless, intracellular components still have immutable role in development of drug resistance.

Key words: glutathione; legumain; MDR transporters; p53; topoisomerases; XRCC1

INTRODUCTION

It is well known that drug resistance is a major obstacle in cancer therapies. Some malignant tumors are intrinsically resistant to standard antineoplastic agents, whereas others respond initially to chemotherapy and then relapse.

Chemotherapy failure may be the result of several factors that could be considered either at the level of the entire organism or at the level of individual cells. At the level of the entire organism a major role play physiologic resistance factors which include absorpsion, distribution, metabolism and elimination of drug. Absorption and distribution are in close relation with drug diffusion or penetration. These factors determine wheather drug really reaches the tumor. They can be affected by changing the way of administration, drug dose and scheduling.

At the level of the individual cells, the microenvironment possibly plays an important role, but the importance of the various factors, e. g. blood supply, cell kinetic factors and pH, is not well understood.

Most information regarding resistance mechanisms derives from *in vitro* models of cells selected by exposure to extremely high levels of cytostatics that are not of clinical relevance. Resistance studies show a lot of mechanisms of resistance and that it is a multifactorial problem. If drug resistance is characterized by resistance to a broad range of structurally and functionally unrelated cytotoxic agents, it is phenomenon, which is called „multiple drug resistance“ (MDR) (42).

Generally, mechanisms which mediate drug resistance can be according their localization divided into the extracellular and intracellular.

Extracellularly or microenvironmentally mediated mechanisms may contribute a much greater role than has been generally considered. Among a broad spectrum of extracellular mechanisms the most important are the following: insufficient drug

Full adress:
Doc. Silvia Rybárová, MVD, PhD
Ústav anatómie Univerzity P. J. Šafárika
Lekárska fakulta
Šrobárova 2
041 80 Košice

*Running title: Resistance mechanisms of cancer cells

diffusion or *penetration*, than *repopulation* of tumor cells between courses of chemotherapy and extracellular expression of novel asparaginyl endopeptidase – *legumain*.

Intracellular mechanisms can be divided into mechanisms localized in the cytoplasmic membrane, structures within the cytoplasm, in the nuclear membrane and inside the nucleus.

Now it is obvious, that cancer cells may possess resistance mechanisms on each one level from the cell microenvironment up to the nucleus. As the target of the most of anticancer drugs is cell nucleus, drugs must penetrate from tumor blood vessels through multiple cell layers to reach the cancer cells and finally to penetrate the cytoplasmic membrane and cytoplasm to achieve the nucleus. As mentioned above, drugs can be eliminate at various cell level by different resistance mechanisms.

The most important intracellular resistance mechanism are components of cytoplasmic membrane – *transporter proteins*. They are divided into ABC (ATP binding cassette) transporters and protein LRP (lung resistance-related protein) which does not belong to ABC family of transporters. Their physiological functions recline in defence of normal cells against xenobiotics. Xenobiotics/drugs are prevented from entering cells, or they are exported outward from the cytoplasm, reducing its levels at their intracellular targets.

The proteins of the ABC group are energy-dependent and act by neutralizing the therapeutic toxic effect of cytotoxic agens. They operate in MDR (multidrug resistance) cells. *Glutathione (GSH)* – the most ubiquitous and abundant nonprotein thiol, is essential in numerous detoxification reactions and is therefore considere as a chemoprotectant.

Topoisomerases – are nuclear enzymes that are involved in every aspect of DNA metabolism. They play important roles in DNA replication and transcription, chromosome condensation and segregation, and repair (3). Topoisomerases form DNA-enzyme complex (the cleavable complex).

X-ray repair cross complementing protein 1 – (XRCC1) – plays an important role in excision repair of DNA after ionizing irradiation. Because radiation therapy exerts its cytotoxic effects through damage to cells, proteins, and DNA, the individual capacity to repair damaged DNA may modify the response of the normal tissue.

The p53 (nuclear protein) – acts as a tumor suppressor, preventing aberrant cellular proliferation in response to various stress signals.

EXTRACELLULAR MECHANISMS

Drug penetration and repopulation of tumor cells

Anticancer drugs reach solid tumors through the blood stream. Drugs have to penetrate from tumor blood vessels through multiple cell layers to gain the cancer cells. Tumors are in a therapeutic disadvantage because the intercapillary distances in tumors are larger than in normal tissues. If drugs do not reach the majority of tumor cells in a potentially lethal concentration, there will be only limited therapeutic effects against the tumor.

Solid tumors form three dimensional aggregates, which are multicellular spheroids. *In vitro* studies compared traditional monolayer to the multicellular spheroids. Spheroids tend to be intrinsically much less sensitive to most chemotherapeutic drugs and radiation (59). These findings are generally more consistent with the *in vivo* behavior of solid tumors to drugs or treatments. The main role in drug penetration play cell adhesion molecules (21). To reduce their impact to drug resistance it is possible to use various anti-adhesive agents as multicellular resistance reversal agents, e. g. neutralizing antibodies to E-cadherin (21) or hyaluronidase (18).

Recently Dalton's group has been studying a phenomenon called CAM-DR, "cell adhesion mediated drug resistance", which can be mediated by other adhesion receptors as are integrins α_5 β_1 (55) suggesting to use the integrin antagonist as possible chemosensitizing agents. Cell adhesion may also serves to protect tumor cells from the effects of anticancer drugs by regulating expression of genes or their proteins which induce drug resistance by inhibition or induction of apoptosis (19) and enzymatic mediators of DNA repair (18). Strategies to improve drug penetration imply continuous drugs delivery and modification of extracellular matrix including adhesion molecules.

Repopulation is a process which determines the frequency of administration of chemotherapy of bone marrow cells. It occurs in surviving tumor cells between treatment and influences outcome of radiotherapy, too. Repopulation seems to be more important in longer intervals between cycles of chemotherapy (11). If repopulation accelerates with time, this will lead to shrinkage and regrowth of tumors (it occurs during fractionated radiotherapy) (49). The outcome of chemotherapy may be improved by selective inhibition of repopulation of tumor cells between cycles od therapy (60). Repopulation may be inhibit by the administration of tumor-selective cytostatic agents (60). The most difficult problem is measurement of rate of repopulation following chemotherapy. It may be impossible at early intervals after drug treatment to separate surviving clonogenic cells from lethally-damaged cells.

Legumain

Legumain is a novel member of the C13 family of cystein proteases (26). It is well conserved in plans and mammals including humans. Legumain was found to be highly expressed in several types of tumors, such as colon, prostate and breast cancers. The patients with colorectal carcinoma that showed both weak and lower percentage of the legumain expression, either in tumor or in stroma, had a better prognosis (45).

On the other hand its expression is not apparent in normal tissues from which the tumors originated (36). Expression of legumain includes both intracellular localization and apears also on the cell surface of tumor cells and tumor associated endothelial cells, where it is colocalized with integrins (45).

It is up-regulated during tumor development *in vivo* and seems to be a stress-responsive gene (36). Cells that

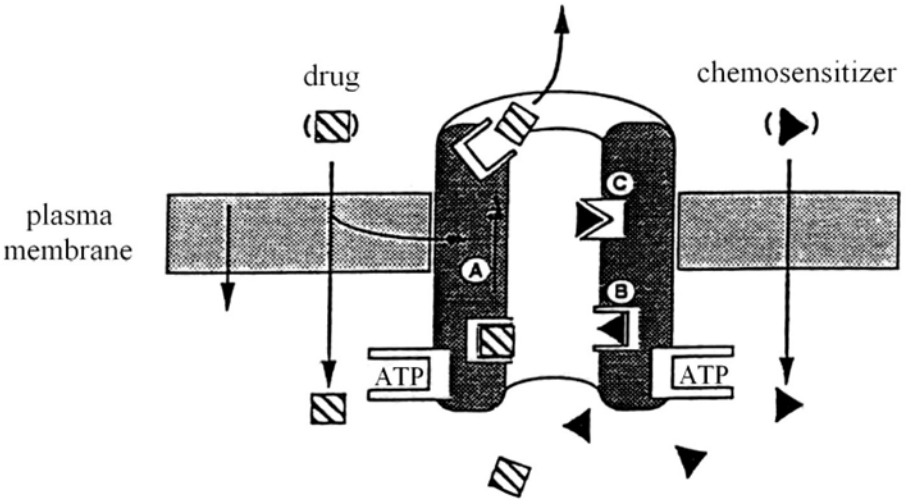


Fig. 1. Mechanism of function of MDR proteins. Functional representation of P-glycoprotein. The model depicts a translocating carrier protein, which utilizes ATP energy to actively transport drug substrate across the plasma membrane (17)

highly express legumain exhibit enhanced migratory and invasive properties, which can be mediated by increased extracellular matrix degradation. Degradation of matrix results from activation of zymogens such as progelatinase A. Legumain activates the gelatinase A zymogen which is an important mediator of extracellular matrix degradation, and thus may be important for tumor cells to adapt more invasive and metastatic phenotype (45). It is well known that tumor microenvironment differs greatly from that of other tissues. It is enriched in proteolytic activity, is acidic and hypoxic. The function of legumain is also in local reduction of pH of the tumor microenvironment (68).

Studies with animal tumor models with higher levels of legumain showed (*in vivo*) more invasive growth and metastasis (36).

Legumain is expressed also by tumor angiogenic endothelial cells and here shows presence in and on tumor-associated macrophages (TAM) (39), and thus presenting multiple local intratumoral cellular target for prodrug activation (16). It is heavily expressed by tumor-associated macrophages in murine breast tumor tissues (38). TAMs have an abundant expression in the tumor stroma (36) and express high level of legumain in this tumor microenvironment. On the other side classical macrophages of the M1 phenotype do not express legumain (4). The authors hypothesized that targeting TAMs which overexpress legumain will reduce their density and than change the tumor microenvironment (38). This could lead to the downregulation of a wide range of tumor growth factors, proangiogenic factors and metalloproteinases, released by these M2 macrophages and suppress angiogenesis of tumors, their growth and metastasis (38).

W u *et al.* (68) were searched for utilizing of the expression of legumain to targeting cell-impermeable prodrug activation to tumor microenvironment. Prodrug activation significantly reduces drug toxicity to normal tissues. The prodrug therapy attacks both tumor and stroma cells through a bystander effects without selectively deleting target cells, and with minimizing drug resistance and toxicity. LEG-3 is a novel doxorubicin prodrug, which is cell-impermeable and activated solely in the tumor microenvironment. The LEG-3 administration resulting in increasing of the end-product of doxorubicin in nuclei of cancer cells, but little in the other tissues (68). The growth of a variety of neoplasms, including multidrug-resistant tumors is completely arrested and survival is significantly prolongated without side-effects (myelosuppression, cardiac toxicity) (68).

INTRACELLULAR MECHANISMS

Transporter proteins

MDR1 – P-glycoprotein

MDR1 transporter protein is a member of the ATP binding cassette transporters (ABC), it belongs to the ABCB subfamily. It operates in multidrug resistant cells by reducing levels of drugs at their intracellular targets, or exported them outward from the cytoplasmic membrane (20). MDR1 mediated drug resistance is characterized by cross-resistance between series of chemically unrelated drugs, decreased drug accumulation (5), increased expression of MDR1 (28), and reversal of phenotype by a variety of different compounds (e. g. verapamil) (62). The drugs most often involved in MDR1- mediated drug resistance are of fungal or plant origin, including anthracyclines (daunorubicin and doxorubicin) and vinca alkaloids. Also epipodophyllotoxins, aktinomycin D, colchicin and taxans are able to induce MDR1-mediated drug resistance.

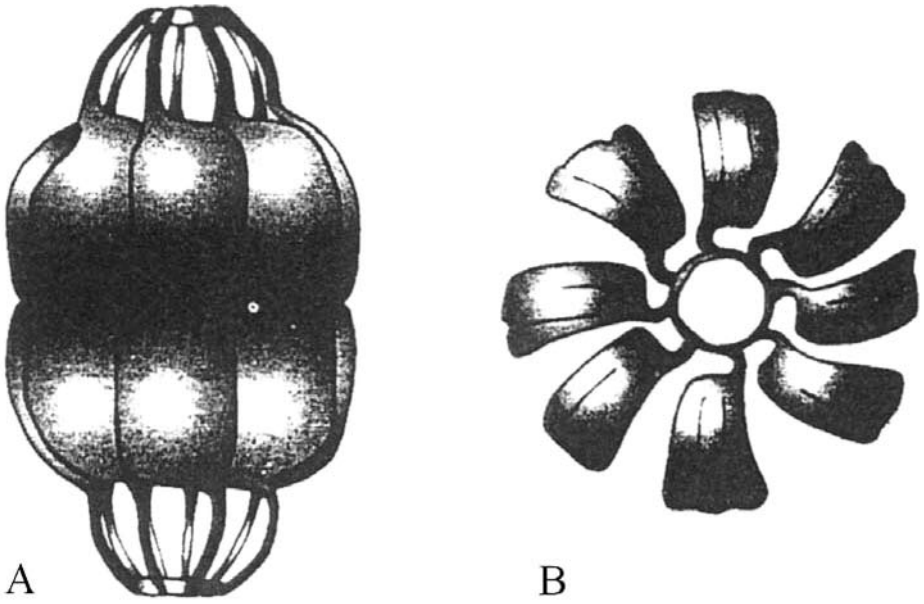


Fig. 2. "Vault" – barrel-like protein, it is a large ribonucleoprotein complex. LRP/MVP is subunit of vault (30)

MRP1 – multidrug resistance-associated protein

Several MDR cell lines with decreased drug accumulation but without expression of MDR1 have been described (46). C o l e *et al.* (9) has revealed a protein named MRP1, which is also a member of ABC transporter proteins. MRP1 belongs to ABCC subfamily with another 12 members (ABCC1-ABCC12) (13). MRP1 is classified as an organic anion transporter, it transports anionic and neutral drugs conjugated to acidic ligands, such as glutathione, glucuronide, or sulphate (37). The human MRP family contains nine members MRP1-MRP9 (6). It has been suggested that also proteins MRP2 (cMOAT) and MRP3 could contribute to drug resistance (10). MRP3 seems to confer resistance to etoposide (VP16) and vincristine (VCR) (69).

MRP1 has been detected in almost every tumor type, both solid tumors (53, 54) and haematological malignancies. It has been demonstrated that expression of MRP1 has implications for prognosis in some solid tumors (48). In contrast to MDR1 protein MRP1 does not transport taxans.

BCRP – breast cancer-resistance protein

This transporter protein gene was discovered in 1998 independently in three laboratories, resulting in three names: *BCRP* (the breast cancer-resistance gene) (14), *MXR* (the mitoxantrone-resistance gene) (41), and *ABCG2* – according its appertaining to ABC transporter proteins and ABCG subfamily (13). It is a half-transporter and is believed to homodimerize to produce an active transport complex (51). The phenotype conferred by BCRP is characterized by high levels of resistance to mitoxantrone and topotecan, moderate resistance to anthracyclines, and lack of resistance to vinca alkaloids, paclitaxel and cisplatin (35). Relatively high expression of this protein has been observed in approximately 30 % of patients with acute myeloid leukaemia, suggesting a potential role for BCRP in drug resistance related to leukaemia (52).

LRP – lung resistance-related protein (MVP/major vault protein)

It was first described in 1993 by S c h e p e r *et al.* (58) in a human MDR lung cancer cell line. It does not belong to the ABC superfamily of transporter proteins. LRP is one of the structural proteins of the vaults – a large ribonucleoprotein particle, with broad distribution in eukaryotic organisms. However, the function of vaults is still unclear. Most of vaults have a cytoplasmic localization (30) and it has been suggested that they play role in nucleocytoplasmic transport of different substrates including cytostatics. Increased expression of LRP has been associated with decreased accumulation of daunorubicin in leukaemic blast cells (40).

Recently, links between vaults/LRP and drug resistance has been critically discussed (43). It is proposed to function as a novel scaffold protein for SHP-2 (tyrosine phosphatase SHP-2) and Erk (extracellular-regulated kinases), two proteins involved in cell signaling (34). Recent studies involving intracellular distribution of drugs have indicated that vaults do not play a direct role in the multidrug-resistant phenotype (63). It appears that LRP/MVP (vaults) is a novel regulator of cellular signaling cascades (32).

Glutathione S-transferases

The glutatione S-transferases (GSTs) are multifunctional enzymes with broad substrate specificities that catalyze glutathion detoxification reactions. In this manner, GSTs protect cellular macromolecules against attack of mutagens, carcinogenes, and other toxic compounds (67). On

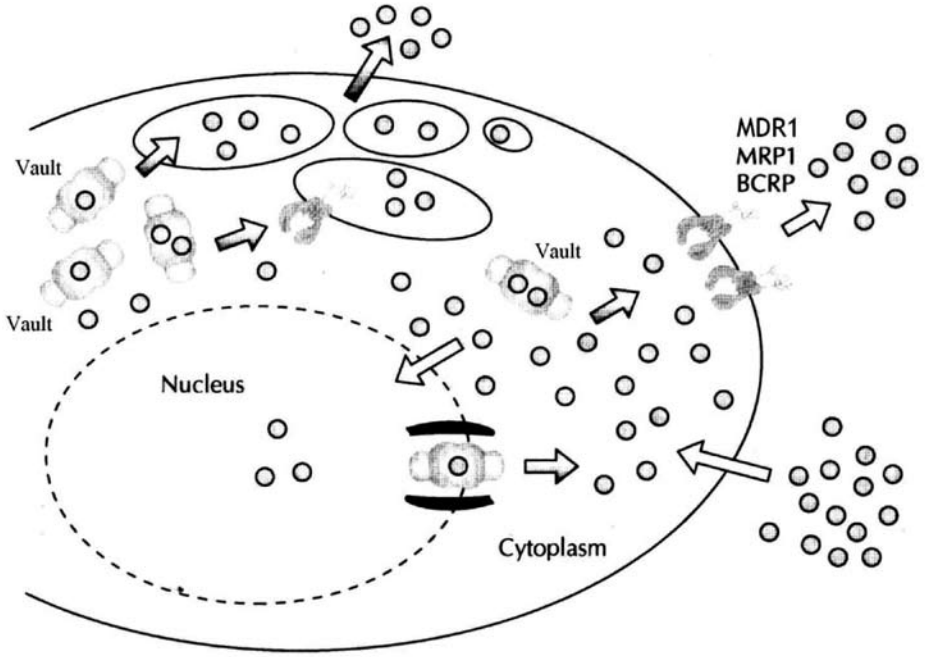


Fig. 3. Hypothetic role of vaults in nucleocytoplasmic transport of drugs (57)

the basis of evolution, substrate and inhibitor specificities, antibody crossreactivities, and primary structures, the mammalian GSTs can be grouped into classes: α (GSTA), ϖ (GSTP), μ (GSTM), ω (GSTO), θ (GSTT), and ξ (GSTZ) (47). Cell lines derived from various human tumors overexpressed GST isoenzymes as well as other GSH (glutatione)-dependent enzymes (8).

Some studies indicate, that overexpression of the GST-α can confirm some drug resistance (22). High levels of the GST-π has frequently been linked to the MDR phenotype. MDR1 expression and GST-π mRNA expression serve as a significant prognostic indicator for breast cancer, thus having guiding significance for assessing prognosis (15). GST-π expression is considered to be an independent prognostic factor for DFS (disease free survival) in patients receiving adjuvant chemotherapy for breast cancer (44).

It is unclear whether GST-ϖ is directly responsible for much of the drug resistance. There have been reports suggesting that there is a direct association between increased GSH and GST-ϖ isoenzyme levels and cisplatin resistance. Although no study has clearly shown that cisplatin is a GST-ϖ substrate. There is evidence that GSH levels are inversely correlated with levels of cisplatin-induced cytotoxicity. Understanding GST expression and polymorphisms within the human population could be a predicitive tool in determining which individuals are susceptible to carcinogens and would benefit from specific drug regimens.

Topoisomerases

Mammalian topoisomerases (TOPO) were classified according to the number of strands of DNA they cleave. TOPO I cleaves only single-strand of DNA, whereas TOPO II acts by cleaving a double-strand of DNA helix. Recently, another mammalian – TOPO III was identified, which cleaves also only one strand of DNA (23). It makes this classification obsolete. Now it is clear, that several natural and synthetic cytotoxic compounds interact specifically with DNA topoisomerases.

TOPO II interacting drugs (e. g. anthracyclines, epipodophyllotoxins) inhibit the rejoining action of the enzyme (complex-forming DNA inhibitors) resulting in DNA-strand breaks (50). Alteration of TOPO II drug activity can be divided into two groups: 1) quantitative changes (decreased levels of TOPO II protein through downregulation of transcription or increased degradation), and 2) qualitative changes (e. g. mutations resulting in an altered drug – DNA – protein interaction, altered enzymatic function).

The best known inhibitor of TOPO I is camptothecin. It is the only one TOPO I inhibitor for which cellular resistance mechanisms have been reported. TOPO I caused drug resistance appears as a result of the following events: 1) removal of the drug from cancer cells, 2) decrease in TOPO I protein, and 3) mutation of TOPO I.

Since topoisomerases appear to be required for most processes involving DNA, it is possible that the DNA damage produced by many anticancer drugs may depend in part on topoisomerase activity for either conversion to lethality or repair.

X-ray repair cross complementing protein 1 – (XRCC1)

X-ray repair cross-complementing protein 1 is required for single-strand break repair in human cells and several polymorphisms in this gene have been implicated in can-

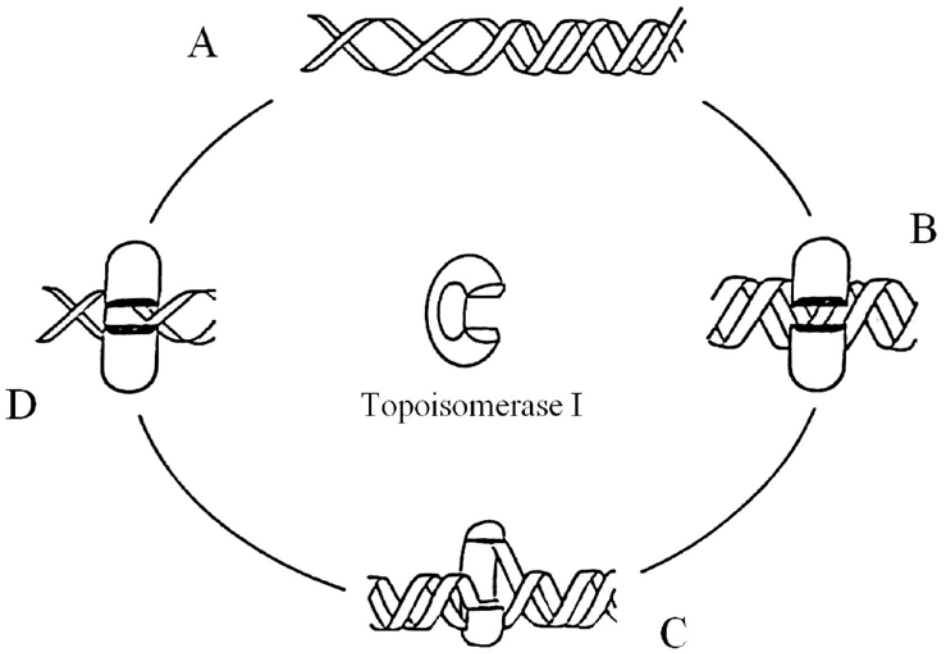


Fig. 4. Topoisomerase I acts by cleaving a single-strand DNA helix (33)

cer risk and clinical prognostic factors. The XRCC1 gene shows three relatively common polymorphisms affecting the amino acid sequence in codon: 194(Arg/Trp), 280(Arg/His), and 399(Arg/Gln) (56). The individual DNA repair capacity consists of several pathways (e. g. nucleotide and BER-base excision repair, homologous recombination, mismatch repair and telomerase metabolism) (24, 12). The XRCC1 protein acts as a scaffold to coordinate other BER proteins at the repair site (24). Several reports indicate that the variant alleles of the repair polymorphisms may affect DNA repair function. DNA repair gene polymorphisms in BER gene (XRCC1 ^{194}Arg and ^{399}Gln) were shown to affect increased mutagen sensitivity after bleomycin treatment, a radiation-mimicking agent that induced double-strand breaks in DNA (66).

The variant alleles in XRCC1 ^{399}Glu were associated with a prolongated cell cycle G_2 delay in response to ionizing radiation (25). Cells defective in XRCC1 have increased sensitivity to ionizing radiation, UV, hydrogen peroxide, and mitomycin (61).

The efficiency of the repair process depends also on the time out for damaged cell to ensure effective repair. On the other side the damaged cell will be eliminated by cell death. It is likely that further DNA repair gene polymorphisms may be associated with ionizing radiation hypersensitivity.

Protein p53

Cells are constantly being exposed to DNA damage. It can occur through a number of different mechanisms, including the induction of mismatches, crosslinks or strand breaks. Especially serious are double-strand breaks in DNA (27). If they are not repaired correctly, broken DNA ends can recombine inappropriately. Double-strand breaks may occur after exposure of extracellular (ionizing radiation, chemotherapeutic agens), or intracellular processes (reactive oxygen species, defects in DNA repair function, ...) (31).

The favourable response to DNA damage is to attempt to repair DNA lesions, which would protect the cellular genome and viability of the cell. In some cases, the cellular response to DNA damage is to attempt cell death – apoptosis. Apoptosis is genetically programmed form of cell death and play an important role in maitenance of homeostasis. The induction of an apoptotic signal by DNA damage very often occurs through the activation of the tumor suppressor protein p53 (wild-type) (64). The cell cycle arrest response is a checkpoint function that allows cells to pause in the cell cycle to prevent reproducing of potentially oncogenic mutations. Cells with some lesions, and which lack p53, may survive and proliferate inappropriately which may lead to development of cancer. In the absence of p53, cells with critically short telomere can survive (1).

Loss of wild-type p53 activity is one of the key factors in the resistance of human cancers to chemotherapy (2). Drug resistance caused by mutation/inactivation of p53 is very often in close relation with multidrug resistance represents by overexpression of transporter proteins MDR1 and MRP1. On the other hand, wild-type p53 represses expression of both MDR1 and MRP1 proteins (65), by what at the same time represents mechanism against development of drug resistance.

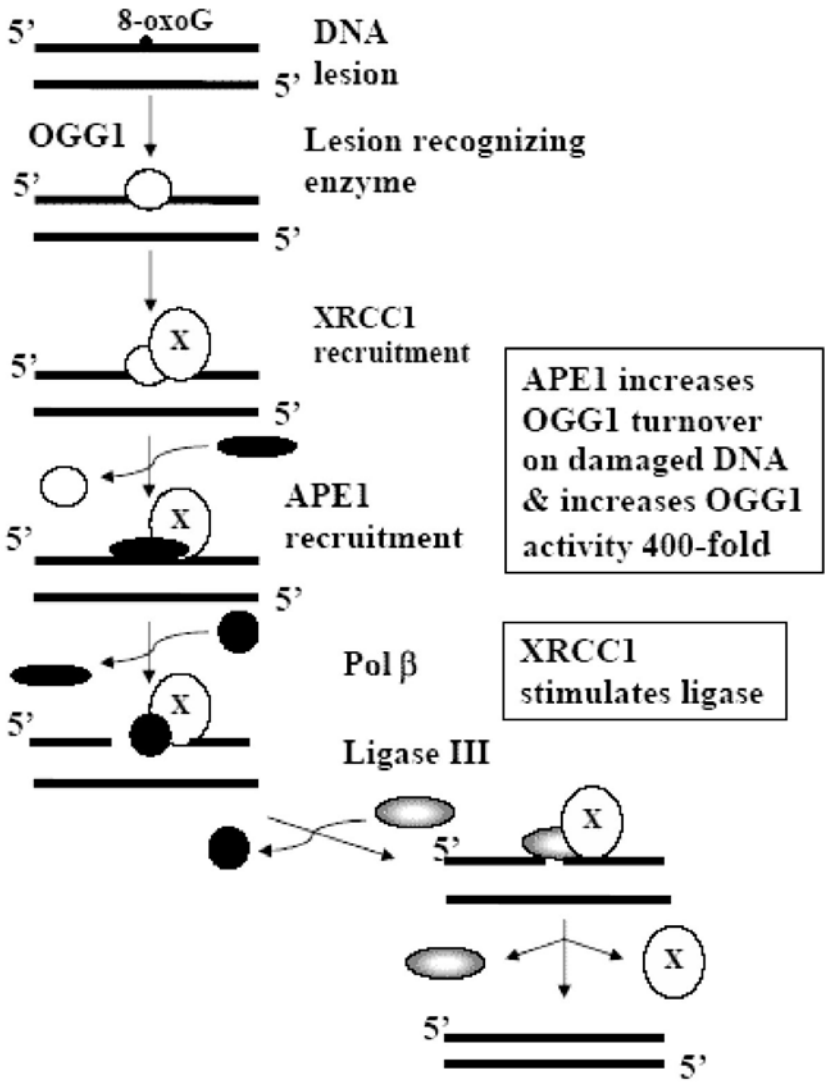


Fig. 5. Coordinative role of XRCC1 protein in BER (base excision repair) (7)

CONCLUSIONS

The major aim of presented study was to give the survey of broad, but not complete, range of mechanisms which are responsible for drug resistance of cancer cells. The same mechanisms which play important physiological roles in normal tissues and organs (e. g. cell protection), under the pathological conditions become unfavourable. In the case of cancer they directly or indirectly protect the cancer cells against cytostatics. Chemotherapy becomes ineffective and fails. Overexpression of some of above mentioned mechanisms in tumors suggests worse prognosis and aggravates overall survival.

The successfull treatment of cancer will benefit most from the development of new anticancer drugs that are more effective, irrespective of the presence of resistance mechanisms. Therefore, in order to effectively treat patients specific modulators should be developed for clinical purposes.

REFERENCES

1. Artandi, S. E., Chang, S., Lee, S. L. ***et al.,*** **2000:** Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature*, 406, 641–645.

2. Attardi, L. D., 2005: The role of p53-mediated apoptosis as a crucial anti-tumor response to genomic instability: lessons from mouse models. *Mutat. Res.*, 569, 145–157.

3. Bakshi, R. P., Galande, S., Muniyappa, K., 2001: Functional and regulatory characteristics of eukaryotic type II DNA topoisomerase. *Crit. Rev. Biochem. Mol. Biol.*, 36, 1–37.

4. Balkwill, F., Charles, K. A., Mantovani, A., 2005: Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell*, 7, 211–217.

5. Biedler, J. I., Riehm, H., 1970: Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: Cross-resistance, radioautographic, and cytogenetic studies. *Cancer Res.*, 30, 1174–1184.

6. Borst, P., Oude Elferink, R., 2002: Mammalian ABC transporters in health and disease. *Annu. Rev. Biochem.*, 71, 537–592.

7. Bu, D., Tomlinson, G., Lewis, Ch., Zhang, C., Kildebeck, E., Euhus, D., 2006: An intronic polymorphism asociated with increased XRCC1 expression reduced apoptosis and familial breast cancer. *Breast Cancer Res Treat.*, 99, 257–265.

8. Castro, V. M., Soderstrom, M., Carlberg, I., *et al.,* 1990: Differences among human tumor cell lines in the expression of glutathione transferases and other glutathione-linked enzymes. *Carcinogenesis*, 11, 1569–1576.

9. Cole, S. P., Bhardwaj, G., Gerlach, J. H., 1992: Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science*, 258, 1650–1654.

10. Cui, Y. H., Konig, J., Buchholz, U. *et al.,* 1999: Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol. Pharmacol.*, 55, 929–937.

11. Davis, A. J., Tannock, I. F., 2000: Repopulation of tumour cells between cycles of chemotherapy: a neglected factor. *Lancet Oncol.*, 1, 86–93.

12. De Boer, J., Hoeijmakers, J. H., 2000: Nucleotide excision repair and human syndromes. *Carcinogenesis*, 21, 453–460.

13. Dean, M., 2002: *The Human ATP-binding Cassette (ABC) Transporter Superfamily*. NCBI Monographs, USA.

14. Doyle, L. A. S., Yang, W. D., Abruzzo, L. V., 1998. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. USA*, 95, 15665–15670.

15. Feng, Y., Hao, X., Mao, H., 2001: Clinical significance of MDR gene expression in malignant pleural effusion and ascites and solid tumors. *Zhonghua Yi Xue Za Zhi*, 81, 1484–1487.

16. Folkman, J., 1995: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.*, 1, 27–31.

17. Ford, J. M., Yang, J. M., Hait, W. N., 1996: P-glycoprotein-mediated multidrug resistance: experimental and clinical strategies for its reversal. *Cancer Treat. Res.*, 87, 3–38.

18. Francia, G., Man, S., Teicher, B. *et al.,* 2004: Gene expression analysis of tumor spheroids reveals a role for suppressed DNA mismatch repair in multicellular resistance to alkylating agens. *Moll. Biol. Cell*, 24, 6837–6849.

19. Frankel, A., Buckman, R., Kerbel, R. S., 1997: Abrogation of taxol-induced G_2-M arrest and apoptosis in human ovarian cancer cells grown as multicellular tumor spheroids. *Cancer Res.*, 57, 2388–2393.

20. Gottesman, M. M., Pastan, I., 1993: Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.*, 62, 385–427.

21. Green, S. K., Francia, G., Isidoro, C. *et al.,* 2004: Anti-adhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy *in vitro*. *Mol. Cancer Ther.*, 3, 149–159.

22. Greenbaum, M., Letourneau, S., Assar, H. *et al.,* 1994: Retrovirus-mediated gene transfer of rat glutathione S-transferase Yc confers alkylating drug resistance in NIH 3T3 mouse fibroblasts. *Cancer Res.*, 54, 4442–4447.

23. Hanai, R., Caron, P. R., Wang, J. C., 1996: Human TOP3: A single-copy gene encoding DNA topoisomerase III. *Proc. Natl. Acad. Sci. USA*, 93, 3653–3657.

24. Hoeijmakers, J. H., 2001: Genome maitenance mechanisms for preventing cancer. *Nature*, 411, 366–374.

25. Hu, J. J., Smith, T. R., Miller, M. S. *et al.,* 2001: Amino acid substitution variants of APE1 and XRCC1 genes associated with ionizing radiation sensitivity. *Carcinogenesis*, 22, 917–922.

26. Ishii, S., 1994: Legumain: asparaginyl endopeptidase. *Methods Enzymol.*, 244, 604–615.

27. Jackson, S. P., 2002: Sensing and repairing DNA double-strand breaks. *Carcinogenesis*, 23, 687–696.

28. Juliano, R. I., Ling, V., 1976: A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta*, 455, 152–162.

29. Kederha, N. I., Rome, L. H., 1986: Isolation and characterization of a novel ribonucleoprotein particle: Large stuctures contain a single species of small RNA. *J. Cell Biol.*, 103, 699–709.

30. Kedersha, N. L., Heuser, J. E., Chugani, D. C., Rome, L. H., 1991: Vaults. III. Vault ribonucleoprotein particles open into flower-like structures with octagonal symmetry. *J. Cell Biol.*, 112, 225–235.

31. Khanna, K. K., Jackson, S. P., 2001: DNA double-strand breaks: signaling, repair and the cancer connection. *Nat. Genet.*, 27, 247–254.

32. Kim, E., Lee, S., Mian, M. F. *et al.,* 2006: Crosstalk between Src and major vault protein in epidermal growth factor-dependent cell signaling. *FEBS J.*, 273, 793–804.

33. Klener, P., 1996: *Antitumorous Chemotherapy* (In Czech). Galén, Praha, 75–76.

34. Kolli, S., Zito, Ch. I., Mossink, M. H. *et al.,* 2004: The major vault protein is a novel substrate for the tyrosine phosphatase SHP-2 and scaffold protein in epidermal growth factor signaling. *J. Biol. Chem.*, 279, 29374–29385.

35. Litman, T., Brangi, M., Hudson, E. e*t al.,* 2000: The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). *J. Cell. Sci.*, 113, 2011–2021.

36. Liu, C., Sun, C., Huang, H. *et al.,* 2003: Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. *Cancer Res.*, 63, 2957–2964.

37. Loe, D. W., Almquist, K. C., Deeley, R. G. *et al.,* 1996: Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles: Demonstration of glutathione-dependent vincristine transport. *J. Biol. Chem.*, 271, 9675–9682.

38. Luo, Y., Zhou, H., Krueger, J. *et al.,* 2006: Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J. Clin. Invest.*, 116, 2132–2141.

39. Mantovani, A., Sozzani, S., Locati, M. *et al.,* 2002: Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.*, 23, 549–555.

40. Michieli, M., Damiani. D., Ermacora, A. *et al.,* 1997: P-glycoprotein (PGP) and lung resistance-related protein (LRP) expression and function in leukaemic blast cells. *Br. J. Haematol.*, 96, 356–365.

41. Miyake, K., Mickley, L., Litman, T., 1999: Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res.*, 59, 8–13.

42. Moscow, J. A., Schneider, E., Ivy, S. P. *et al.,* 1997: Multidrug resistance. *Cancer Chemother. Biol. Response Modif.*, 17, 139–77.

43. Mossink, M. H., Van Zon A., Scheper, R. J. *et al.,* 2003: Vaults: a ribonucleoprotein particle involved in drug resistance? *Oncogene*, 22, 7458–7467.

44. Moureau-Zabotto, L., Ricci, S., Lefranc, J. P. *et al.,* 2006: Prognostic impact of multidrug resistance gene expression on the management of breast cancer in the context of adjuvant therapy based on a series of 171 patients. *Br. J. Cancer*, 94, 473–480.

45. Murthy, R. V., Arbman, G., Gao, J. *et al.,* 2005: Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer. *Clin. Cancer Res.*, 11, 2293–2299.

46. Nielsen, D., Skovsgaard, T., 1992: P-glycoprotein as multidrug transporter: A critical review of current multidrug resistant cell lines. *Biochim. Biophys. Acta*, 1139, 169–183.

47. Nicholson, D. W., Vaillancourt, J. P., Calaycay, J. R. *et al.,* 1993: Purification to homogenity and the N-terminal sequence of human leukotriene C_4 synthase: A homodimeric glutathione S-transferase composed of 18 kD subunits. *Proc. Natl. Acad. Sci. USA*, 90, 2015–2019.

48. Nooter, K., Brutel, de la Riviere, G., Look, M. P. *et al.,* 1997: The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer. *Br. J. Cancer*, 76, 486–793.

49. Norton, L., Simon, R., 1977: Tumor size, sensitivity to therapy, and design of treatment schedules. *Cancer Treat. Rep.*, 61, 1307–1317.

50. Osheroff, N., Zechiedrich, E. L., Gale, K. C., 1991: Catalytic function of DNA topoisomerase II. *BioEssays*, 13, 269–273.

51. Ozvegy, C., Litman, T., Szakacs, G. *et al.,* 2001: Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells. *Biochem. Biophys. Res. Commun.*, 285, 111–117.

52. Ross, D. D., 2000: Novel mechanisms of drug resistance in leukemia. *Leukemia*, 14, 467–473.

53. Rybárová, S., Hajduková, M., Hodorová, I. *et al.,* 2004: Expression of multidrug resistance-associated protein 1 (MRP1) and the lung resistance-related protein (LRP) in human lung cancer. *Neoplasma*, 51, 169–174.

54. Rybárová, S., Hodorová, I., Hajduková, M. *et al.,* 2006: Expression of MDR proteins in breast cancer and its correlation with some clinical and pathological parameters. *Neoplasma*, 53, 128–135.

55. Shain, K. H., Dalton, W. S., 2001: Cell adhesion is a key determinant in *de novo* multidrug resistance (MDR): new targets for the prevention of acquired MDR. *Mol. Cancer Ther.*, 1, 69–78.

56. Shen, M. R., Jones, I. M., Mohrenweiser, H., 1998: Nonconservative amino acid substitution variants exist at polymorphic frequency in DNA repair genes in healthy humans. *Cancer Res.*, 58, 604–608.

57. Scheffer G. L., Scheper, R. J., 2002: Drug resistance molecules: lessons from oncology. *Wiley, Chichester (Novartis Foundation Symposium)*, 243, 19–37.

58. Scheper, R. J., Broxterman, H. J., Scheffer, G. L. *et al.,* 1993: Overexpression of a Mr 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Res.*, 53, 1475–1479.

59. Sutherland, R. M., 1988: Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science*, 240, 177–184.

60. Tannock, I. F., 2005: Limited drug access and repopulation of surviving tumor cells between courses of chemotherapy: important and neglected causes of clinical drug resistance. In *AACR 96th Annual Meeting, AACR Education Book*, 103–107.

61. Thompson, L. H., West, M. G., 2000: XRCC1 keeps DNA from getting stranded. *Mutat. Res.*, 459, 1–18.

62. Tsuruo, T., Iida, H., Tsukagoshi, S. *et al.,* 1981: Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.*, 41, 1967–1972.

63. Van Zon A., Mossink, M. H., Schoester, M. *et al.,* 2004: Efflux kinetics and intracellular distribution of daunorubicin are not affected by major vault protein/lung resistance-related protein (vault) expression. *Cancer Res.*, 64, 4887–4892.

64. Vousden, K. H., Lu, X., 2002: Live or let die: the cell's response to p53. *Nat. Rev. Cancer*, 2, 594–604.

65. Wang, Q., Beck, W. T., 1998: Transcriptional suppression of multidrug resistance-associated protein (MRP) gene expression by wild-type p53. *Cancer Res.*, 58, 5762–5769.

66. Wang, Y., Spitz, M. R., Zhu, Y. *et al.,* 2003: From genotype to phenotype: correlating XRCC1 polymorphisms with mutagen sensititvity. *DNA Repair (Amst.)*, 2, 901–908.

67. Wilce, M. C. J., Parker, M. W., 1994: Structure and function of glutathione S-transferases. *Biochim. Biophys. Acta*, 1205, 1–18.

68. Wu, W., Luo, Y., Sun, C. *et al.,* 2006: Targeting cell-impermeable prodrug activation to tumor microenvironment eradicates multiple drug-resistant neoplasms. *Cancer Res.*, 66, 970–980.

69. Zeng, H., Bain, L. J., Belinsky, M. G. *et al.,* 1999: Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. *Cancer Res.*, 59, 5964–5967.

Received January 30, 2008

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 51, 3–4: 217–220, 2007

GUIDANCE FOR CONTRIBUTORS

All articles, which conform to the Uniform Requirements for Manuscripts submitted to Biomedical Journals, will be reviewed.

The house style, which differs in respect of References, is set out below.

Folia Veterinaria is issued quarterly and distributed worldwide. Original research papers on all aspects of veterinary science together with clinical case studies, notes and short communications, review articles, and correspondence are welcomed on the understanding that the manuscript, in part or in whole, has not been published or submitted for publication elsewhere. Each author is responsible for the originality of the work and the correctness of its content. In the case of joint authorship, it will be assumed that all the authors agree to the submission. Manuscripts are considered on the proviso that consent to publication has been given by the head(s) of department(s) where this is required. Papers will be subject to peer review and if accepted for publication, they become the copyright property of *Folia Veterinaria*. In return for the assignment of copyright, the author will be given twenty off prints of the article, and will have no restriction placed on his/her personal freedom to use the information in the article in publications elsewhere. Manuscripts not recommended for publication will be returned.

Manuscripts. Papers should be written in English and the spelling should follow the preferred form in *The New Shorter Oxford English Dictionary* (1993). Three copies of the text (preference: CD ROM, secondrate: disc-form) and one copy of the photographs and illustrations, tables or graphs (in digital form) should be sent to the Executive Editor, *Folia Veterinaria*, The University of Veterinary Medicine, Komenského 73, 041 81 Košice, The Slovak Republic.

The text should be double-spaced and typewritten on one side of A4 paper, 30 lines, 60 strokes, with margins of at least 25 mm.

Where papers cited are "in press", copies should accompany the manuscript submitted. The editor reserves the right to make literary corrections: texts will be returned to the author(s) for major rectification, in line with the recommendations of the referees.

Authorship. In accordance with the criteria for authorship recommended by the International Committee of Medical Journal Editors (*JAMA* 1997; 277, 927–934; *Can. Med. Assoc. J.* 1997; 156: 270–7 and 1997; 156: 571–574) each author must have (a) participated substantially in the conception and execution of the work, (b) contributed significantly to the drafting and/or revision of the manuscript, and (c) agreed with the final version, in order to accept public responsibility for the article. In cases of multiple authorship, authors should provide a description of what each contributed. This information may be published. The order of authorship on the by-line should be a joint decision of the co-authors. Authors should be prepared to explain this order.

Acknowledgements. Those who have given technical assistance, or moral or financial support, or supplied equipment or materials, or engaged in translation or general supervision, etc. should be recognized in the Acknowledgements (cf also McNab, S. M. Coping with Clutter in a Scientific Paper. *European Science Editing*, 1992; 45: 8)

Conflict of Interest. If a study evaluates a pharmaceutical product, a medical or scientific device, or any other commercial manufacture, the authors must disclose, in a confidential covering letter to the editor, any and every financial interest (e. g. employment, consultancy, share-holding, board membership, etc.) they may have in the company that manufactures the product discussed or in a rival firm and/or commodity.

References. Only the work used should be mentioned. At the end, the references should be listed in alphabetical order by the first author's surname. List the first six authors followed by *et al.* References should be set out thus.

Journals: Surname(s) and initial(s) of the author(s), year of publication, full title of the paper, title of the journal (in *italics*), volume, and relevant pages (See examples below). The issue number should be quoted in parentheses only if the pagination of the journal is by issue rather than by volume.

Books: Surname(s) and initial(s) of the author(s) and/or editor(s), year of publication, full title of the book and

edition (if not the first), publisher and place of publication, and pages.

These references should then be numbered. In the text, these numbers are used instead of names and dates for citations, e.g. "All space-flight embryos... showed normal embryogenesis (3, 6) and post-hatch development (5)." Only if the writer's name is a necessary part of the sentence should it be used, e.g. "J o n e s (7) discovered that...". If the date is essential, it too should form part of the text, e.g. "Then in 1997 J o n e s (7) made a breakthrough." This alphabetical – numerical style for references is to make the text flow: to separate the science from the customary clusters of nominal citations.

Examples:

Ahlborg, B., Ekelund, L. C., Nilsson, C. G., 1968: Effect of potassium-magnesium aspartate on the capacity of prolonged exercise in man. *Acta Physiol. Scand.*, 74, 238–245.

Black, H., Duganzich, D., 1995: A field evaluation of two vaccines against ovine pneumonic pasteurellosis. *New Zeal. Vet. J.*, 43, 60–63.

Brown, L. W., Johnson, E. M., 1989: Enzymatic evidence of alkaline phophatase. In **Caster, A. R.:** *Enzymology.* Plenum Press, New York, 99–101.

Ikuta, K., Shibata, N., Blake, J. S., Dahl, M. V., Nelson, R. D., Hisamichi, K. *et al.,* 1997: NMR study of the galactomannaus of *Trichophyton mentagrophytes* and *Trichophyton rubrum. Biochem. J.*, 323, 297–305.

Language Style. Be prepared to use the first person ("I" or "We"), but do not overuse it. (e.g. "We studied 24 Slovak Merino ewes.")

The excessive use of the passive voice is a principal cause of dullness in scientific writing. Use it sparingly, and prefer the active voice ("We conclude that...") to the passive ("It can be concluded that...") whenever justifiable.

Use the past tense for reporting observations, completed actions, and specific results ("We observed no significant changes.")

Use the present tense or the present perfect for generalizations and generalized discussion. ("This suggests that...")

Employ the specialist vocabulary of your discipline(s), but do not allow this technical jargon to turn into gobbledegook. "The dynamic development of biological sciences has... had a positive influence on the current knowledge of the activated mechanisms... in the case of human and animal organisms" can be rendered succinctly as "The rapid growth of biological science has enabled us to understand the functions of human and animal bodies better." Convoluted and roundabout expression does not impress and may well irritate the reader.

Remember that many readers will not be native-speakers of English. If you are an ESL (English as Second Language) author, apply the principles of English style and syntax when writing, and be mindful that the correct word order is important in English sentences. Make sure that your sentences are sentences: do not lose control of their structure.

Be simple and concise; where possible use verbs instead of abstract nouns. Break up long noun clusters and "stacked modifiers" (strings of adjectives before nouns without clues about which modifies which). Logically ordered and lucidly expressed ideas will make your meaning clear: obfuscation will not assist the reader.

Avoid "dictionary" and "computer English" – transverbation based upon an incorrect choice of words in a dictionary or word bank. (One computer produced this: "Natural immunity is not bound on antecedent individual skill by your leave pathogen and him close non-pathogenic microorganism").

Units of Measurement. Measurements of length, height, weight, and volume should be reported in metric units.

Temperatures should be given in degrees Celsius; blood pressures in millimetres of mercury.

All haematological and clinical chemistry measurements should be recorded in the metric system or in the terms of the International System of Units (SI).

Abbreviations and Symbols. Use only standard abbreviations. Avoid abbreviations in the title and abstract. Abbreviations and acronyms should be used only if they are repeated frequently. The full term for which an abbreviation stands should precede its first use in the text unless it is a standard unit of measurement, e.g. positron emission tomography (PET).

Numerals and Dates. Whole numbers from one to ten should be written as words in the text, not as numerals, e.g. "Experiments were carried out on four male Rhine geese..." Numerals should be used for numbers above ten, except in the titles of papers and at the beginning of sentences, where they must appear as words. Dates in the text should be written as follows: 29 September 2000.

Nomenclature and Terminology. Medicines must be shown by there generic name followed by the proprietary name and manufacturer in parentheses when they are first mentioned, e.g. Apramycin (Apralan 200; Elanco, Austria).

Authors should respect international rules of nomenclature. For animal species and organisms, the recommendations of the International Code of Zoological Nomenclature, London 1999, should be observed. Linnaean names should be used for plant species. Anatomical terminology should agree with the nomenclature published in the *Nomina Anatomica Veterinaria* 4th edn. (1994) ed. Habel, R. E., Frewein, J., and Sack, W. O., World Association of Veterinary Anatomists, Zurich and Ithaca, New York.

Latin terms and other non-English words should be italicised in the manuscript. Use the British Standard 2979:1958 for the transliterations of Cyrillic characters in the references as well as the text.

Photographs and Illustrations. These should be on separate sheets, each with a label pasted on its back, bearing the author's name, the figure number, and an arrow indicating the top of the figure. Black-and-white photographs should be clear and sharp, suitable for reproduction. Photomicrographs must state the magnification and stain technique. Illustrations should be drawn in black ink on white paper in a form suitable for photographic reproduction. The main objects, changes, and

findings should be shown by an arrow or some other symbol explained in the legend.

Graphs and Tables should contain essential data not given in the text. In the journal, graphs will have an overall width of no more than 8.5 cm and be drawn on pages 17.5 cm wide. The size of the letters in legends should suit these dimensions. Computer-drawn graphs are preferred-laser-printed on diamond paper. Statistics (with tables of parameters) must be enclosed. Captions for figures, including graphs and photographs, and all legends should be subscribed. Tables should be typed with double-spacing on separate sheets of paper and numbered consecutively in the order of their citation in the text. Within each table, lines should separate only the headings from the body of the table, and the body of the table from any totals, averages, etc. Titles for tables should be superscribed, and explanatory matter placed in footnotes, using the symbols and sequence recommended. Number explanatory texts to graphs, figures, and tables with Roman letters or Arabic numerals. Each such text should be enclosed on a separate sheet of paper and placed in order. The author should indicate with pencil in the left-hand margin of the manuscript where the supporting material should be inserted.

Ethical Considerations. When reporting experiments on animals indicate whether the Institution's or Research Council's Guide for, or any national law on, the care and use of laboratory animals were followed. Manuscripts should describe the measures taken to minimize or eliminate pain and distress in animals during experiments and procedures. If the editors deem that animals have been subjected to suffering unjustified by the scientific value of the information sought, they will reject the paper on ethical grounds.

The journal encourages integrity in science. Questionable and fraudulent claims will not be entertained.

Experimental Hazards. Authors should draw attention to any dangers involved in carrying out their experiments, and should detail the precautions taken to guard against such hazards.

Statistics. Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the results reported. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty. Discuss the eligibility of experimental subjects. Give details about randomisation. (Cf. the statistical guidelines for authors in *The Australian Veterinary Journal* Vol. 76, No. 12, December 1998, p. 828.)

Types of Papers. Please state clearly which category of paper is being submitted. (If an author believes that his/her article or short communication is of outstanding topicality and importance, he/she should indicate this in a covering letter. It may merit fast-track publications.)

Standard Full Length Papers. Full papers should be concise. They should not exceed 12 pages (A4) including tables, graphs, illustrations, photographs, and references.

The Title Page. The papers should be headed with the full title, which should accurately and concisely describe the topic in no more than two lines. The surname(s) and initials of the author(s) and the name and place(s) of their employment should follow this. (If the work was carried out in an institution other than the place of employment, this should be noted in the body of the text.) Acknowledgements (see above) should be typed in a separate section, separated by a reasonable space. A short title (the running head) of no more than forty characters (counting letters and spaces) should be included at the foot of the page. Each manuscript should be thematically complete: serialization is discouraged.

The Abstract. The second page should carry an abstract, which should be self-contained and not exceed 250 words. It should briefly incorporate the purpose and relevance to veterinary science of the work, basic procedures, the main findings, and principal conclusions. It should emphasize new and important aspects of the study or observations.

Key words. Key words should be listed below the abstract, from which they are separated by a one-line space. They should consist of three to ten words in alphabetical order, written in lower case and separated by semi-colons.

The Introduction. State the purpose of the article and summarize the rationale for the study or observation. Give only strictly pertinent references and do not include data or conclusions from the work being reported.

Material and Methods. Describe your selection of observational or experimental subjects (including controls) clearly. Identify the age, sex, state of health, and other important characteristics of the subjects.

Identify the methods, apparatus (with the manufacturer's name and address in parentheses), and procedures in sufficient detail for other workers to reproduce the experiment. Quote established methods, including statistical methods; provide references and brief descriptions for methods that have been published but are not well known; describe new or substantially modified methods in full; give reasons for using them, and evaluate their limitations. Precisely identify all drugs and chemicals used, including generic name, dose, and route of administration.

Results. These should be as succinct as possible and presented in a logical sequence in the text, with graphs and tables. Emphasize or summarize only the important observations in the text. Do not duplicate in the text all the data in the graphs and tables.

Discussion. Emphasize the new and important aspects of the study and the conclusions that follow from them. Do not repeat in detail data or other material given in the Introduction or the Results sections. Include in the Discussion section the implications of the findings and the limitations, together with their significance for future research. Relate the observations to other relevant studies.

Link the conclusions with the aims of the study, but avoid unqualified statements and conclusions not completely sup-

ported by the data. Avoid claiming priority and alluding to work that has not been completed. Recommendations, when appropriate, may be included.

Notes and Short Communications. Such manuscripts should have the same form as full papers, but are much shorter. Separate headings are needed only for the Acknowledgements, Key Words, Abstract, Main Text, and References. These scripts fall under the following main headings and should be marked accordingly.

Technical Notes. Such notes should record a new method, technique, or procedure of interest to veterinary scientists. They should include the reason(s) for the new procedure, a comparison of results obtained by the new method with those from other methods, together with a discussion of the advantages and disadvantages of the new technique. A technical note should not exceed six printed pages, including figures and tables.

Research Communications. These are short articles, no more than four printed pages, which should introduce novel and significant findings to the commonwealth of veterinarians.

Observations. Research of this kind contributes to knowledge, but not to the advancement of ideas or the development of concepts. In some cases, these papers underpin what may seem obvious, with statistical data. Such communications should not exceed four type-set pages.

Current Issues. Papers that deal with issues of topical interest to veterinary scientists will be considered. Issues may include items on environmental concerns, legislative proposals, etc.

Review Articles. These should provide a substantial survey, with an appropriate historical perspective, of the literature on some aspect of veterinary medicine. Alternatively, such articles may review a topic of veterinary interest, which may not come within the normal purview of many veterinarians (e.g. Asefa Asmare, A., 2000: The Camel,..., *Folia Veterinaria,* 44, 4, 215–221). Authors submitting review manuscripts should include a section describing the methods used for locating, selecting, extracting, and synthesizing data. These methods should be summarized in the abstract.

Book. Reviews may be submitted. They should bring a new text to the readership and evaluate it.

Letters to the Editor. These are items of scientific correspondence, designed to offer readers the chance to discuss or comment on published material and for authors to advance new ideas. Should a letter be polemical, a reply or replies for simultaneous publication may be sought from interested parties.

Editorial Board

FOLIA VETERINARIA, 51, 1–2, 2007

CONTENTS

No. 1

No. 2

FOLIA VETERINARIA, 51, 3–4, 2007

CONTENTS