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EFFECT OF ORGANIC AND INORGANIC ZINC SUPPLEMENTATION ON THE MORPHOLOGY OF THE TESTIS IN ASSAM GOAT *(Capra hircus)* KIDS

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ABSTRACT

Thirty six healthy local Assam male goats, three months of age, between 3 to 4 kg body weight were utilized in this study. The kids were divided into three groups according to their feeding regime: group I (control group) in which animals received no zinc supplementation; group II and III animals received inorganic (zinc sulphate, 120 mg/kid/day) and organic forms (zinc propionate, 40 mg/kid/day) of zinc supplements, respectively, with a concentrate mixture. The study revealed both treatment and age wise variations in respect to the histomorphological parameters of the testes. At 4 months of age, elongated spermatids were observed in the adluminal portion of the Sertoli cells of kids of the organic zinc-supplemented group; however spermatozoa did not appeared in the lumen of the seminiferous tubules until 5 months of age. Only spermatocytes and round spermatids were observed in the epithelium as well as in the lumen of the seminiferous tubules at 4 months of age in the cases of the inorganic and control groups. In the inorganic group, spermatozoa appeared in the tubular lumen at 5 months of age; however the concentration was lower as compared to the organic group. From 6 months onwards, spermatozoa were seen in the lumen in all groups of kids, however, the maximum concentrations of tubular spermatozoa were observed in the organic zinc-supplemented group. All of the micrometrical parameters i.e. thickness of the tunica albuginea; diameter of the seminiferous tubule; diameter of the lumen of the seminiferous tubule; and height of the epithelium of the seminiferous tubule increased with advancing age. The largest diameter of the seminiferous tubules was found in the organic group from 4 to 7 months of age, followed by the inorganic and then the control group at all ages. The height of the epithelium of the seminiferous tubules of the organic group was significantly higher ($P < 0.05$) than the control group in all the ages, whereas in the inorganic group, it showed significantly higher values at 7 month of age as compared to the control group.

Key words: goat; histomorphology; micrometry; testis, zinc supplementation

INTRODUCTION

Minerals have attained a global focus as being a versatile food component and health promoter in both humans and animals, because they are involved in a large number of physiological, digestive and biosynthetic processes in the body. As many as sixty four macro and micro minerals have been found to be vital for the body functions, including the maintenance and nightly rebuilding processes. Therefore, this multifaceted mineral's domain has stimulated scientists to investigation the possible prospects for the improvement of the physiological maximization of animal's growth, reproduction and productivity. The evaluation of the fluctuating demands of minerals for different stages of reproduction (like growth and maturation of genital organs, pubertal and cyclic reproductive phases and pregnancy (13, 16, 6, 14, 19)), constitutes valuable scientific endeavours. The role of zinc in male reproduction have been explored for decades by numerous investigators, but still some controversies persist. However, the key role of zinc in spermatogenesis and sperm

motility (8, 2) and morphology (20) and their metabolism, tubular secretion of male hormones etc. has been well documented (12) and have led to their use in the form of biomarkers. The high concentration of zinc in organs like the testes (3), prostate and spermatozoa themselves (23) clearly signifies its importance in male reproduction. Earlier studies on zinc substantiate that it is directly involved in anatomical development and the normal function of male reproductive organs. A deficiency in the diet may lead to a delay in testicular development, reduced testosterone production and possibly stop spermatogenesis (23). The use of organic minerals in livestock nutrition has gained considerable interest over the past decade. Zinc supplementation either in the organic or inorganic form in the diet has been shown to improve the qualitative and quantitative attributes of the semen and it is also necessary for the normal development of the male reproductive organs (9). Rojas *et al.* have suggested that organic zinc in ruminant diets might be metabolized differently upon absorption compared with inorganic zinc (18). These investigators have shown further that the organic sources of minerals were more biologically available than inorganic sources of the minerals.

The testicular cellular architecture along with the seminiferous tubules and the epididymal lumen's micrometric studies have also been considered important parameters in monitoring the growth process of the genital tract of young males towards puberty. Available reports suggested that there was inadequate growth of the male genital tract in young males as a result of zinc deficiency (13). Thus, it could be anticipated that zinc supplementation would optimize the growth rate of the young male genital system including testosterone secretion from the Leydig cells. In view of the above, the present investigation was taken up to study the effect of zinc supplementation in the feed with a view to assess its effects on the testis morphology.

MATERIAL AND METHODS

A total of thirty six local Assam male kids of three months of age and between 3 to 4 kg of body weight were included in this experiment. They were divided into three groups:

Group-I (control group): Kids fed without zinc supplementation
Group-II (inorganic group): Kids fed with inorganic zinc supplementation
Group-III (organic group): Kids fed with organic zinc supplementation

All the animals were reared under a semi-intensive system in the Experimental animal shed belonging to the Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati. Standard management practices were adopted with regards to the rearing of the experimental animals. The experimental goats received a concentrate mixture at a dose of 50 g/day/goat up to 5 months and then 100 gm/day/goat up to 7 months in addition to their free range grazing. Group I (control) animals received no zinc supplementation while the Group II and III animals received the inorganic and organic form of zinc supplements, respectively along with the concentrate mixture. The concentrate mixture contained 2 per cent mineral mixture – the AAUVETMIN without zinc which was supplied by AICRP project (Improvement of Feed Resources and Nutrient Utilization in Raising Animal Production). The kids belonging to Group II and III received 120 mg of inorganic zinc supplement (zinc sulphate) and 40 mg of organic zinc supplement (Bioplex zinc, i.e. zinc propionate), respectively. The tissue pieces from the testes of twelve animals (from each group) were fixed in Bouin's solution (11). All the tissues were processed for paraffin sections by the alcohol- xylene method using cedar wood oil. Sections were cut at 5 µ thickness using a rotary microtome (Thermo, Germany) and stained by Haematoxylin & Eosin (11) for histological and micrometrical studies. The thickness of the tunica albuginea, diameter of the seminiferous tubules, diameter of the lumen of seminiferous tubules and height of the seminiferous tubular epithelium were measured in twenty apparently round seminiferous tubules per testicles by micrometrical methods (12). The Nikon image analyzer and Image Pro software (Nikon, Germany) were used.

For all the observed data in the present experiment, the standard statistical procedures recommended by Snedecor and Cochran (22) have been followed. The data were presented by the showing of the mean and standard error. The significant differences of values for different parameters studied were assessed by the test of analysis of variance, while the critical difference test was performed

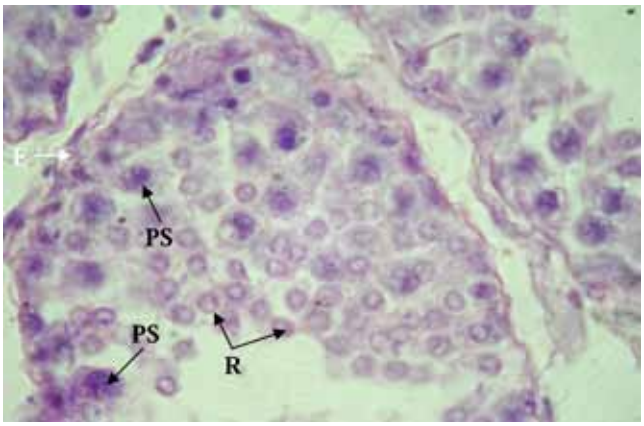

Fig. 1. Photomicrograph of the testis in a four months old kid (inorganic group) showing primary spermatocytes (PS) and round spermatids (R) in the seminiferous epithelium. H & E. Magn. ×1000

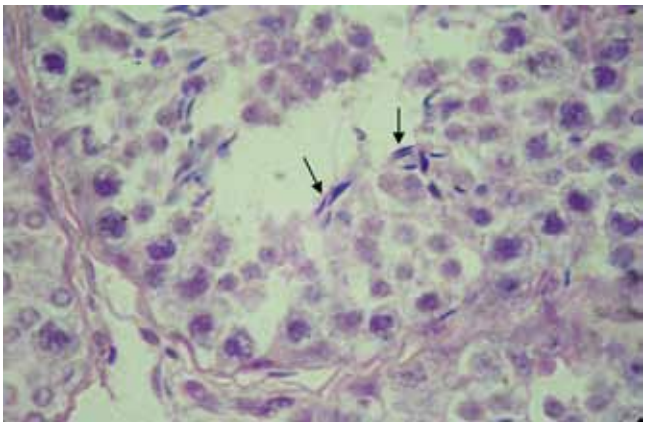

Fig. 2. Photomicrograph of the testis of a four months old kid (organic group) showing elongated spermatids (arrows) in the ad luminal border of the Sertoli cells of the seminiferous epithelium. H & E. Magn.×1000

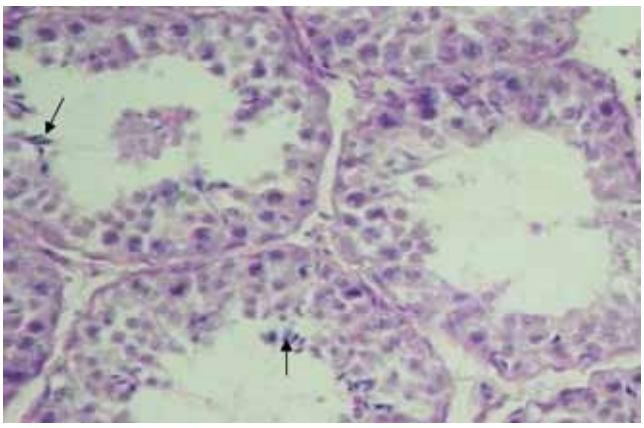

Fig. 3. Photomicrograph of the testis of a five months old (Inorganic group) kid showing the presence of mature sperm cells (arrows) in thelumen of the seminiferous tubule. H & E. Magn. ×400

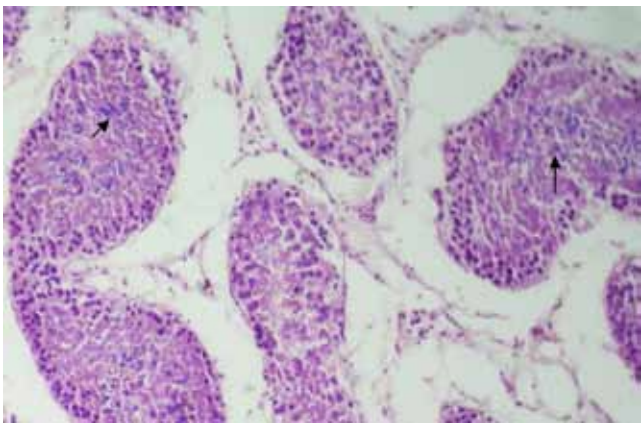

Fig. 4. Photomicrograph of the testis of a five months old kid (Organic group) showing the presence of mature sperm cells (arrows) in the lumen of the seminiferous tubule. H & E. Magn. ×100

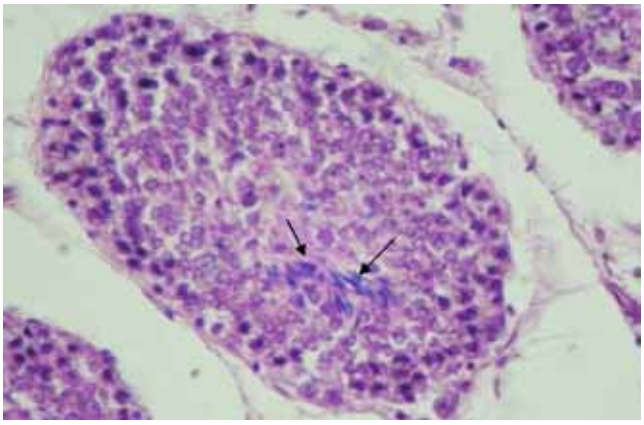

Fig. 5. Photomicrograph of the testis of six month old kid (Control group) showing the presence of mature sperm cells (arrows) in the lumen of the seminiferous tubules. H & E. Magn. ×400

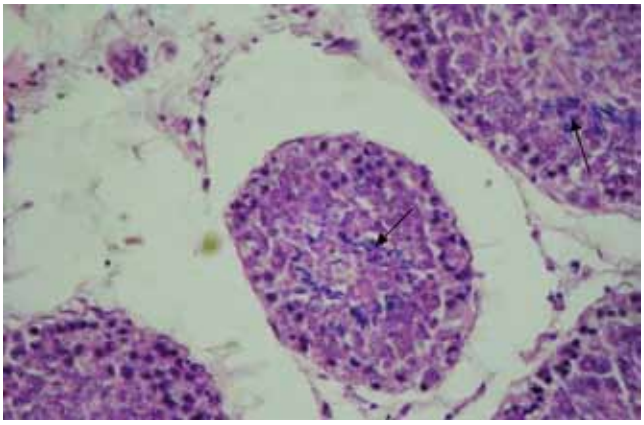

Fig. 6. Photomicrograph of the testis of a six month old kid (Inorganic group) showing the presence of mature sperm cells (arrows) in the lumen of the seminiferous tubules. H & E. Magn. ×100

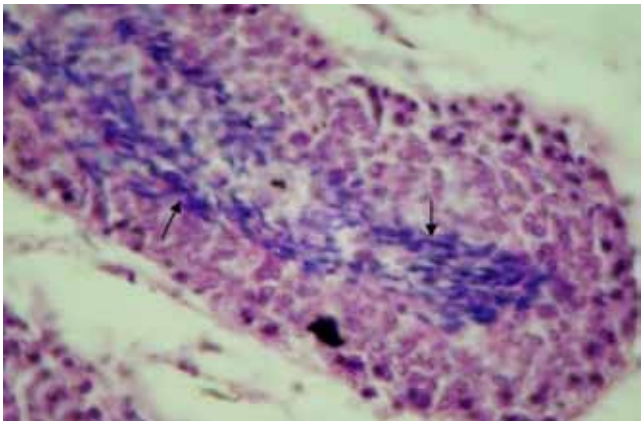

Fig. 7. Photomicrograph of the testis of a seven month old kid (Organic group) showing the presence of mature sperm cells (arrows) in the lumen of the seminiferous tubule. H & E. Magn. ×400

to evaluate the significant difference among the treatment groups. All the above calculations were carried out using SPSS software version 11.5.

RESULTS

Histology of testis

Histological studies of the seminiferous tubules of the testis revealed that at 4 months of age, the tubules were compactly arranged in all the treatment groups (control, inorganic and organic zinc-supplemented groups). In all three groups, the seminiferous tubules had distinct lumina. In the kids of the inorganic and control groups, no elongated spermatozoa was observed in the seminiferous epithelium or in the lumen of the seminiferous tubules and only spermatocytes and round spermatids were visible in the seminiferous epithelium (Fig. 1). However, in the organic group, the se-

miniferous epithelium showed elongated spermatids in the adluminal portion of the Sertoli cells (Fig. 2). But, no spermatozoa were seen in the lumen of the seminiferous tubules.

By 5 months of age, almost similar histological features (as above) were seen in the control group of animals, i.e., spermatozoa were not observed in either the seminiferous epithelium or in the lumen of the seminiferous tubules. However, in both the inorganic (Fig. 3) and organic groups (Fig. 4) of animals, the presence of spermatozoa were evident in the epithelium as well as in the lumen of the seminiferous tubules.

At 6 and 7 months of age, spermatozoa were seen in the lumen of the seminiferous tubules in all of the kids, of all groups (Fig. 5 and 6). However, the maximum populations of spermatozoa were seen in 6 and 7 months old kids of the organic group (Fig. 7).

Micrometry of testis

Table 1 represents the micrometry of the testis in the local Assam goats of the control, inorganic and organic zinc-supplemented groups from 4 months to 7 months of age at monthly interval.

Thickness of tunica albuginea

The thickness of the tunica albuginea was increased with advancing age in all the treatment groups. From 5 months onwards, higher values were found in both zinc-supplemented groups compared with that of control group (Table 1). The values differed significantly ($P < 0.05$) between groups and between ages ($P < 0.01$), but not due to interaction between groups and ages (Table 2). The results of the critical difference test for the thickness of the tunica albuginea of the kids in the different groups and at different ages can be studied from Table 1. This revealed that at 4 and 6 months of age, the thickness of the tunica albuginea was found to be significantly higher ($P < 0.05$) in the organic group of kids, when compared to inorganic group. However, at 5 and 7 months of age, no significant difference was found between the three treatment groups, but, the highest thickness was found in the organic group than in the inorganic zinc-supplemented or the control groups. Similarly, more thickness was found in the inorganic zinc-supplemented group than the control group of kids from 5 to 7 months.

Diameter of the seminiferous tubules

In all the treatment groups, the external diameter of seminiferous tubule was increased with age advanced. The external diameter of the seminiferous tubules was found higher in organic group than the other two groups (Table 1). The external diameter of seminiferous tubule varied significantly ($P < 0.01$) between groups and ages, but not due to interaction between group and ages (Table 2). Table 1 presents the

Table 1. Testes micrometry (μ, mean ± SD) in male goat kids of the controls compared with those receiving zinc supplementation

Age in months	Group	Micrometry of testes			
		Thickness of tunica albuginea	External diameter of sem. tub.	Diameter of lumen of sem. tub.	Height of epithelium of sem. tub.
4	Control	$194.64^{ab} \pm 3.63$	$171.34^{a} \pm 1.27$	$136.22^{a} \pm 0.45$	$40.11^{a} \pm 1.70$
	Inorganic	$191.30^{b} \pm 5.95$	$173.12^{ab} \pm 3.84$	$136.55^{a} \pm 1.42$	$40.85^{ab} \pm 1.18$
	Organic	$203.10^{a} \pm 6.56$	$176.07^{b} \pm 1.32$	$137.54^{a} \pm 1.23$	$42.50^{b} \pm 0.50$
5	Control	$230.96^{a} \pm 2.34$	$181.33^{a} \pm 0.36$	$137.91^{a} \pm 1.23$	$43.20^{a} \pm 0.62$
	Inorganic	$232.42^{a} \pm 1.80$	$185.85^{b} \pm 0.85$	$140.62^{a} \pm 0.93$	$44.74^{ab} \pm 0.81$
	Organic	$237.22^{a} \pm 2.36$	$187.86^{b} \pm 1.23$	$141.43^{a} \pm 1.53$	$45.63^{b} \pm 0.23$
6	Control	$282.64^{a} \pm 1.78$	$188.82^{a} \pm 0.87$	$141.26^{a} \pm 0.65$	$48.12^{a} \pm 0.25$
	Inorganic	$287.91^{a} \pm 3.34$	$192.79^{ab} \pm 1.41$	$142.08^{a} \pm 0.92$	$51.85^{a} \pm 0.73$
	Organic	$299.57^{b} \pm 3.33$	$193.52^{b} \pm 0.87$	$143.92^{a} \pm 1.60$	$54.25^{b} \pm 0.38$
7	Control	$320.95^{a} \pm 1.79$	$193.66^{a} \pm 0.93$	$139.57^{a} \pm 1.29$	$60.16^{a} \pm 0.51$
	Inorganic	$324.12^{a} \pm 3.85$	$201.89^{b} \pm 0.74$	$140.97^{b} \pm 1.18$	$65.12^{b} \pm 0.54$
	Organic	$323.45^{a} \pm 5.28$	$204.94^{b} \pm 1.05$	$139.21^{ab} \pm 2.85$	$69.25^{c} \pm 0.42$

Means bearing similar superscript in a column at different months do not differ significantly

Table 2. Analysis of variance for testicular micrometry in different groups of male goat kids (including the controls) following zinc supplementation

Source of variance	d.f.	Mean square			
		Thickness of tunica albuginea	Diameter of seminiferous tubule	Diameter of lumen of seminiferous tubule	Height of epithelium of seminiferous tubule
Group	2	246.239*	145.060**	19.3513^{NS}	75.5486**
Age	6	28804.5**	1139.80**	131.359**	988.694**
Group × Age	12	43.4696^{NS}	8.95098^{NS}	10.5457^{NS}	8.09193**
Error	123	44.3145	6.62457	5.85436	1.77588

** – $P<0.01$; * – $P<0.05$; ^{NS} – $P>0.05$

critical difference of the external diameter of the seminiferous tubule of kids under different groups and at different ages. The values of the inorganic zinc-supplemented group were found significantly higher ($P<0.05$) than that of the control group from 5 to 7 months of age. Higher values were found in the organic group than that of inorganic group in all ages.

Diameter of the lumen of the seminiferous tubule

In all the treatment groups, the diameter of the lumen of the seminiferous tubule was increased with advancing age (Table 1). The diameter of the lumen of the seminiferous tubule varied significantly ($P<0.01$) between ages but not due to groups or due to the interaction between groups and ages (Table 2). Table 1 presents the critical difference of the diameter of the lumen of the seminiferous tubules of the kids in different groups and at different ages and revealed that there was no significant difference found between the three groups of kids from 4 months to 6 months of age. However, at 7 months of age, the lumen diameter of the inorganic group of kids was found significantly higher ($P<0.05$) than the other two groups (Group I and III).

Height of epithelium of seminiferous tubules

The height of the epithelium of the seminiferous tubule was increased with advancing age in all the treatment groups (Table 1). The values were found higher in the organic zinc-supplemented group than the inorganic zinc-supplemented or the control group of kids in all of the ages studied. Interaction between treatment and age was also found highly significant (Table 2). The values of the organic zinc-supplemented groups were significantly higher ($P<0.05$) than the other two groups in all the ages. However, the corresponding values of inorganic zinc-supplemented groups were significantly higher than that of control group at 7 months of age. The organic zinc-supplemented group showed significantly higher values than that of the inorganic zinc-supplemented group at 6 and 7 months of age.

The external diameter, luminal diameter and epithelial cell height of seminiferous tubule all showed an increasing pattern of growth with advancing age in all the experimental kids (Table 1). The organic zinc-fed kids showed a significant increase of the external diameter of the seminiferous tubule beginning from 4 months until 6 months of age, while the highest significant value of the luminal diameter was observed in the inorganic-fed group at 7 months of age. In all the parameters, including the epithelial height of the seminiferous tubules, the highest measures were obtained in the organic zinc-fed kids, followed by the inorganic-fed and the control kids.

DISCUSSION

From the present findings it could be observed that the development of the process of spermatogenesis was different in the zinc-supplemented group of kids when compared with that of the control kids. While spermatozoa were found in the epithelium and lumen of the seminiferous tubules in zinc-treated kids at 5 months of age (Fig. 3 and 4), no spermatozoa could be located in the same site in the control kids (Fig. 1). Although kids of the inorganic zinc-supplemented and the control group did not differ in regard to the presence of spermatozoa at 4 months of age, the organic group kids were marked by the existence of elongated spermatids in the adluminal portion of the Sertoli cells (Fig. 2). The extent of the development of the testes was at variance in kids as reported by various workers. The development of the testes and the onset of puberty were studied histologically by Mishra *et al.* (14) who reported that bulls of 3 months of age produce spermatozoa in the testes. According to Lee *et al.* (10), spermatogenesis started from 8 weeks of age in Korean native goats and spermatozoa appeared in the seminiferous tubule at 20 weeks. Spermatogenesis was completed at 6 to 7 months of age in the goat (4) which supports the findings in our control group of kids (Fig. 5).

The developmental difference in the process of spermatogenesis in the treated groups could be attributed to the function of zinc, because zinc is an important mineral for normal testicular development and maintenance of germinal epithe-

lium (2) and it is vital for the spermatogenesis process (5). The spermatogenesis process in the male is impacted by the dietary zinc levels (12). Zinc acts as a cofactor for several enzymes which helps in the steroidogenesis processes in the body.

The maximum population of spermatozoa seen in the seminiferous tubular lumen in the organic group (Fig. 7) as compared to inorganic zinc treated group could be ascribed to higher bioavailability of zinc in influencing the testicular function of spermatogenesis.

In this study, the thickness of the tunica albuginea was increased with advancing age in all the treatment groups. A slight increase in its normal thickness was evident for the zinc-supplemented animals. Under normal conditions there is a suggestion that the increased thickness of the tunica albuginea with advancing age, is due to the need to possibly combat the increase intra-testicular pressure as reported earlier (7). Similarly, the external diameter of the seminiferous tubules was found to be larger in the organic zinc supplemented groups than the inorganic and control groups in all age groups. In addition, the values for the height of the seminiferous epithelium in organic zinc-supplemented groups were significantly higher ($P < 0.05$) than the control group in all the ages shows the efficacy of the organic form of zinc in enhancing the anatomical growth of the seminiferous tubules in the male goats. However, the dimensions of the lumina of the tubules exhibited no such conclusions.

The growth promoting effect of zinc was seen in regard to the histological parameters of the testes under study. The available literature, also suggested that the increasing dimensions of these parameters with advancing age are conspicuous (21, 17, 1, 15, 24). However, published studies addressing the morphological variations of the seminiferous tubules related to zinc supplementation are scanty.

REFERENCES

1. Allrich, R. D., Christenson, R. K., Ford, J. J., Zimmerman, D. R., 1983: Pubertal development of the boar: Age related changes in Testicular morphology and in- vitro production of testosterone and Estradiol 17 β. *Biol. Reprod.,* 28, 902–909.

2. Anderson, M. B., Lepak, K., Farinas, V., George, W. J., 1993: Protective action of zinc against cobalt induced testicular damage in the mouse. *Reprod. Toxicol.,* 7, 49–54.

3. Bedwal, R. S., Bahuguna, A., 1994: Zinc, copper and selenium in reproduction. *Cellular and Molecular Life Sciences,* 50, 626–640.

4. Bongso, T. A., Jainudeen, M. R., Zahrah, Siti, A., 1982: Relationship of scrotal circumference to age, body weight and onset of spermatogenesis in goats. *Theriogenology,* 18, 513–524.

5. Davies, S., 1985: Zinc, nutrition and health. In *1984–85 Yearbook of Nutritional Medicine.* **Bland, J.** (ed.), 113–152, Keats Publishing, New Canaan, Conn.

6. Gruzdev, N. V., 1975: Effect of proportion of zinc in the diet on sperm production in bulls. *Zhivotnovodstvo,* 6, 55–56.

7. Karmore, S. K., Bhamburker, V. R., Dalvi, R. S., Banubakode, S. B., Nandeswar, N. C., 2003: Histomorphology of testes of goat *(Capra hircus). Indian J. Anim. Sci.,* 73, 187–189.

8. Kuludin, I., 1983: Animal Breeding Abstract, 52, 4697 (c. f. Sudheer, S., 2000, Relationship between testicular size and seminal attributes in crossbred buls. *Int. J. Animal. Res.,* 34, 159–160).

9. Kumar, N., Verma, R. P., Singh, L. P., Varshney, V. P., Dass, R. S., 2006: Effect of different levels and sources of zinc supplementation on quantitative and qualitative semen attributes and serum testosterone level in crossbred cattle *(Bos indicus × Bos taurus) bulls. Reprod. Nutr. Dev.,* 46, 663–675.

10. Lee, J., Lee, B., Lee, S. 2004: Studies on spermatogenesis in Korean native goat. *Korean J. Vet. Res.,* 25, 91–101.

11. McDowell, L. R., Conrad, J. H., Hembry, R., 1993: *Minerals for Grazing Ruminants in Tropical Regions,* 2nd edn., Animal Science Department, University of Florida, 122–134.

12. Miller, M. J., Fisher, M. I., Elocote, P. V., Mawson, C. A., 1958: The effects of dietary zinc deficiency on the reproductive system of male rats. *Canadian J. Biochem. Physiol.,* 36, 557.

13. Miller, W. J., Clifton, C. M., Fowler, P. R., 1965: Influence of high levels of dietary zinc on zinc on milk, performance and biochemistry of lactating cows. *J. Dairy Sci.,* 48, 450.

14. Mishra, T. P., Saxena, V. B., Tripathi, S. S., 1989: Trace elements in semen plasma of crossbred bulls. *Indian J. Anim. Sci.,* 59, 1245–1248.

15. Nishimura, S., Okano, K., Yasukouchi, K., Gotoh, T., Tabata, S., Iwamoto, H., 2000: Testes development and puberty in the male Tokara *(Japanese native)* goat. *Anim. Reprod. Sci.,* 64, 127–131.

16. Pitts, W. J., Miller, W. J., Fosgate, O. T., Morton, J. D., Clifton, C. M., 1966: Effect of zinc deficiency, and restricted feeding from two to five months of age on Reproduction in Holstein bulls. *J. Dairy Sciences,* 49, 995–1000.

17. Raja, C. K. S. V., Rao, A. R., 1982: Note on histological development of the testis in Brown Swiss crossbred bulls. *Indian J. Anim. Sci.,* 52, 1241–1243.

18. Rojas, L. X., McDowell, L. R., Martin, F. G., Wilkinson, N. S., Johnson, A. B., Njeru, C. A., 1996: Relative bioavailability of zinc methionine and two inorganic zinc sources fed to cattle. *J. Trace Elem. Med. Biol.,* 10, 205–209.

19. Saleh, P., 1992: Role of minerals in Reproduction. A review. *Indian J. Dairy Sci.* 45, 4.

20. Sarlos, P. R., Molnar, L., Ratky, A., Jozsef, 2005: Role of zinc supplement in weight gain, testicle development and semen characteristics of young rams. *REU Technical Series 50: Sheep and Goat Production in Central and Eastern European Countries,* 1–4.

21. Sergeev, N. I., Zaboltskii, V. A., 1976: Age changes in the development of the testis in rams. *Doklady Vesoyuzuoi Akademii Selskokhozyaistvennykh Nauk.,* 12, 26–28. (fide *Vet. Bull.,* 47, 4723).

22. Snedecor, G. W., Cochran, W. G., 1994: *Statistical Methods,* 1st East-West Press edn., Affiliated East-West Private Ltd., New Delhi, 55–67.

23. Underwood, E. J., Somers, M., 1969: Studies of zinc nutrition in sheep. I. The relation of zinc to growth, testicular development, and spermatogenesis in young rams. *Australian J. Agr. Res.,* 20, 889.

24. Zahid, I. A., Lodhi, L. A., Rehman, N. U., Akhtar, M. S., 2002: Effects of gossypol on micrometry of testis of Teddy male goat. *Pakistan Vet. J.,* 27, 55–62.

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RABBIT AS AN ANIMAL MODEL IN SPINAL CORD INJURY

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ABSTRACT

Experimental studies on animals, such as the detailed knowledge of the anatomy of the blood supply to the spinal cord including all existing variations, can contribute to the enhanced treatment and protection of the spinal cord. We, therefore, observed the morphological variations of the blood supply to the spinal cord. Investigations were carried out on 10 adult New Zealand rabbits. We prepared corrosion casts of the blood supply to the spinal cord by using Batson's corrosion casting kit No. 17. The presence of branches entering the *arteria spinalis ventralis* in the thoracic region was observed in 71% of cases on the left side and in 29% on the right side. In the lumbar region, left-sided branches were observed in 52% of the cases and right-sided in 48% of the cases. Along the entire thoracic and lumbar spinal regions we observed left-sided branches in 62.5% and right-sided in 37.5% of the cases, which is most likely related to the left-sided localisation of the aorta. The artery of Adamkiewicz was present in all cases. In 50% of the cases, this artery was left-sided and in 50% right-sided. Documenting the anatomical variations in the spinal cord blood supply in the rabbit will aid in the planning of future experimental studies and determining the clinical relevance of such studies.

Key words: blood supply; rabbit; spinal cord; the artery of Adamkiewicz

INTRODUCTION

Thoracic surgery poses a rare, yet potentially tragic threat to the integrity of the spinal cord circulation. Many diseases can cause spinal cord ischemic injury; i.e. scoliosis, thrombosis, embolism and arterio-venous malformations. Iatrogenic intraoperative spinal cord ischemic injury is also multifactorial, with mechanisms including perioperative hypotension, acute thrombo-embolic episodes, aortic cross clamping, increased cerebrospinal fluid pressure, and the interruption or compression of intercostal and lumbar arterial supply (4).

Spinal cord ischemic injury generally results from the interruption of the spinal cord blood supply. In addition to considerable variations in the normal anatomy, occlusion of segmental arteries due to mural thrombus or artherosclerotic change has made it difficult to elucidate the pathogenesis of spinal cord ischemia. This is why there are conflicting views regarding the appropriate strategy for reconstruction of the segmental arteries in thoracoabdominal surgical repair (8). The anatomy of the spinal cord circulation and its marked variations must be appreciated and the post-operative clinical presentations must be clarified for appropriate follow-up care.

Experimental studies on animals and detailed knowledge of the anatomical variations of the blood supply to the spinal cord may contribute to better treatment of the spinal cord. Rabbits are laboratory animals frequently used in studies of spinal cord ischemic injuries. Few studies have dealt with the arterial supply of the spinal cord in the rabbit (12). The high variability of spinal cord feeding arteries was infallibly demonstrated in several species but has been probably best documented in man (1, 3, 5, 6, 9).

The aim of this study was to contribute to the knowledge of the arterial supply of the spinal cord of rabbits with a focus on the thoracic and lumbar regions in which the surgical procedures may be associated with the risk of potentially serious neurological damage. At the same time we would like to illustrate some variations in the segmental arterial supply of the spinal cord.

MATERIALS AND METHODS

The study was carried out on 10 adult (age = 140 days) New Zealand white rabbits (breed HY+), females (n = 5) and males (n = 5), of mean weight 2.5–3 kg, in an accredited experimental laboratory at the University of Veterinary Medicine in Kosice, SR. The animals were kept in cages under standard conditions (temperature 15–20 °C, relative humidity 45 %, 12 hour light period) and fed granular mixed feed (O-10 NORM TYP). Drinking water was provided *ad libitum*. The animals were euthanized by prolonged inhalation anaesthesia with ether. Immediately after euthanasia, the vascular network was perfused with saline. During the manual injections through an ascending aorta, the right vestibule was opened in order to lower the pressure in the vessels to ensure thorough profusions (2). Batson's corrosion casting kit No. 17 in the quantity of 50 ml (Dione, the Czech Republic) was used as a casting medium. After polymerisation of the medium (1 hour), 10 % formalin was injected into the vertebral canal between the last lumbar vertebra and sacrum in order to fix the spinal cord. After 24 h fixation the vertebral canal was opened by removing vertebral arches in the thoracic, lumbar and sacral spinal regions. The prepared spinal cord was fixed in 10 % formaldehyde.

RESULTS

The spinal cord receives blood from the *arteria spinalis ventralis* (in humans it is known as the *arteria spinalis ante-*

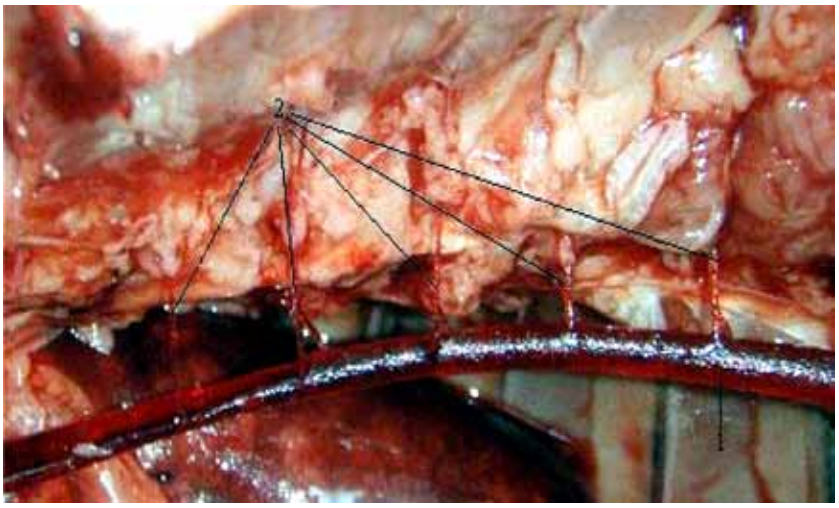

Fig. 1. ***Arteriae intercostales dorsales*** **as segmental branches of** ***aorta thoracica*****.**
1. ***aorta thoracica,*** **2.** ***arteriae intercostales dorsales*****. Lateral view. Macroscopic image**

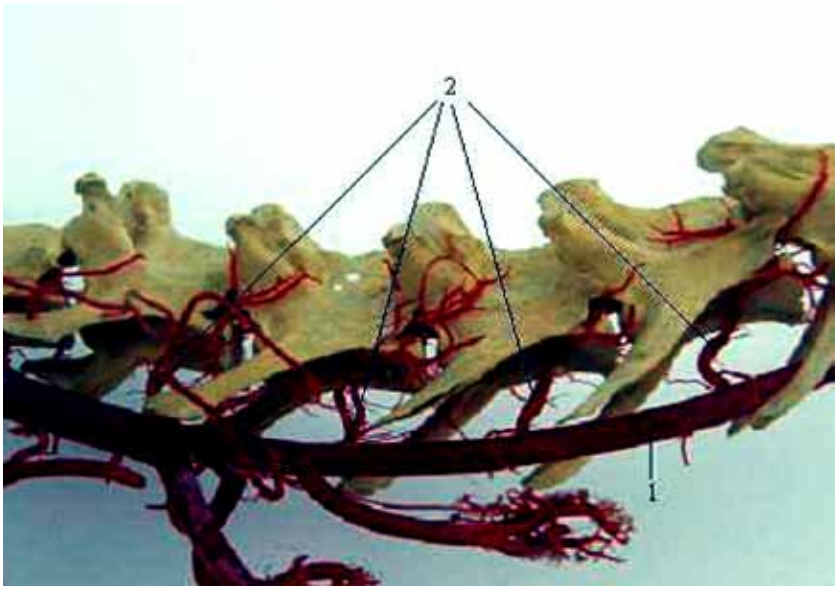

Fig. 2. ***Arteriae lumbales*** **as segmental branches of** ***aorta abdominalis*****.**
1. ***aorta abdominalis,*** **2.** ***arteriae lumbale*****s. Lateral view. Macroscopic image**

Fig. 3. Segmental branches entering *arteria spinalis ventralis*
1. *arteria spinalis ventralis,* 2. ventral branch of *ramus spinalis arteriae intercostalis dorsalis V sinistrae,* 3. ventral branch of *ramus spinalis arteriae intercostalis dorsalis VI sinistrae,* 4. ventral branch of *ramus spinalis arteriae intercostalis dorsalis VII dextrae,* 5. ventral branch of *ramus spinalis arteriae intercostalis dorsalis IX dextrae,* 6. ventral branch of *ramus spinalis arteriae intercostalis dorsalis XI sinistrae,* 7. ventral branch of *ramus spinalis arteriae intercostalis dorsalis XIII dextrae,* 8. ventral branch of *ramus spinalis arteriae intercostalis dorsalis XIII sinistrae*.
Ventral view. Macroscopic image

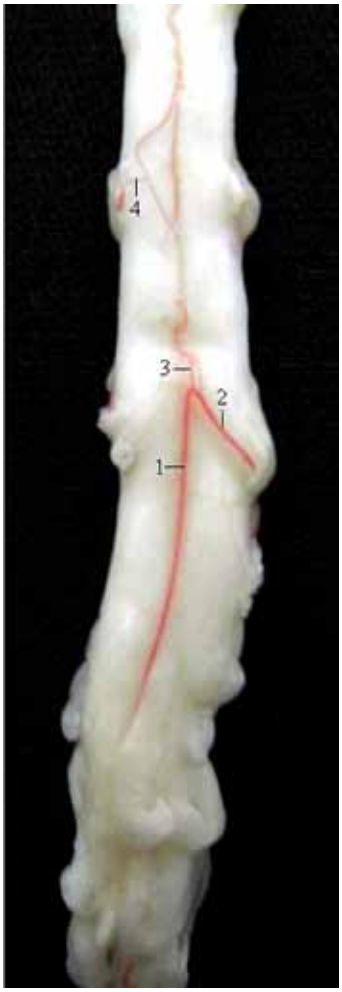

Fig. 4. Left-sided localisation of Adamkiewicz artery. 1. *arteria spinalis ventralis,* 2. Adamkiewicz artery, 3. branch of Adamkiewicz artery running cranially, 4. ventral branch of *ramus spinalis arteria lumbalis V dextrae*.
Ventral view. Macroscopic image

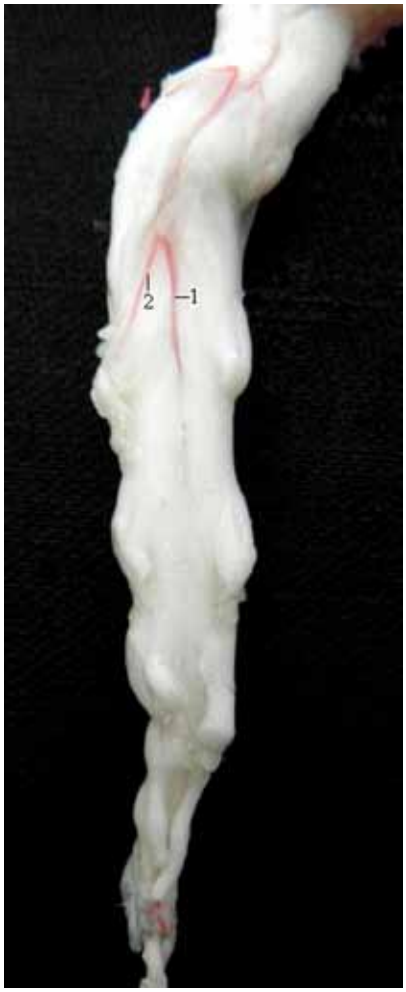

Fig. 5. Right-sided localisation of Adamkiewicz artery. 1. *arteria spinalis ventralis,* 2. Adamkiewicz artery. Ventral view. Macroscopic image

rior). It runs subdurally in the ventral median fissure of the spinal cord. In the thoracic and lumbar regions it receives strengthening branches from the intercostal and lumbar arteries (Fig. 1, 2). They enter the vertebral canal through the *foramen intervertebrale* in association with the respective spinal nerve roots. After entering the vertebral canal they flow to the spinal cord *ramus spinalis,* which is divided into dorsal and ventral branches. The ventral branch enters the *arteria spinalis ventralis*. The frequency of occurrence of individual segmental arteries is shown in Table 1. The presence of branches entering arteria *spinalis ventralis* in the thoracic region was observed in 71 % of the cases on the left side and in 29 % on the right side. In the lumbar region, left-sided branches were observed in 52 % of the cases and right-sided in 48 % of the cases. Along the entire thoracic and lumbar spinal regions, we observed left-sided branches in 62.5 % and right-sided in 37.5 % of the cases (Fig. 3), which is most likely related to the left-sided localisation of the aorta.

In addition to relatively small and weak segmental spinal arteries, we observed also a bigger feeding artery arising from the spinal branch of the sixth lumbar artery, entering the vertebral canal through the *foramen intervertebrale* and passing into *arteria spinalis ventralis*. This artery, termed *arteria radicularis magna* or the artery of Adamkiewicz, was present in all cases. In 50 % of the cases this artery was left-sided (Fig. 4) and in 50 % right-sided (Fig. 5). The artery of Adamkiewicz supplies the spinal cord caudally from the point of narrowing of the *arteria spinalis ventralis* in the lumbar region. After reaching the *fissura mediana ventralis* it runs caudally replacing *arteria spinalis ventralis* and sends an important branch cranially to the thinning *arteria spinalis ventralis* (Fig. 4).

Table 1. Frequency of occurrence of spinal arteries in the thoracic and lumbar regions of spinal cord

Frequency of occurrence of spinal arteries (%)		
Level	**Right**	**Left**
Th 1	**0**	**50**
Th 2	**25**	**50**
Th 3	**25**	**0**
Th 4	**0**	**50**
Th 5	**0**	**75**
Th 6	**25**	**75**
Th 7	**25**	**25**
Th 8	**0**	**50**
Th 9	**25**	**0**
Th 10	**25**	**50**
Th 11	**25**	**0**
Th 12	**25**	**25**
Th 13	**25**	**75**
L 1	**50**	**50**
L 2	**25**	**25**
L 3	**50**	**50**
L 4	**75**	**75**
L 5	**50**	**75**
L 6	**50**	**50**

Th – thoracic spinal cord region; L – lumbar spinal cord region

DISCUSSION

Observations in the rabbit confirmed the high variability of the segmental arteries supplying blood to the spinal cord. On the left side, they occurred in higher number with more uniform distribution. Arteries in the thoracic and lumbar regions ensured the segmental supply of the respective sections of *arteria spinalis ventralis* and this supplies the spinal cord caudally from the point of narrowing. This is the reason why the rabbit can be partially used for simulation of spinal cord ischemia in man. Segmental arteries in the thoracic region occurred irregularly and their absence was noted more frequently than in the lumbar region which allowed us to assume a higher risk of irreparable ischemic damage to the thoracic region of the spinal cord in the rabbit.

The artery of Adamkiewicz, regularly observed in man in which it supplies the caudal two thirds of the spinal cord, was present in all the cases (7). While in humans this artery arises typically between the eighth thoracic and second lumbar vertebrae (3, 10), in all rabbits examined in our study it originated more caudally; namely at the sixth lumbar vertebral level. An extensive angiographic study in humans revealed that the most caudal origin of Adamkiewicz artery was observed at the level of fourth lumbar vertebra in 30 % of the cases (5).

Spinal cord ischaemia in the rabbit is induced by the simple occlusion of the aorta caudally from the origin of the *arteriae renales,* contrary to the animals with heterosegmental blood supply to the aorta, for example the dog, in which double ligature of aorta is necessary – caudally from the origin of the arteria subclavia sinistra and cranially close to the *hiatus aorticus* of the diaphragm, which is considerably a more demanding surgical procedure.

CONCLUSIONS

The presence of the artery of Adamkiewicz in all our studied animals and its more caudal origin explains the use of the rabbit as a preferred simple model of ischaemic dam-

age to the caudal half of the spinal cord. The use of the rabbit in the study of spinal cord ischemic injury is technically more advantageous than in the mouse or rat. This is because of the larger size of the rabbit and there have been some studies which make the presence of the artery of Adamkiewicz in rats questionable (12). Also, compared with dogs, using rabbits in such experiments is less expensive.

Documenting the anatomical variations in the spinal cord blood supply in the rabbit will aid in the planning of future experimental studies and may shed light on determining the clinical relevance of such studies.

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REFERENCES

1. Alleyne, C. H., Cawley, C. M., Shengelaia, G. G., 1998: Microsurgical anatomy of the artery of Adamkiewicz and its segmental artery. *J. Neurosurg.,* 89, 791–795.

2. Flešárová, S., Tesfaye, A., Danko, J., Bátor, R., Dorko, F., Barta, A., 2003: Morphological variations of arcus aortae in rabbits. *Acta Vet. Brno,* 72, S20, Supplementum, 116.

3. Koshino, T., Murakami, G., Morishita, K., 1999: Does the Adamkiewicz artery originate from the larger segmental arteries? *J. Thorac. Cardiovasc. Surg.,* 117, 898–905.

4. Laschinger, J. C., Izumoto, H., Kouchoukos, N. T., 1987: Evolving concepts in prevention of spinal cord injury during operations on the descending thoracic and thoracoabdominal aorta. *Ann. Thorac. Surg.,* 44, 667–674.

5. Lo, D., Valleé, J. N., Spelle, L., 2002: Unusual origin of the artery of Adamkiewicz from the fourth lumbar artery. *Neuroradiology,* 44, 153–157.

6. Malikov, S., Rosset, E., Paraskevas, N., 2002: Extra-anatomical revascularization of the artery of Adamkiewicz: anatomical study. *Ann. Vasc. Surg.,* 16, 723–729.

7. Milen, M. T., Bloom, D. A., Culligan, J., 1999: Albert Adamkiewicz (1850–1921) – his artery and its significance for the retroperitoneal surgeon. *World. J. Urol.,* 17, 168–170.

8. Morishita, K., Murakami, G., Fujisawa, Y., Kawaharada, N., Fukada, J., Saito, T., Abe, T., 2003: Anatomical study of blood supply to the spinal cord. *Ann. Thorac. Surg.,* 76, 1967–1971.

9. Nijenhuis, R. J., Leiner, T., Cornips, E. M., 2004: Spinal cord feeding arteries at MR angiography for thoracoscopic spinal surgery: feasibility study and implications for surgical approach. *Radiology,* 233, 541–547.

10. Rodriguez-Baeza, A., Muset-Lara, A., Rodriguez-Pazos, M., 1991: The arterial supply of the human spinal cord: a new approach to the arteria radicularis magna of Adamkiewicz. *Acta Neurochir.,* 109, 57–62.

11. Schievink, W. I., Luyendijk, W., Los, J. A., 1988: Does the artery of Adamkiewicz exist in the albino rat? *J. Anat.,* 161,95–101.

12. Soutoul, J. H., Gouaz'e, A., Castaing, J., 1964: The spinal cord arteries of experimental animals. 3. Comparative study of the rat, guinea-pig, rabbit, cat, dog, orang-outang, chimpanzee, with man and fetus. *Pathol. Biol.,* 12, 950–962.

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MORPHOLOGICAL CHANGES IN THE SPONGY BONE OF JAPANESE QUAILS AFTER 90 DAYS OF HYPODYNAMY

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ABSTRACT

One of the major well known consequences of prolonged weightlessness in animals is a significant depression in the quality and quantity of the musculoskeletal mass. We investigated the morphological changes in the spongy bone of Japanese quails following their exposure to the ground based model simulated microgravity (hypodynamy). The primary structural alterations of the spongy bone consisted of a marked increase in the number and activity of the osteoclasts when compared with the controls. In addition, we also observed decreasing numbers of the bone forming cells (osteoblasts) in the treated group compared with the controls.

Key words: hypodynamy; Japanese quails; spongy bone

INTRODUCTION

The Japanese quail *(Coturnix coturnix japonica)* is a bird with: a low body weight; high productive and reproductive abilities; short individual development; and a high tolerance for crowded conditions (10). Due to these unique experimental advantages and its distinctive adaptability to microgravity (17), it was considered a good prospect for the space programme, where it could possibly ensure production of animal proteins for astronauts (2, 3) and/or provide suitable animal model experiments (1). Although a variety of physiological alterations during true spaceflights or simulated unloaded conditions have been detected, their cytological mechanisms remain unclear. Because experiments in space are demanding and expensive, it is far less costly to use animal model experiments on the earth, even though it is not possible to simulate completely such a unique space environment. Using a well established avian model for simulation of microgravity on the ground conditions, we have investigated the possible changes of the microscopic and ultramicroscopic histology of spongy bone following 90 days of hypodynamy in Japanese quail.

MATERIALS AND METHODS

The experiment was carried out at the Institute of Animal Biochemistry and Genetics of SAS in Ivanka pri Dunaji. On the second day after hatching six male chicks were placed into individual slings suspended by a flexible device in such a way that their legs could not touch the floor. The Japanese quails were kept under microclimatic conditions favourable for their growth and welfare. At the same time, an equal number of quail chicks of the control group were placed in a rearing box and were kept at the same respective conditions until 92 days of age. The birds were fed a complete mixed feed HYD–13 throughout the experiment. Feed and water were provided *ad libitum.* The care and use of animals complied with the laws and regulations of the Slovak Republic (11) and were approved by the Ethical Committee of the Institute of Animal Biochemistry and Genetics.

The samples of spongy bone harvested from the femur were: immediately fixed by immersion in 3 % glutaraldehyde buffered in 0.1 M cacodylate buffer; decalcified in buffered EDTA at pH 7.2; then postfixed by 1 % OsO_4 in 0.1 M cacodylate buffer; dehydrated

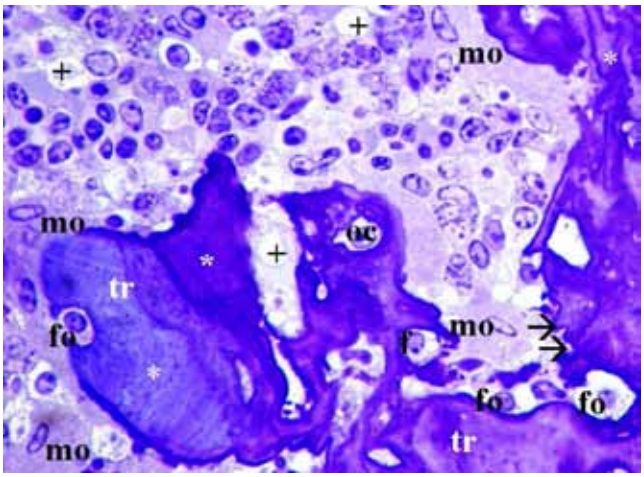

Fig. 1. Spongy bone of Japanese quail after 90 days hypodynamy (semithin section). Magn. ×1000.
mo – multinucleated osteoclasts with foamy cytoplasm; oc – osteocytes within the trabeculae; fo – free osteocytes; arrows – foveolated trabeculae; + – degenerated blood cells; * – injured matrix

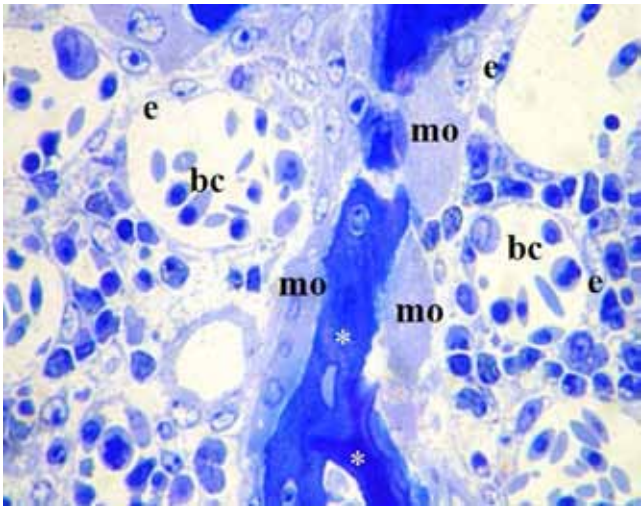

Fig. 2. Spongy bone of Japanese quail after 90 days hypodynamy (semithin section). Magn. ×1000.
bc – blood cells; e – endothelium; mo – multinucleated osteoclasts with foamy cytoplasm; * – injured matrix

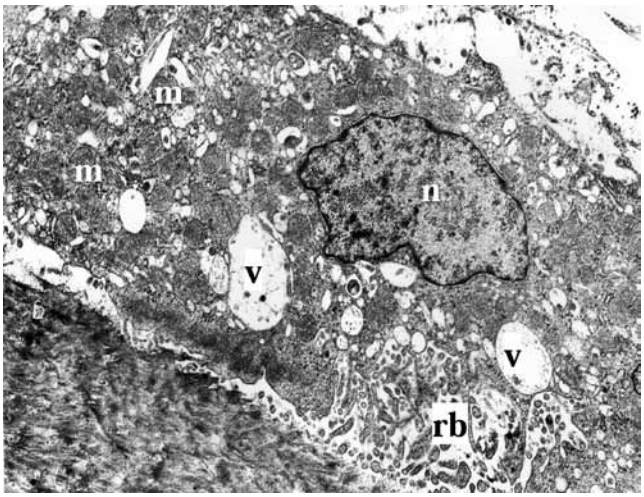

Fig. 3. Electron micrograph of osteoclast in spongy bone of Japanese quail after 90 days of hypodynamy(ultrathin section). Magn. ×6200.
rb – ruffled border; v – vacuoles and vesicles; n – nucleus; m – mitochondria

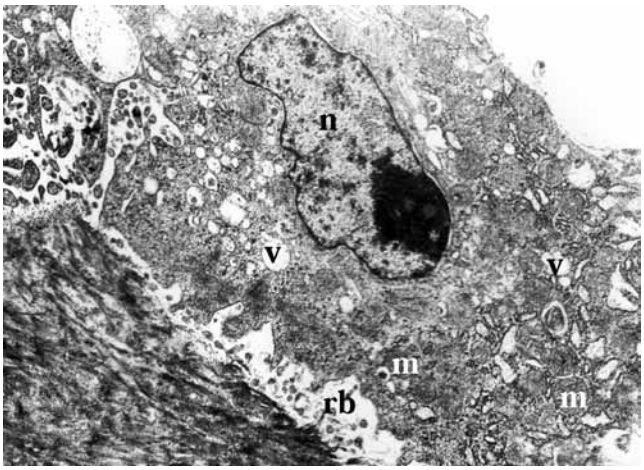

Fig. 4. Electron micrograph of osteoclast in the spongy bone of Japanese quail after 90 days of hypodynamy (ultrathin section). Magn. ×6800.
rb – ruffled border; v – vacuoles and vesicles; n – nucleus; m – mitochondria

in acetone; and embedded in Durcupan ACM. One μm semithin sections for light microscopic examination were carried out by use of the ultramicrotome Tesla BS 490, and immediately stained by toluidin blue; the ultrathin sections for transmission electron microscopic examination were contrasted with uranyl acetate and lead citrate (13). Photodocumentation was performed by the light microscope Jenamed and the electron microscope Tesla BS 500.

RESULTS

The ninety day exposure of the experimental Japonese quail to hypodynamy caused significant microscopic and ultramicroscopic changes to their spongy bone. One of the major changes in the structure of the bone included a strong elevation in the number and activity patterns of the osteoclasts (bone resorbing cells) in comparison with the controls. These multinucleated giant cells covered much of the bone trabeculae and contained from 6 to 12 oval shaped nuclei. The cytoplasm of these cells was strongly vacuolated which caused its foamy appearance (Fig. 1, Fig. 2). The trabeculae showed a highly foveolated surface and strongly reduced thickness in response to the exuberant activity of the osteoclasts. There were evident disruptions in the structure of the intercellular matrix as a result of the failure in mineralization and osteosynthesis (Fig. 1, Fig. 2). Concomitantly,

the osteoblasts (bone forming cells) completely disappeared from the bone tissue. Some osteocytes lived normally inside the irregular shaped lacunae within the trabeculae, but others were found beside the trabeculae lying freely on their surfaces (Fig. 1). We surmise that these cells are osteocytes exposed from the trabeculae following extensive osteoclastic bone resorption.

The bone marrow contained blood forming elements in different stages of their differentiation. Occasionally dead blood forming elements or even small and irregular empty spaces between the developing blood cells could be seen, which could be signs of the degeneration of these cells (Fig. 1, Fig. 2). There were no adipocytes in the inter-trabecular spaces. The endothelium of the blood vessels remained intact (Fig. 2).

The ultrastructure of the osteoclasts testified to their very strong resorptive action. Noticeable was the fully developed ruffled border consisting of many highly folded microvilli. The cytoplasm contained many vacuoles and vesicles of different sizes, confirming their robust vesicular transport activity as a sign of elevated resorption as compared to controls. The nuclei were highly euchromatic and often in close association with the Golgi complex. The endoplasmic reticulum and mitochondria were conspicuous as well (Fig. 3, Fig. 4).

DISCUSSION

We recorded an obvious unfavourable stress induced deleterious effect of prolonged hypodynamy on the bone tissue in the experimental quails. Light microscopy revealed significant changes between the structure of the spongy bone tissue of the experimental and the control birds. As the most dominant impact of the experimental microgravity, we consider the elevated bone resorption due to very strong activation of the osteoclasts at the expense of bone formation.

An adverse impact of true or simulated unloaded conditions on the skeletal system has been proven by many authors. They noted the negative effect of long-duration space flight on: calcium metabolism (16, 18); disturbed bone development in simulated microgravity (7, 15); or changes in the ultrastructure of bone cells (14). In spite of many scientific findings, cytological mechanisms of gravity dependent changes in bone remain still quite unclear. Recent experiments using several osteoblastic cell types confirmed the significant alterations in the cell morphology and gene expression of different factors under microgravity conditions (4). Together, these results suggest some sort of disturbed cellular function.

The effects of simulated microgravity on Japanese quail have been studied by many authors. They generally concluded that hypodynamy acts as a high stress factor (6, 8), even though the beneficial finding is that Japanese quails are able gradually to adapt to hypodynamy (17). Hypodynamy and microgravity also induced some disorders on the reproductive organs (5), and morphological changes were found in small intestinal cells (12), liver, lungs, bone marrow (20) and kidneys (5). Regarding the skeleton and muscle systems they respond to the microgravity by atrophy of the muscles of the limbs (9). According to Zibrín *et al.* (19), long-term experimental hypodynamy caused the activation of osteoclasts and loss of spongy bone in an adult Japanese quails. Our observations in this paper support these findings.

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REFERENCES

1. Baumgartner, J., Hetényi, L., 2001: *Japanese Quail* (In Slovak). VÚŽV, Publ. house Slovenský chov, Nitra, 75 pp.

2. Boďa, K., 1984: *Cosmic Biology. Gravitation Physiology* (In Slovak). Publ. house SAV, Košice, 9–12.

3. Boďa K., 1997: Cosmic biology and veterinary medicine (In Slovak). *Slov. Vet. Čas. (Slovak Veterinary Journal),* 22, 12–126.

4. Carmeliet, G., Bouillon, R., 1999: The effect of microgravity on morphology and gene expression of osteoblasts *in vitro. FASEB J.,* 13, 129–134.

5. Cigánková, V., Kočišová, J., Boďa, K., Tomajková, E., Dadasheva, O. A., 1993: Morphological changes in tissue of the testes and kidneys in Japanese quail exposed to microgravity. *Acta Vet. Brno,* 62, 69–71.

6. Cigánková, V., Zibrín, M., Boďa, K., Holovská, K., 2005: Effect of long-term experimental hypodynamy on the adrenal glands of Japanese quails: An ultrastructural study. *Bull. Vet. Inst. Pulawy,* 49, 449–453.

7. Fleming, O. S., Reddy, S., Mantalaris, A., 2002: Advances in the study of bone development in simulated microgravity. *European Cells and Materials,* 4, 123–124.

8. Juráni, M., Boďa, K., Výboh, P., Zeman, M., Lamošová, D., Somogyiová, E. *et al.,* 1991: An endocrine response to short-term hypodynamy in Japanese quail selected for resistance to hypodynamy. *Physiologist,* 34, 129–131.

9. Kočišová, J., Cigánková, V., Zibrín, M., Tomajková, E., Sabo, V., Boďa, K. *et al.,* 2002: Morphological study of gastrocnemius muscle in Japanese quail chicks hatched out in space flight. *Aviakosm Ekolog. Med,* 36, 6–9.

10. Koréneková, B., Skalická, M., Naď, P., Korének, M., 2004: Breeding and history of Japanese quails (In Slovak). *Magazín chovateľa (Breeder's Magazine),* 8, 46–47.

11. Korim, P., Bugarský, A., Juriš, P., Hadbavný, M., Korimová, M., 2003: Protection of experimental animals (In Slovak). *Slov. Vet. Čas. (Slovak Veterinary Journal),* 28, 10–12.

12. Lenhardt, Ľ., Cigánková, V., Zibrín, M., Kočišová, J., Tomajková, E., Sabo, V. *et al.,* 2001: Functional development of small intestine of Japanese quail hatched on Mir orbital station. *Acta Vet. Brno,* 70, 127–131.

13. Mráz, P., Polónyi, J., 1988: *Methods of Electron Microscopy of Animal Tissues* (In Slovak). Publ. house SAV-VEDA, 152–161.

14. Rodionova, N. V., Oganov, V. S., Zolotov, N. V., 2002: Ultrastructural changes in osteocytes in microgravity conditions. *Adv. Space Res.,* 4, 765–770.

15. Saxena, R., Pan, G., McDonald, J. M., 2007: Osteoblast and osteoclast differentiation in modeled microgravity. *Ann. NY Acad., Sci.,* 1116, 494–498.

16. Smith, S. M., Wastney, M. E., Morukov, B. V., Larina, I. M., Nyquist, L. E., Abrams, S. A. *et al.,* 1999: Calcium metabolism before, during, and after a 3-month space flight: kinetic and biochemical changes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* 277, 1–10.

17. Škrobánek, P., Hrančová, M., 2003: Adaptability of Japanese quail chicks to conditions of simulated weightlessness. *Acta Vet, Brno,* 2, 347–351.

18. Whedon, G. D., Rambaut, P. C., 2005: Effects of long-duration space flight on calcium metabolism: Review of human studies from Skylab to the present. *Acta Astronautica,* 58, 59 81.

19. Zibrín, M., Boďa, K., Cigánková, V., Kočišová, J., Tomajková, E., Komorová, T. *et al.,* 2003: Long-term experimental hypodynamy affects the structure of spongy bone and osteoclasts in Japanese quail. *Acta Vet. Brno,* 72, 143–149.

20. Zibrín, M., Cigánková, V., Kočišová, J., Tomajková, E., Komorová, T., Boďa, K. *et al.,* 2005: Effect of short-term space flight on structure of liver, lungs, bone and bone marrow of Japanese Quail hatched on orbital station Mir. *Acta Vet. Brno,* 74, 167–174.

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MAMMARY GLAND TUMOURS: HORMONE DEPENDENCY AND STROMA REACTIONS IN TUMOUR PROGRESSION AND PROGNOSIS (A Review)

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ABSTRACT

Breast cancer is a highly heterogeneous disease represented by tumours that have a diverse natural history, complex histology and a variable response to therapy. Breast cancer was recognized to be a hormone-dependent malignancy as early as 1896, when Beatson reported that the removal of the ovaries caused the regression of disseminated breast cancer. Estrogens are important for the development of the mammary gland and strongly associated with oncogenesis in this tissue. The biological effects of estrogens are mediated through the Estrogens Receptor (ER), a member of the nuclear receptor superfamily. The estrogens signalling pathway plays a central role in the mammary gland development regulating the expression and activity of other growth factors and their receptors.

Transforming Growth Factor-β (TGF-β) plays a central role in embryonic development, organogenesis and physiologic connective tissue remodelling during wound healing and tissue repair, as well as in carcinogenesis. It acts as a potent inhibitor of tumour progression, owing to an induction of growth arrest and apoptosis. However, in many carcinomas, the levels of TGF-β are elevated. Breast cancer, in particular, express high levels of TGF-β1 and the level of expression is correlated with the rate of diseases progression.

During tumour evolution, the stroma, composed of inflammatory cells, small vessels, fibroblastic and myofibroblastic cells, and extracellular matrix (ECM) components, reflects the disturbed interactions between the neoplastic population and their surroundings. Myofibroblasts differentiate from resident stromal fibroblasts. This process is induced also by the well known cytokine – TGF-β. Fibroblasts and myofibroblasts have been suggested to represent important players in the development of the invasive process.

This review describes the different roles, relationship and sequence of estrogen receptors, transforming growth factors (TGF) and myofibroblasts under physiological conditions as well as in the process of mammary gland tumorigenesis.

Key words: mammary tumours; prognosis; progression

INTRODUCTION

The mammary gland, a specialized accessory gland of the skin that characterizes the mammalian species, is a frequent source of tumours and neoplasms. Worldwide, breast cancer comprises 10.4 % of all cancer incidences among women, making it the second most common type of non-skin cancer (after lung cancer) and the fifth most common cause of cancer death. The incidence is continuously increasing; most of all, in the industrialized parts of the world (26).

Although breast cancer develops in women as the result of a combination of external and endogenous factors such as exposure to ionizing radiation, diet, socioeconomic status and endocrinologic, familial or genetic factors, no specific agent(s) or the mechanism responsible for the initiation of the disease have been clearly identified as yet. Thus, experimental models that exhibit the same complex interactions are needed for testing various mechanisms

and for assessing the carcinogenic potential of given chemicals. Rodent mammary carcinomas represent such a model to a great extent because in these species, mammary cancer is a multistep complex process that can be induced either by chemicals, radiations, viruses or genetic factors (19).

In mammals estrogens play a crucial role in a wide range of physiological processes, such as reproduction and behaviour. However, estrogens are also involved in the development and progression of diseases. Thus, they are considered as risk factors for tumourigenesis, especially in tissues that respond to sex steroids with receptor mediated proliferation, namely the breast epithelium and endometrium. In their capacity to regulate the development and function of reproductive organs, they are often committed to interactions with growth factors, especially TGF-β. Unfortunately, there exists only scarce information regarding this question (1).

As TGF-β exerts positive as well as negative influences on cancer cells, it is considered as both a promoter and a suppressor of tumour progression. The growth factor negatively regulates the proliferation of normal and transformed epithelial cells, including those of the mammary gland, by arrest in the G1 phase of the cell cycle and by promoting apoptosis. During cancer development, the cells become less sensitive to the antiproliferative and proapoptotic effects of the chemokine. Concomitantly, the tumour promoting properties, like induction of Extra Cellular Matrix (ECM) and cell migration, gain in importance. The migratory potency of cancer cells demonstrably increases in response to estrogens and growth factors and substantially accounts for metastatic dissemination of a tumour from its primary site (1).

All carcinomas arise as complex mixtures of a variety of cell types (fibroblasts, endothelial cells, smooth muscle cells, and inflammatory cells), which are summarily termed stroma (6). The predominant stromal cells type in most carcinomas are the myofibroblasts which transdifferentiate from fibroblasts. This process is induced by paracrine signals; one of the most potent one being TGF-β. The presence of myofibroblasts in tumour stroma prevent invasion of immune and inflammatory cells into tumours which represent the worse prognosis of the neoplastic process (8).

This review describes the different roles, relationship and sequence of estrogen receptors, transforming growth factors (TGF) and myofibroblasts under physiological conditions as well as in the process of mammary gland tumorigenesis.

Pathogenesis of mammary gland neoplasia

Cancer is a genetic disease. Damage to the cellular genome is a common feature for virtually all neoplasm, despite the fact that neoplasm arise in a broad variety of tissues and that diverse agents such as viruses, mutagenic chemicals and radiation induce their outgrowth. The genetic damage produced by carcinogens is believed to be random, and many mutations may be inconsequentional (3).

Some genes are known to increase cellular transformation (TGF-α) or growth by over expression (EGF – epidermal growth factor) or by mutation (p53). Gene amplifications and mutations have recently been reported in canine mammary gland carcinoma. Relatively little is known about the time of initiation or what agents may cause mammary cancer. Experimental studies in the rat have demonstrated that the types of mammary lesion that appeared after administration of dimethylbenz(a)antracene (DBMA) were associated with the mammary gland development and hence with hormonal status. Mammary carcinomas arose in the terminal end buds in the most primitive end buds in the most primitive ductal structures in the very young animals. Adenomas, fibroadenomas, and cysts arose from more differentiated structures, such as alveolar buds, in slightly older animals (18).

Growth hormone production was demonstrated in structures resembling terminal end buds in dogs under endogenous or exogenous progesterone stimulation, possibly indicating autocrine or paracrine induction of proliferation. From the sparing effect of ovariectomy and the stimulating effect of progestins, it seems likely that hormones play a promoter role in mammary carcinogenesis in the dog and cat. Growth hormone and other growth factors produced either in the epithelium or in the stroma may play a joint role (3).

Role of estrogen receptors in mammary gland physiology and in the process of tumorigenesis

Russo and Russo (19) mentioned in their study that breast cancer was recognized to be a hormone-dependent malignancy as early as in 1896 by Beatson who reported that the removal of the ovaries caused the regression of disseminated breast cancer.

Estrogens are important for the development of the mammary gland and strongly associated with oncogenesis in this tissue. Referring to their respective position in biosynthesis, estrogens are termed E1 for estrone, E2 for 17β-estradiol and E3 for estriol, with E2 being the most potent, biological active form (16).

In general, estrogen effects are mediated by two distinct receptors, Estrogen Receptor α (ERα) and Estrogen Receptor β (ERβ), which are encoded by two discrete genes (7). The estrogen receptor is a ligand-activated transcriptional factor that is a member of the nuclear receptor superfamily (14). In the nervous and cardiovascular systems as well as in bone and urogenital tract, both subtypes occur simultaneously, but to different extents. Other organs reveal a more distinct distribution of ERs. Thus, ERα is almost exclusively expressed in the liver, breast, and uterus, whereas only ERβ could be detected in the gastrointestinal tract. It is widely accepted that ERα and ERβ account for dissimilar functions, according to their distribution and cellular context. Moreover, evidence is accumulating that in tissues where both receptors appear, they reveal a different, oppositional mode of action. On account of its dominant occurrence in the breast and uterus, regulation of reproductive processes is attributed to ERα. Due to its proliferative effect, ERα has been implicated in the development of breast cancer, while it has been proposed that in the normal mammary gland, ERβ exhibits protective properties by modulating the effects of ERα on cell growth. With respect to tumourigenesis in breast tissues, it is of interest that the expression ratio of ERα and ERβ in malignant tissues is shifted in favour of ERα, which is suggested to be the first step of transition from a normal into a malignant state (13, 21).

Transforming growth factor in normal and neoplastic cells

Cellular homeostasis is maintained by the orchestrated functions of a variety of growth regulatory factors, such as peptides and lipophilic hormones. Interactions between growth factor and estrogen signalling pathways at the level of gene expression, play important roles in maintaining the reproductive homeostasis (14) and a central role in mammary gland development (8, 3).

Transforming growth factor (sometimes referred as Tumour Growth Factor, TGF) is used to describe two classes of polypep-

tide growth factors: TGF-α and TGF-β. The name transforming growth factor is somewhat arbitrary, since two classes of TGFs are not structurally or genetically related to one another, and they act through different receptor mechanisms. Furthermore, they do not always induce cellular transformation, and are not the only growth factor that induces cellular transformation. TGF-α is up regulated in some human cancers. It is produced by macrophages, brain cells and keratinocytes, and induces epithelial development (1).

TGF-β acting in normal cells through its signalling pathway stops the cell cycle at G1 phase to stop proliferation, induce differentiation, or promote apoptosis. When a cell is transformed into a cancer cell, parts of TGF-β signalling pathway are mutated, and TGF-β no longer controls the cell. These cancer cells proliferate. The surrounding stromal cells (fibroblasts) also proliferate. Both cells increase their production of TGF-β. This TGF-β acts on surrounding stromal cells, immune cells, endothelial and smooth muscle cells. It causes immunosuppresion and angiogenesis, which makes the cancer more invasive. TGF-β also converts effector T-cells, which normally attack cancer with an inflammatory (immune) reaction, into regulatory (suppressor) T-cells which turn off the inflammatory reaction. Finally, TGF-β will often suppress early tumorigenesis and later enhance tumour progression (1). TGF-β signalling is mediated through transmembrane receptors (I and II) located at the cell surface which are serine/threonine kinases (14).

In mammalian cells, three different TGF-β isoforms are expressed, termed TGF-β 1, 2, and 3. TGF-β1 is the most frequently expressed species in cell lines and tissues of breast cancer. The level of its expression is positively correlated with the progression of the disease. The notion that increased expression of TGF-β1 by tumour cells stimulates tumorigenesis has been demonstrated by experiments in which the inhibition of TGF-β1 or a reduced TGF-β1 gene dosage, inhibited tumour incidence and metastasis (11).

In breast cancers, TGF-β1 is expressed preferentially at the advancing edges of primary tumours and in lymph node metastases, suggesting that it plays a role in interacting with neighbouring host stromal cells. It has also been shown that TGF-β1 stimulate fibroblast-myofibroblast differentiation as it is capable of upregulating α SMA (Smooth Muscle Actin) in fibroblasts both *in vivo* and *in vitro* (22, 8).

Transforming growth factor, which is also the Stromal cell Derived Factor – 1 (SDF-1), is supposed to be associated with the prognosis and survival in breast cancers. SDF-1 has the remarkable capacity to induce sustained signaling through its chemokine receptor 4 (CXCR4). SDF-1 is constitutively expressed in various organs including bone, lung, liver, brain, thymus and lymph nodes (12), but SDF-1 is mainly produced by stromal cells such as ostoeclasts, fibroblasts and endothelial cells in the bone marrow (20, 15). This factor is supposed to also play an important role in the chemotaxis of cancer cells and in tumour metastasis. The significant correlation between its expression and overall breast carcinoma-free survival has been demonstrated. The high level of SDF-1 expression suggests that there is a high likelihood of node metastasis, local recurrence and death from breast cancer. The expression pattern of CXCR4 was found to be significantly correlated with the degree of lymph node metastasis but not with haematogenous metastases. Therefore, SDF-1 with its receptor CXC4 may have potential value when assessing long-term clinical outcome in breast cancer (12).

Myofibroblasts in cancer stroma and their prognostic value

Fibroblasts are the most abundant cell type in normal connective tissue. Fibroblasts normally express only two actin isoforms (β and γ). Recent studies, however, have demonstrated that in areas of fibrosis, a subgroup of fibroblasts exists which express the smooth muscle isoform α-actin (α SMA) that is normally expressed constitutively only in smooth muscle (8, 4). The name myofibroblasts has been established for these cells. They are immunohistochemically defined by their expression of vimentin, α-smooth muscle actin, smooth muscle myosin heavy chain, desmin, calponin and α1-integrin (22).

Although genetic mutations in epithelial cells can cause their abnormal proliferation, tumours that derive from these cells will, almost invariably, not be composed exclusively of these genetically altered cells. Instead, virtually all carcinomas arise as complex mixture of a variety of cell types (fibroblasts, endothelial cells, smooth muscle cells and inflammatory cells), which are summarily termed stroma (6).

The predominant stromal cell type in most carcinomas is the myofibroblast (24). There is an increased rate of proliferation, expression of different Extra Cellular Matrix (ECM) proteins, and increased expression of certain growth factors in these cells. It also has been hypothesized that cancer-associated myofibroblasts are endowed with enhanced activities that promote the growth of the nearby carcinoma cells. Evidence supporting this notion has come from prostate tumour xenograft model in which human tumour-associated fibroblasts and normal fibroblasts were compared for their tumour-promotion abilities (17). The result indicates that the activated fibroblasts derived from tumours, which presumably have undergone conversion to myofibroblasts, had acquired an enhanced tumour-promotion activity in comparison to the fibroblasts present in normal prostate gland (6).

Myofibroblasts are found in the human embryo from gestational week 13 on, and are co-opted by remodelling tissues. In postembryonic stages, differentiation from resident stromal fibroblasts in myofibroblasts is induced by paracrine signals generated by different pathological processes including cancers. Among these signals, TGF-β is the most potent one (4). This well described cancer cell-derived cytokine directly transdifferentiates fibroblasts into myofibroblasts, and it also stimulates angiogenesis *in vitro* and *in vivo*. TGF-β mediated angiogenesis combines both direct and indirect effects. Indirectly, TGF-β may recruit monocytes and myofibroblasts, which are both powerful factories for cytokines, proteinases and ECM proteins necessary for angiogenesis. Angiogenetic factors induced by TGF-β are Vascular Endothelial Growth Factor (VEGF) family members, which directly act on endothelial cells to stimulate their proliferation, migration, and capillary tube formation at least *in vitro* (5).

One of the aspects of neoplastic process prognosis is supposed to be in connection with the presence of myofibroblasts in the surrounding area, because these cells may shield cancer cells from the immune response. *In vitro* experiments, with fibroblast and myofibroblast containing collagen cells, indicate that the contractive properties and probably also the surrounding ECM of cancer associated myofibroblasts prevent invasion of immune and inflammatory cells into tumours. In this way cancer associated myofibroblasts prevent physical contact between cancer cells and immune cells, an essential phenomenon for cancer cell destruction (5).

According to these last studies, there is a correlation between the appearance of myofibroblasts and the expression of tenascin-C

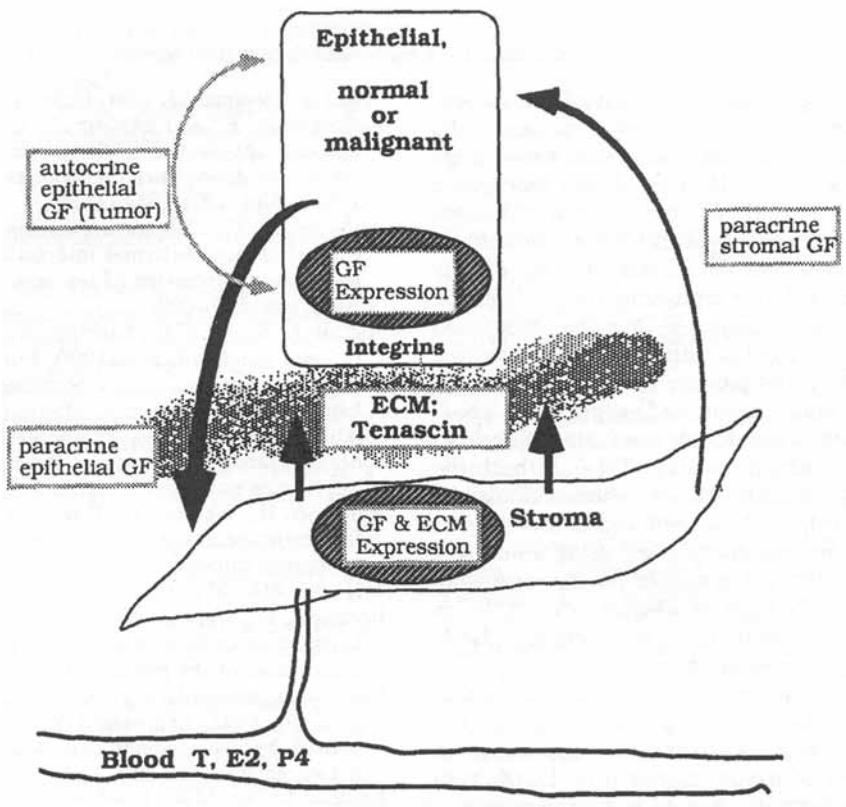

Fig. 1. Epithelial-mesenchymal interactions.
Potential pathways of cellular interactions in glandular organs composed of glandular epithelial cells and mesenchymal stroma are summarized.
T – testosterone, E2 – estradiol, P4 – progesterone, GF – growth factor, ECM – extracellular matrix (23)

(TN-C) in canine mammary gland tumors. Tenascin-C is a large (180–300 kDa), hexameric multidomain glycoprotein located mainly in the ECM that is involved in tissue interactions during embryogenesis, wound healing, inflammation and oncogenesis. The most prominent function of TN-C includes antiadhesion effects, favouring cell motility and growth promotion. These proposed activities suggest a potential role for TN-C in the regulation of tumour cells proliferation, invasion and metastasis (10).

The distribution of stromal TN-C correlates with the presence of myofibroblasts. The appearance of stromal myofibroblasts and expression of TN-C were significantly correlated with higher histological grades of malignancy and vascular/lymphatic invasion in simple mammary gland carcinomas. Stromal myofibroblasts appear to be a major cellular source of TN-C and play an important role in the development of canine mammary tumours (25). Figure 1 illustrates and summarizes the major epithelial-mesenchymal interactions presented in this paper.

CONCLUSION

Estrogens are considered as risk factors for tumorigenesis, especially in tissues that respond to sex steroids with receptor mediated proliferation; namely breast epithelium and endometrium. In their capacity to regulate the development and function of reproductive organs, they are often committed to interactions with growth factors, especially TGF-β.

The TGF-β plays a key role in a wide range of biological processes, including cell proliferation, development, wound healing, inflammation and angiogenesis. TGF-β acts as a potent inhibitor of tumour progression, owing to an induction of growth arrest and apoptosis. However, in many carcinomas the levels of TGF-β are elevated. Breast cancer, in particular, express high levels of TGF-β1 and the level of expression is correlated with the rate of disease progression. The balance between tumour promoting and suppressing effects may be destabilised in late stage tumours when the sensibility of cells towards the antiproliferative and proapoptotic effects of the chemokines declines.

The stroma represents an integral part of the cancer. The main stromal cells are myofibroblasts which appear early during cancer development. Their origin remains controversial although fibroblasts are considered to be the main progenitor cells. The process of transdifferentiation is induced by paracrine signals among which the TGF-β is the most potent one. The mutual interaction between cancer cells and myofibroblasts is essential for invasive growth and contributes to a poor clinical prognosis.

The relationship between estrogen receptors, transforming growth factors and the ability of TGF-β to induce the cellular changes in tumour stroma at different time of cancer development, represent a great challenge for the study of mammary gland carcinomas in animal experimental models.

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REFERENCES

1. Bierie, B., Moses, H., 2006: TGF-β and cancer. *Cytokine Growth Factor Rev.,* 17, 29–40.

2. Bocchinfuso, W. P., Korach, K. S., 2004: Mammary gland development and tumorigenesis in estrogen receptor knockout mice. *J. Mammary Gland Biol. Neoplasia,* 2, 323–334.

3. Cullen, J. M., Page, R., Misdorp, W., 2002: An overview of cancer pathogenesis, diagnosis and management. In **Meuten, D. J.:** *Tumours in Domestic Animals,* 4th edn., Iowa State Press, 3–44.

4. De Wever, O., Demeter, P., Mareel, M., Bracke, M., 2008: Stromal myofibroblasts are drivers of invasive cancer growth. *Int. J. Cancer,* 123, 2229–2238.

5. De Wever, O., Mareel, M., 2003: Role of tissue stroma in cancer cell invasion. *J. Pathology,* 200, 429–447.

6. Elenbaas, B., Weinberg, R. A., 2001: Heterotypic signaling between epithelial tumour cells and fibroblasts in carcinoma formation. *Exp. Cell Res.,* 264, 169–184.

7. Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G. *et al.*, 1997: Human estrogen receptor β-gene structure, chromosomal localization and expression pattern. *J. Clin. Endocrinol. Metab.,* 82, 4258–4262.

8. Evans, R. A., Tian, Y. C., Steadman, R., Philips, A. O., 2003: TGF-β1-mediated fibroblast-myofibroblast terminal differentiation – the role of small proteins. *Exp. Cell Res.,* 282, 90–100.

9. Ewan, K. B., Shyamala, G., Ravani, A., Tang, Y., Akhurst, R., Wakefield, L. *et al.*, 2002: Latent transforming growth factor-β activation in mammary gland. *Amer. J. Pathol.,* 160, 2081–2093.

10. Hancox, R. A., Allen, M. D., Holliday, D. L., Edwards, D. R., Pennington, C. J., Guttery, D. S. *et al.*, **2009:** Tumour-associated tenascin-C isoforms promote breast cancer cell invasion and growth by matrix metalloproteinase-dependent and independent mechanisms. *Breast Cancer Res.,* 11, 24–37.

11. Li, S., Han, B., Liu, G., Li, S., Ouellet, J. L., Pelletier, G., 2010: Immunocytochemical localization of sex steroid hormone receptors in normal human mammary gland. *J. Histochem. Cytochem.,* 58, 509–515.

12. Kang, K. H., Watkins, G., Parr, Ch., Douglas-Jones, A., Mansel, R. E. *et al.*, 2005: Stromal cell derived factor-1: its influence in invasiveness and migration of breast cancer cells *in vitro*, and its association with prognosis and survival in human breast cancer. *Breast Cancer Res.,* 7, 402–410.

13. Leygue, E., Dotzlaw, H., Watson, P. H., Murphy, L. C., 1998: Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. *Cancer Res.,* 58, 3197–3201.

14. Matsuda, T., Yamamoto, T., Muraguchi, A., Saatcioglu, F., 2001: Cross-talk between transforming growth factor-β and estrogen receptor signaling trough smad3*. *J. Biol. Chem.,* 276, 42908–42914.

15. Murdoch, C., 2000: CXCR4: chemokine receptor extraordinaire. *Immuno. Rev.,* 177, 175–184.

16. Muramatsu, M., Inoue, S., 2000: Estrogen receptors: how do they control reproductive and nonreproductive functions? *Biochem. Biophys. Res. Commun.,* 270, 1–10.

17. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carrol, P. R., Tlsty, T. D., Cunha, G. R., 1999: Carcinoma-associated fibroblasts direct tumour progression of initiated human prostatic epithelium. *Cancer Res.,* 59, 1287–1303.

18. Russo, J., Russo, I. H., 1987: Biology of disease. Biological and molecular basis of mammary carcinogenesis. *Lab. Invest.,* 75, 112–137.

19. Russo, I. H., Russo, J., 1996: Mammary gland neoplasia in long-term rodent studies. *Environ. Health Perspect.,* 104, 938–967.

20. Rossi, D., Zlotnik, A., 2000: The biology of chemokines and their receptors. *Annu. Rev. Immunol.,* 18, 217–242.

21. Speirs, V., Parkers, A. T., Kerin, M. J., Walton, D. S., Carleton, P. J., Fox, J. N. *et al.*, **1999:** Coexpression of estrogen receptor alpha and beta: poor prognostic factors in human breast cancer? *Cancer Res.,* 59, 525–528.

22. Vaughan, M. B., Howard, E. W., Tomasek, J. J., 2000: Transforming growth factor-β1 promotes the morphological and functional differentiation of the myofibroblast. *Exp. Cell Res.,* 257, 180–189.

23. Vollmer, G., 1974: Expression of tenascine during carcinogenesis an involution of hormone dependent tissues. *Biochem. Cell Biol.,* 72, 505–514.

24. Walker, R. A., 2001: The complexities of breast cancer desmoplasia. *Breast Cancer Res.,* 3, 143–145.

25. Yoshimura, H., Michishita, M., Ohkusu-Tsukada, K., Takahashi, K., 2011: Increased presence of stromal myofibroblasts and Tenascin-C with malignant progression in canine mammary tumors. *Vet. Pathol.,* 48, 313–321.

26. Zhang, X., Podsypanina, K., Huang, S., Mohsin, S. K., Chamnes, G. C., Hatsel, S. *et al.*, 2005: Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations. *Oncogene,* 24, 4220–4231.

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INFECTIVITY OF DUCK EMBRYO-PROPAGATED INFECTIOUS BURSAL DISEASE VIRUS (SEROTYPE 1)

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ABSTRACT

The isolation of infectious bursal disease (IBD) virus in the absence of specific-antibody-free embryonated chicken eggs and cell culture facilities is a challenge in developing countries. The suitability of embryonated duck eggs for the isolation of IBD virus was therefore investigated. IBD virus (serotype 1) inoculum was prepared using the bursae of Fabricius harvested from a clinical IBD outbreak. Viral suspension ($1.5 \times 10^{5.5}$ LD_{50}) was inoculated into 11-day old embryonated duck eggs via the allantoic sac route, incubated at 37.5 °C and the allantoic fluid (AF) was harvested after 48 hours. The AF was tested for IBDV antigen and titrated using an AGID test. Ninety microlitre of 5 $\log_2$ AF was inoculated into 11 chickens (5 week-old) via conjunctival instillation and observed for morbidity and mortality. A group of four chickens served as uninoculated controls. The chickens were bled weekly post-inoculation (pi), sera were harvested and tested for IBDV antibody using the AGID test. The inoculated chickens showed clinical signs of dullness and ruffled feathers with no mortality. IBDV-specific antibodies were detected from 3 weeks pi. This study has shown that embryonated duck eggs are suitable for propagation of IBDV and could be used for attenuation purposes in the production of live IBD vaccines.

Key words: attenuation; embryonated duck eggs; IBD virus; infectivity; virus propagation

INTRODUCTION

Infectious bursal disease (IBD) virus is the etiological agent of an immunosuppressive disease of young chickens of 3 to 6 weeks of age. It is a member of the genus *Avibirnavirus* belonging to the family *Birnaviridae*. The virus causes the destruction of lymphoid tissues, especially the bursa of Fabricius (8). Two distinct serotypes of IBD virus (IBDV) have been recognized. Serotype 1 displays a wide variation in pathogenic potential, while serotype 2 is apathogenic (4, 6).

Although IBD is essentially a disease of chickens, evidence of natural and experimental infections have been reported in other poultry species such as turkeys and ducks via virus isolation and or detection of antigen or antibody (2, 3, 6, 11, 12, 13, 15). However, ducks have been observed to either seroconvert poorly or not to produce antibody when exposed to IBD virus (6, 10, 11). Typical clinical IBD was absent in all these instances. However, duck embryo cells have been used successfully in cell culture for the propagation of IBD virus (7).

For the propagation of IBDV in embryonated chicken eggs, specific-antibody-free eggs are required which are not readily available in developing countries due to the endemicity of IBD and unavailability of biosecured facility for their production. The use of duck embryos as an alternative was therefore considered since IBDV-antibody free embryonated duck eggs are readily available and McNulty *et al.* (7) had reported the successful propagation of IBDV in duck embryo cell culture. This study was therefore carried out to determine the suitability of embryonated duck eggs for the propagation of IBDV for virological studies.

MATERIALS AND METHODS

IBD virus (Serotype 1)

The bursae of Fabricius was harvested from necropsied chickens obtained from an outbreak of IBD with about a 40 % mortality rate. A 50 % bursal homogenate in PBS was prepared by the addition of 1ml bursal homogenate to 1 ml PBS, vigorously mixed and clarified by centrifugation at 4000 rpm for 5 minutes. The supernatant was decanted and confirmed to contain IBDV by using AGID test as described by Oladele (11). The supernatant was then kept at –20 °C for further use.

Virus Propagation

Ten duck eggs from a free ranging flock were set in a laboratory incubator at 37.5 °C. At 11 days of incubation, six eggs were found to be fertile and alive and were used for virus propagation. The broad end of each of four embryonated eggs was cleaned with alcohol, the air space was marked with pencil and a tiny hole was drilled into the air space. IBD virus inoculum (0.5 ml) containing 10,000 IU penicillin, 10 mg streptomycin per ml (16) of bursal supernate was inoculated into the allantoic cavity of each of the 4 eggs. The remaining 2 embryonated eggs were uninoculated and served as controls. The openings on the eggs were sealed with paper tape and they were further incubated. Candling was done twice daily for 48 hours post-inoculation (pi). The eggs were then removed from the incubator and placed in a refrigerator overnight. Allantoic fluid (AF) was harvested from each egg into appropriately labeled test tubes using sterile syringes and needles. Samples of the AF were subjected to AGID test for the detection of IBDV antigen. AF samples from the 4 inoculated eggs were pooled and titrated using quantitative AGID test (14).

Experimental Infection

Fifteen unvaccinated 5 week-old cockerels (Isa hybrid) which had been shown to be negative for IBDV antibodies were used for experimental infections following the necessary institutional guidelines for animal experimentation. Ninety microliters ($5 \log_2$) of IBD-positive duck AF was inoculated into each of 11 cockerels via conjunctival instillation. The remaining four cockerels which served as uninoculated controls were housed in a separate cage. The chickens were observed daily for clinical signs of IBD and mortality, and they were bled weekly for serum. The harvested sera were screened weekly for IBDV antibodies using the AGID test as described by Oladele (11) with IBDV serotype 1 antigen and purified agar. The results were read after 24 hours. A positive result was shown by the presence of a precipitin line within the space between the central antigen well and any of the peripheral wells.

RESULTS

The qualitative AGID test carried out on the duck AF showed that all 4 samples were positive for IBDV antigen and the titre of the pooled allantoic fluids was $5 \log_2$ by quantitative AGID test. AF samples from the uninoculated duck embryonated eggs were negative for IBD virus antigens.

At 48 hours pi, the infected birds showed signs of dullness and ruffled feathers which extended to 8 days pi. However, no mortality was observed until the experiment was terminated 30 days pi. The AGID test carried out on sera harvested weekly from the infected chickens showed precipitin lines at 3 and 4 weeks pi (Table 1). While sera harvested at 3 weeks pi were 81.8 % positive for IBDV antibody, those harvested at 4 weeks pi were 90.9 % positive.

Table 1. Antibody response in chickens inoculated with duck embryo-propagated IBD virus (serotype 1)

Sample No.	1 week (pi)	2 weeks (pi)	3 weeks (pi)	4 weeks (pi)
1	–	–	+	+
2	–	–	+	+
3	–	–	+	+
4	–	–	+	+
5	–	–	+	+
6	–	–	+	+
7	–	–	–	+
8	–	–	–	–
9	–	–	+	+
10	–	–	+	+
11	–	–	+	+
% positive	**0 %**	**0 %**	**81.8 %**	**90.9 %**

pi – post-inoculation

DISCUSSION

The detection of IBD virus antigen in allantoic fluids harvested from the inoculated duck embryos shows that the virus was able to successfully replicate and get propagated in this medium. Although duck embryo cells have been used in cell culture for the isolation of IBD virus (7), embryonated duck eggs have rarely been used. Since ducks do not readily produce antibody to IBDV infection (9, 11, 12), maternal antibody transfer to eggs is therefore unlikely. Thus, embryonated duck eggs have been shown in this study to be suitable alternatives to embryonated chicken eggs for the propagation of IBDV for virological studies.

The mild clinical signs and absence of mortality observed in the experimental chicken's post-IBD virus infection suggest attenuation of the virus in duck embryo. This is in spite of the fact that the virus was obtained from a severe IBD outbreak in a nine week old pullet flock. Although earlier workers had reported reduced virulence of IBDV by passaging in embryo-

nated chicken eggs (1), the loss of virulence in this study was quite fast, i.e. after only one passage. The detection of IBDV antibody in the flock 3 weeks pi indicates that the virus was still infective although less virulent. This shows that it still retained its immunogenicity, thus making it a good candidate for vaccine production as proposed by Yamaguchi *et al*. (17) for embryonated chicken eggs. Although earlier studies have shown that the AGID test is a highly specific immunologic test, it is less sensitive than other tests such as the enzyme-linked immunosorbent assay (5, 14). Therefore, it is possible that seroconversion had occurred in these chickens earlier than the 3 weeks pi detected by the AGID test in this study.

We conclude that embryonated duck eggs are suitable for propagation of IBD virus and could be used for attenuation purposes in the production of live IBD vaccines.

REFERENCES

1. Abdel-Alim, G. A., Saif, Y. M., 2002: Pathogenicity of embryo adapted serotype 2 OH strain of infectious bursal disease virus in chickens and turkeys. *Avian Dis.,* 46, 1000–1006.

2. Barnes, H. J., Wheeler, J., Reed, D., 1982: Serological evidence of infectious bursal disease virus infection in Iowa Turkeys. *Avian Dis.,* 26, 560–565.

3. Giambrone, J. J., Fletcher, O. J., Lukert, P. D., Page, P. K., Eidson, C. E., 1978: Experimental infection of turkeys with infectious bursal disease virus. *Avian Dis*., 22, 451–458.

4. Jackwood, D. J., Saif, Y. M., Hughes, J. H., 1982: Characteristics and serologic studies of two serotypes of infectious bursal disease virus in turkeys. *Avian Dis*., 26, 871–882.

5. Marquardt, W., Johnson, R. B., Odenwald, W. F., Schlothober, B. A., 1980: An indirect enzyme-linked immunosorbent assay (ELISA) for measuring antibodies in chickens infected with infectious bursal disease virus. *Avian Dis*., 24, 375–385.

6. McFerran, J. B., McMulty, M. S., Killop, E. R., Connor, T. J., McCracken, R. M., Collins, P. S., Allan, G. M., 1980: Isolation and serological studies with infections bursal disease virus from fowl, turkeys and ducks: demonstration of a second serotype. *Avian Pathol.*, 9, 395– 404.

7. McNulty, M. S., Allan, G. M., McFerran, J. B., 1979: Isolation of infectious bursal disease virus from turkeys. *Avian Pathol*., 8, 205–212.

8. Muller, R., Kaufer, J., Reinacher, M., Weiss, E., 1979: Immunoflourescent studies of early virus propagation after oral infection with infectious bursal disease virus (IBDV). *Zbl. Vet. Med. (B),* 26, 345–352.

9. Nawathe, D. R., Onunkwo, O., Smith, I. M., 1978: Serological evidence of infections with the virus of infectious bursal disease in wild and domestic birds in Nigeria. *Vet. Rec.,* 102, 444.

10. Okoye, J. O., Iguomu, E. P., Nwosuh, C., 1990: Pathogenicity of an isolate of infectious bursal disease virus in local Nigerian duck. *Trop. Anim. Hlth. Prod.*, 22, 160–162.

11. Oladele, O. A., 2001: Comparison of seroprevalence rates of infectious bursal disease antibody in village chickens and ducks in South West Nigeria. *African Journal of Clinical Experimental Microbiology*, 2, 49–51.

12. Oladele, O. A., 2010: Chronological studies on experimental infectious bursal disease virus infections in chickens, turkeys and ducks. *JCVA,* 26, 23–28.

13. Oladele, S. B., Kazeem, H. M., Raji, M. A., 1996: Survey for antibodies to infectious bursal disease, Newcastle disease and fowl pox in ducks, pigeons and guinea fowls in Zaria. *Nigerian Veterinary Journal* (special edn.), 1, 85–87.

14. Oladele, O. A., Emikpe, B. O., Ohore, O. G., Oluwayelu, D. O., Agboola, O. O., 2007: Comparison of the antibody response of Nigerian indigenous and exotic chickens to infectious bursal disease virus infection. *Folia Veterinaria*, 51, 38–42.

15. Reddy, S. K., Silim, A., 1991: Isolation of infectious bursal disease virus from turkeys with arthritic and respiratory symptoms in commercial farms in Quebec. *Avian Dis*., 35, 3–7.

16. Rodgers, S. J., Vanhooser, S. L., Welsh, R. D., Silkwood, T. G., 1994: Preliminary studies of primary ostrich fibroblasts for the isolation of Ratite viruses. *Avian Dis.,* 38, 866–872.

17. Yamaguchi, T., Ogawa, M., Inoshima, Y., Miyoshi, M., Fukushi, H., Hirai, K., 1996: Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. *Virology,* 223, 219–223.

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USING MESENCHYMAL STEM CELLS AND BIOMATERIALS IN HARD TISSUE RECONSTRUCTION

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ABSTRACT

Regenerative medicine is a new multidisciplinary area of medicine and offers new therapeutic methods for replacement or regeneration of the cells/tissues which are not functional due to disease, injury or congenital anomalies. It covers the methods of cell treatment and tissue engineering, and uses a range of new technological advances that provide benefits beyond simple traditional transplantations. Recently, the focus of attention has been concentrated upon implant preparation with mesenchymal stem cells seeded on a scaffold. Bone formation comprises a complex set of events that begins with the recruitment and proliferation of osteoprogenitors, followed by cell differentiation, osteoid formation, and mineralization. It has been recognized that bone is unique and has a vast potential for regeneration from stem cells. A number of reports have indicated there are a number of potential uses for mesenchymal stem cell transplantations from bone marrow in a variety of skeletal disorders. Mesenchymal stem cells have significance for veterinary medicine in basic theoretical disciplines, as well as for clinical use in large and small animal forms of regenerative applications, such as in the digestive and respiratory systems. The initial contact of the cells with the implant surface is of critical importance for adhesion of the bone cells that influence osseointegration. One strategy to improve osseointegration that has been tried involves the pre-coating of implants with an extracellular matrix protein for use as a scaffold, which enables specific cell-extracellular matrix interactions, along with the use of growth factors to facilitate the differentiation of the osteoblasts.

Key words: biomaterials; mesenchymal stem cells; regenerative medicine; tissue engineering

REGENERATIVE MEDICINE AND TISSUE ENGINEERING

The regenerative capacity of mesenchymal stem cells may be affected by damage or the neutralization of chronic degenerative diseases when fibrotic repair starts (e.g. damage of the articular cartilage, corium, pancreas, spinal cord, most of the brain, retina and lens of the eye, myocardium, lung and kidney glomeruli). The response of the organism is regarded as a reparative process because incomplete cells arise (26).

Tissue engineering, which is a part of regenerative medicine, combines medical science with engineering for the development of biological replacements of tissues or organs (24). Excellent reconstruction results may be achieved by minimizing unwanted complications. The goal of tissue engineering in the craniofacial region is to develop abiological substitutes that promote tissue regeneration (27). One of the most remarkable advantages is that the regeneration of only a small amount of tissue can be highly beneficial to the patient, particularly in the field of periodontal tissue regeneration (28). Tissue engineering, which includes bed structures for osteogenesis in dental implantations, makes use of the advantage of combining cultured living cells and an implant body that can be used as a scaffold (2, 9, 10). Previous attempts to tissue engineer bone or cartilage reconstruction around implants have utilized osteoblasts or chondrocytes encapsulated in various hydrogels, which can survive *in vitro* fabrication and be able to synthesize cell-associated scaffold extracellular matrices (6, 42).

Therefore, researchers are very interested in a tissue engineering procedure that uses autologous Mesenchymal Stem Cells (MSC) combined with an osteoconductive scaffold as a live bone substitute (18). The current tissue engineering principles utilize the seeding of

bone marrow mesenchymal stem cells onto biodegradable porous scaffolds to be placed around the titanium implant fixture (28).

MESENCHYMAL STEM CELLS

The application of stem cells in regenerative medicine has the same significance as the discovery of antibiotics. MSC are a population of multipotent, non-haematopoietic, marrow-derived cells, which are easily expanded in culture and differentiate into cells with an osteogenic phenotype (5, 16).

Generally, stem cells are the original cells, common to all multicellular organisms that retain the ability to expand through cell division and can differentiate into different cell types (26). MSC obtain a leading position among the various types of adult cells for reparation of connective tissues such as bone, cartilage and bone marrow stroma. Mesenchymal stem cells can be differentiated into several cell types and due to the fact that they produce important growth factors and cytokines may provide important signals for cell survival in the damaged tissues with or without their participation in long-term tissue reparation. Therefore, they become the most important cell candidates suitable for treatment in an ever-expanding range of tissues (35). MSC play a central role in osteogenesis and in all bone reparative mechanisms, including those underlying implant osteointegration, and represent for this reason, an ideal tool of all those experimentations aimed at investigating the interaction between bone cells and implant surfaces (19).

Mesenchymal stem cells are one of the few normal cell types, which may be obtained in large enough quantities which is required for therapy. Mesenchymal stem cells also have the ability to change the responses of the immune system. While the hypothesis of plasticity of mesenchymal stem cells is still under discussion, it is well known that an adult bone marrow contains several populations of multipotent stem cells. The characteristic of mesenchymal stem cells isolated from bone marrow is their ability to differentiate into all cell types of connective tissue, involving bone, cartilage, fat and muscle. MSC support the growth and differentiation of hematopoietic stem cells in their roles in the bone marrow (35).

Studies of stem cells are currently one of the most promising areas of biomedical research. Recent findings that MSC can regenerate critical-size bone defects when combined with bone substitutes (11, 13) and improve bone allograft integration (33) have increased interest in their clinical application. Previous studies have indicated that without blood vessel ingrowth, bone formation is generated only at the surface of the construct (1). It is well known that MSC are able to influence endothelial cell behaviour and vice versa (21). In addition, MSC secrete Vascular Endothelial Growth Factor (VEGF), which exhibits elevated expression during osteogenesis (30).

Platelets are collected in a small volume of plasma called Platelet-Rich Plasma (PRP), which plays a fundamental role in haemostasis and is a natural source of growth factors and adhesive glycoproteins (7, 34). It is well known that seeded MSC can provide an osteogenic cell source for new bone formation, and PRP improves expansion of MSC and helps their differentiation and invivo bone formation capacity (33, 39). Therefore, MSC and PRP are an effective approach for enhancing the osseointegration of dental implants when applied to a mineral or a collagen scaffold in mandibular bone defects (20). The transplantation of MSC and PRP in a fluorohydroxyapatite (FH) scaffold, compared with FH alone, can significantly enhance bone formation, and can significantly improve the osseointegration of endosseous implants placed in the augmented sinus (33).

THE ADVANTAGE OF BIOMATERIALS

Biomaterial is defined as a lifeless material that comes into interaction with the biological system. In this sense, significant progress has been made with the production of innovative surfaces able to promote and accelerate osseointegration (19). Biomaterials for reconstruction of the hard tissues usually include three basic components: scaffold, stem cells and signal molecules (growth factors). For production of the biomaterial microstructure, it is important to choose a specific structure of substitute tissues that will form an extracellular matrix that will support cell proliferation and differentiation for the necessary cell lines. Tissue engineering helps develop the necessary osteoinductive and osteoconductive properties of the hydroxyapatite and other calcium phosphates. An osseointegrated implant may closely resemble a natural tooth; however, the absence of the periodontal ligament and connective tissue via cementum results in fundamental differences in the adaptation of the implant to occlusal forces (28). The stabilization and subsequent osseointegration of endoprostheses is crucial with regard to the clinical results for dental implantation. In order to accelerate and enhance the bone growth and to strengthen the osseous fixation, implant surfaces have been modified, and in particular, there has been increased use in surface coatings of functional proteins (32).

Presently, autogenous bone grafts are considered to be the "gold standard", although their disadvantages (low availability of intraoral bone tissue, high surgery costs, and post-operative morbidity) have stimulated the search for alternative sources (31, 38). With the development of new types of biomaterials, it is necessary to focus attention on the research of biocomposite systems with biopolymers as one component, which represents an appropriate solution for the replacement and reconstruction of hard tissues. Biopolymers, depending on the type applied, increases the activity of osteoblasts and other types of morphologically and biologically different cells. Biochemical processes during the healing may gradually degrade the composite matrix, thus enabling subsequent new bone tissue growth into the implant.

The most frequent complication of dental implantation is bone resorption that is initiated by an inflammatory response at the implant site. Recent studies have demonstrated that titanium is the most frequent component of dental implants, and it is this material that is responsible for inducing apoptosis and suppressing the expression of the osteoblastic phenotype in human MSC (40, 41).

EXTRACELLULAR MATRIX AND ITS PROTEINS

The extracellular matrix (EM) is a complex network of macromolecules located in the extracellular space. In all tissues, it plays an important role in regulating basic cellular processes including proliferation, differentiation, migration and the interaction between cells. The macromolecular network of the EM consists of collagen, elastin, glycoproteins (fibronectin, laminin) and proteoglycans (hy-

aluronic acid, chondroitin-sulphuric acid, etc.). This network creates a three-dimensional gelatin environment into which cells are embedded. Integrins are transmembrane proteins that provide binding between EM proteins and cytoskeletal proteins. This provides communication between the extracellular and the intracellular space and developes into fibrous structures which will be more compatible with the structure of the bone tissue. EM proteins bind to the cell surface and transmit signals which result in tension and compression of the cells. Therefore, it is thought that the dental implants binding to the extracellular matrix proteins such as fibronectin, is necessary for successful tissue regeneration. Extracellular matrices, such as collagen type I and fibronectin, are effective in promoting cellular adhesion and spreading, and they possess a RGD (arginine-glycine-aspartic acid) sequence, which is recognized by integrins. Signaling occurs when integrins bind to the EM. This results in changes in the expression of selected genes that control the initial preosteoblast/osteoblast adhesion, proliferation and maturation (8, 12, 29). Early cell adhesion was found on fluoride treated surfaces with respect to titanium dental implants (19). Fluoride is known to stimulate bone cell proliferation (14, 25) and there is evidence that it may act primarily on osteoprogenitor cells rather than on mature osteoblasts (4, 22), by an acute increase of the intracellular calcium levels (43).

In parallel with the increased cell growth, bone marrow-derived mesenchymal stem cells (BM-MSC) cultured on fluoride-modified grit-blasted (F-TiO) surfaces showed higher levels of type I collagen synthesis with respect to titanium grit-blasted (TiO). Type I collagen is the most abundant protein component of the bone extracellular matrix.. It is secreted early by stromal cells during their differentiation into osteoblasts, forming a network which will constitute the final scaffold of the bone matrix (15). An increased production of this protein by BM-MSC cultured on F-TiO may be interpreted as an expression of an enhanced attitude to synthesize an organized extracellular matrix, which represents the first step of the osteogenic process (19).

The animal model of sinus augmentation in minipigs is used frequently in studies because the minipig sinus has a well-defined ostium, the Schneider membrane is thin, and the size of the sinus is comparable with human sinuses (17, 23, 36). Furthermore, minipigs have close similarities to humans in terms of platelet count, clotting parameters, metabolic rate, bone structure, and MSC characteristics (3, 37). Studies show that the surgical procedure applied is comparable with that performed in humans. All these factors are relevant to further human clinical application (33).

Pathologically changed tissues in animals are possible to be restored by the regenerative processes and thus ensure longer utilization of livestock for production, social use and improved survival.

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REFERENCES

1. Abukawa, H., Shin, M., Williams, W. B., Vacanti, J. P., Kaban, L. B., Troulis, M. J., 2004: Reconstruction of mandibular defects with autologous tissue-engineered bone. *J. Oral Maxillofac. Surg.,* 62, 601–606.

2. Adell, R., Eriksson, B., Lekholm, U., Bränemark, P. I., Jemt, T. A., 1990: A long term follow up study of osseointegrated implants in the treatment of totally edentulous jaws. *International Journal of Oral and Maxillofacial Implants,* 5, 347–359.

3. Aerssens, J., Boonen, S., Lowet, G., Dequeker, J., 1998: Interspecies differences in bone composition, density, and quality: potential implications for *in vivo* bone research. *Endocrinology,* 139, 663–670.

4. Bellows, C. G., Heersche, J. N., Aubin, J. E., 1990: The effects of fluoride on osteoblast progenitors *in vitro*. *J. Bone Miner. Res.,* 5, 101–105.

5. Bruder, S. P., Kraus, K. H., Goldberg, V. M., Kadiyala, S., 1998: The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J. Bone Joint Surg.* (Am), 80, 985–996.

6. Cassinelli, C., Morra, M., Bruzzone, G., Carp, A., Di Santi, G., Giardino, R., Fini, M., 2003: Surface chemistry effects of topographic modification of titanium dental implant surface: 2. *In vitro* experiments. *International Journal of Oral and Maxillofacial Implants,* 18, 46–52.

7. Christgau, M., Moder, D., Hiller, K. A., Dada, A., Schmitz, G., Schmalz, G., 2006: Growth factors and cytokines in autologous platelet concentrate and their correlation to periodontal regeneration outcomes. *J. Clin. Periodontol.,* 33, 837–845.

8. Cooper, L. F., Handelman, B., McCormack, S. M., Guckes, A. D., 1993: Binding of murine osteoblastic cells to titanium disks and collagen I gels: implications for alternative interpretations of osseointegration. *International Journal of Oral and Maxillofacial Implants,* 8, 264–272.

9. Cronin, R. J. jr., Oesterle L. J., 1994: Mandibular implants and growing patients. *International Journal of Oral and Maxillofacial Implants,* 55–62.

10. Cronin, R. J. jr., Oesterle L. J., 1998: Implant use in growing patients, treatment concerns. *Dent. Clin. North. Am.,* 42, 1–34.

11. Dallari, D., Fini, M., Stagni, C., Torricelli, P., Nicoli Aldini, N., Giavaresi, G., et al., 2006: *In vivo* study on the healing of bone defects treated with bone marrow stromal cells, platelet-rich plasma, and freeze-dried bone allografts, alone and in combination. *J. Orthop. Res.,* 24, 877–888.

12. Degasne, I., Baslé, M. F., Demais, V., Huré, G., Lesourd, M., Grolleau, B., Mercier, L., Chappard, D., 1999: Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. *Calcif. Tissue Int.,* 64, 499–507.

13. De Kok, I. J., Drapeau, S. J., Young, R., Cooper, L. F., 2005: Evaluation of mesenchymal stem cells following implantation in alveolar sockets: a canine safety study. *International Journal of Oral and Maxillofacial Implants,* 20, 511–518.

14. Farley, J. R., Wergedal, J. E., Baylink, D. J., 1983: Fluoride directly stimulates proliferation and alkaline phosphatase activity in bone-forming cells. *Science,* 222, 330–332.

15. Franceschi, R. T., Iyer, B. S., 1992: Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J. Bone Miner. Res., 7, 235–246.

16. Friedenstein, A. J., Chailakhjan, R. K. and Lalykina, K. S., 1970: The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell and Tissue Kinetics,* 3, 393–403.

17. Fuerst, G., Gruber, R., Tangl, S., Zechner, W., Haas, R., Mailath, G., Sanroman, F., Watzek, G., 2003: Sinus grafting with autogenous platelet-rich plasma and bovine hydroxyapatite. A histomorphometric study in minipigs. *Clin. Oral Implants Res.,* 14, 500–508.

18. Gronthos, S., Akintoye, S. O., Wang, C. Y., Shi, S., 2006: Bone marrow stromal stem cells for tissue engineering. *Periodontology* 2000, 41, 188–195.

19. Guida, L., Annunziata, M., Rocci, A., Contaldo, M., Rullo, R, Oliva, A., 2010: Biological response of human bone marrow mesenchymal stem cells to fluoride-modified titanium surfaces. *Clin. Oral Implants Res.,* xx, 000–000.

20. Ito, K., Yamada, Y., Naiki, T., Ueda, M., 2006: Simultaneous implant placement and bone regeneration around dental implants using tissue-engineered bone with fibrin glue, mesenchymal stem cells and platelet rich plasma. *Clin. Oral Implants Res.,* 17, 579–586.

21. Kaigler, D., Krebsbach, P. H., Wang, Z., West, E. R., Horger, K., Mooney, D. J., 2006: Transplanted endothelial cells enhance orthotopic bone regeneration. *J. Dent. Res.,* 85, 633–637.

22. Kassem, M., Mosekilde, L., Eriksen, E. F., 1994: Effects of fluoride on human bone cells *in vitro*: differences in responsiveness between stromal osteoblast precursors and mature osteoblasts. *Eur. J. Endocrinol.,* 130, 381–386.

23. Klongnoi, B., Rupprecht, S., Kessler, P., Zimmermann, R., Thorwarth, M., Pongsiri, S., Neukam, F. W., Wiltfang, J., Schlegel, K. A., 2006: Lack of beneficial effects of platelet-rich plasma on sinus augmentation using a fluorohydroxyapatite or autogenous bone: an explorative study. *J. Clin. Periodontol.,* 33, 500–509.

24. Koller, J., 2008: Transplantation of tissues and cells and tissue engineering in regenerative medicine. In **Lešník, F., Ostró, A.:** *Biological Aspects of Regenerative Medicine.* Publ. house Olomouc, Olomouc, 135–147.

25. Lau, K. H., Baylink, D. J., 1998: Molecular mechanism of action of fluoride on bone cells. *J. Bone Miner. Res.,* 13, 1660–1667.

26. Lešník, F., Ostró, A., 2008: Biological aspects of regenerative medicine. In **Lešník, F., Ostró, A.:** *Biological Aspects of Regenerative Medicine.* Publ. house Olomouc, Olomouc, 9–16.

27. Mao, J. J., Giannobile, W. V., Helms, J. A., Hollister, S. J., Krebsbach, P. H., Longaker, M. T., Shi, S., 2006: Craniofacial tissue engineering by stem cells. *J. Dent. Res.,* 85, 966–979.

28. Marei, M. K., Saad, M. M., El-Ashwah, A. M., Ei-Backly, R. M., Al-Khodary, M. A., 2009: Experimental formation of periodontal structure around titanium implants utilizing bone marrow mesenchymal stem cells: a pilot study. *Journal of Oral Implantology,* 35, 106–29.

29. Masi, L., Franchi, A., Santucci, M., Danielli, D., Arganini, L., Giannone, V. *et al.*, 1992: Adhesion, growth, and matrix production by osteoblasts on collagen substrata. *Calcif. Tissue Int.,* 51, 202–212.

30. Mayer, H., Bertram, H., Lindenmaier, W., Korff, T., Weber, H., Weich, H., 2005: Vascular endothelial growth factor (VEGFA) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation. *J. Cel. Biochem.,* 95, 827–839.

31. Nkenke, E., Weisbach, V., Winckler, E., Kessler, P., Schultze-Mosgau, S., Wiltfang, J., Neukam, F. W., 2004: Morbidity of harvesting of bone grafts from the iliac crest for preprosthetic augmentation procedures: a prospective study. *Int. J. Oral Maxillofac. Surg.,* 33, 157–163.

32. Ogura, N., Kawada, M., Chang, W. J., Zhang, Q., Lee, S. Y., Kondoh, T., Abiko, Y., 2004: Differentiation of the human mesenchymal stem cells derived from bone marrow and enhancement of cell attachment by fibronectin. *Journal of Oral Science,* 46, 207–213.

33. Pieri, F., Lucarelli, E., Corinaldesi, G., Iezzi, G., Piattelli, A., Giardino, R. *et al.*, 2008: Mesenchymal stem cells and platelet-rich plasma enhance bone formation in sinus grafting: a histomorphometric study in minipigs. *J. Clin. Periodontol.,* 35, 539–546.

34. Pryor, M. E., Polimeni, G., Koo, K. T., Hartman, M. J., Gross, H., April, M. *et al.*, 2005: Analysis of rat calvaria defects implanted with a platelet-rich plasma preparation: histologic and histometric observations. *J. Clin. Periodontol.,* 32, 966–972.

35. Rosocha, J., Cibur, P., Hulman, M., Holovska, V., Lehocka, 2008: Human mesenchymal stem cells. In **Lešník, F., Ostró, A.:** *Biological Aspects of Regenerative Medicine.* Publ. house Olomouc, Olomouc, 52–77.

36. Terheyden, H., Jepsen, S., Mőller, B., Tucker, M. M., Rueger, D. C., 1999: Sinus floor augmentation with simultaneous placement of dental implants using a combination of deproteinized bone xenografts and recombinant human osteogenic protein-1. A histometric study in miniature pigs. *Clin. Oral Implants Res.,* 10, 510–521.

37. Thorwarth, M., Schultze-Mosgau, S., Kessler, P., Wiltfang, J., Schlegel, K. A., 2005: Bone regeneration in osseous defects using a resorbable nanoparticular hydroxyapatite. *J. Oral Maxillofac. Surg.,* 63, 1626–1633.

38. Van den Bergh, J. P., ten Bruggenkate, C. M., Groeneveld, H. H., Burger, E. H., Tuinzing, D. B., 2000: Recombinant human bone morphogenetic protein-7 in maxillary sinus floor elevation surgery in 3 patients compared to autogenous bone grafts. A clinical pilot study. *J. Clin. Periodontol.,* 27, 627–636.

39. Vogel, J. P., Szalay, K., Geiger, F., Kramer, M., Richter, W., Kasten, P., 2006: Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and *in vivo* bone formation in calcium phosphate ceramics. *Platelets,* 17, 462–469.

40. Wang, M. L., Nesti, L. J., Tuli, R., Lazatin, J., Danielson, K. G., Sharkey, P. F., Tuan, R. S., 2002: Titanium particles suppress expression of osteoblastic phenotype in human mesenchymal stem cells. *J. Orthop. Res.,* 20, 1175–1184.

41. Wang, M. L., Tuli, R., Manner, P. A., Sharkey, P. F., Hall, D. J., Tuan, R. S., 2003: Direct and indirect induction of apoptosis in human mesenchymal stem cells in response to titanium particles. *J. Orthop. Res.,* 21, 697–707.

42. Weng, Y., Cao, Y., Silva, C. A, Vacanti, M. P., Vacanti, C. A., 2001: Tissue-engineered composites of bone and cartilage for mandible condylar reconstruction. *J. Oral Maxillofac. Surg.,* 59, 185–190.

43. Zerwekh, J. E., Morris, A. C., Padalino, P. K., Gottschalk, F., Pak, C. Y., 1990: Fluoride rapidly and transiently raises intracellular calcium in human osteoblasts. *J. Bone Miner. Res.,* 5, 131–136.

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HISTOPATHOLOGY AND IMMUNODETECTION OF *MANNHEIMIA HEMOLYTICA* ANTIGENS IN EXPERIMENTAL CAPRINE PNEUMONIA

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ABSTRACT

Mannheimiosis was experimentally induced in 15 six-month-old goats by intratracheal inoculation of 10^9 colony forming units of four hour log-phase *Mannheimia hemolytica* (MH) serotype A2 and five goats served as control. The histopathological and immunohistochemical investigations were performed on the tissues collected using the standard techniques. Histologically, the acute inflammatory reaction which had developed by 8 days post inoculation was characterised by flooding of the alveoli with oedema, hemorrhage and neutrophils (oat cells). This progressed to an acute exudative fibrinous pneumonia with extensive involvement of the interlobular septa and often with pleurisy. Subsequently, these pulmonary lesions became walled off by fibrous tissue which became infiltrated by macrophages, plasma cells and lymphocytes. At this stage, antigens were located in the necrotic center, necrotic alveolar wall, fibrin, exudate, and degenerated leukocyte. The bacterial colonies among these leukocytes, macrophages, necrotic bronchial and bronchiolar epithelia with the Bronchial Associated Lymphoid Tissue (BALT) had strong immunostaining. The strong immunostaining in the BALT showed its importance in antigen processing and this phenomenon may be indicative that the use of intranasal vaccines may help to curtail caprine pneumonia. This study further showed that immunohistochemical investigations might be helpful in retrospective studies and where microbiological examination is relatively difficult.

Key words: experimental infection; goat; immunohistochemistry; *Mannheimia hemolytica;* pneumonia

INTRODUCTION

Sheep and goats from West and Central Africa constitute about 37 % and 21 % of the livestock population in Africa, respectively. These animals require a low capital outlay for their production. Over 80 % of rural families keep ruminants, especially sheep and goats with women being more involved in their upkeep then other family members (12).

Over the past 15 years there has been a steady growth in goat production. Goats therefore have a higher population than the sheep in most African countries (1). Goats are considered to be one of the most important protein producing animals in Nigeria and they provide 30–36 % of the total meat consumption of the Nigerian populace annually (1).

Previous investigations have shown that Mycoplasma spp. and *Mannheimia haemolytica* (MH) are the most important non-viral agents associated with caprine pneumonia (13). The studies on MH infection in African small ruminant population is very scanty as the few reports available are on the serotypes of the bacteria available and their comparative pathogenicity especially in sheep (9, 13, 14); with very little information on the pathogenesis of the disease in goats, including the immunodetection of MH antigens in the cells of the respiratory tract.

In an attempt to understand the disease, there is a need to study the sequential histopathological changes that occur as well as the immunohistochemical detection of the antigen in the experimental form of this disease. The detailed immunohistochemical (IHC) studies on pneumonic lesions of calves naturally infected with

MH or induced by MH have been reported (10, 12), while that of goats are scanty in the literature (17) especially concerning the experimental MH infection. Although Zamri-Saad *et al.* (18) have described the pathology in transport stressed and dexamethasone treated goats, the aim of this study was to evaluate the sequential histopathological changes and the immunohistochemical detection of the antigens in experimentally MH infected goats not subjected to the stress of transport or dexamethasone treatment in order to further elucidate the possible role of stress in the pathology of manheimiosis in goats.

MATERIALS AND METHODS

Study location

The small ruminant pens of the Veterinary Pathology Department, in the experimental animal unit of the Faculty of Veterinary Medicine, University of Ibadan were used for this study.

Experimental animals

The details of the experimental procedures has been described earlier (6). Twenty apparently normal West Africa Dwarf goats (WAD), six months of age were used for the experiments. The animals were divided into two groups and housed in well partitioned pens. Group A had 15 goats, while group B with 5 goats served as controls. They were conditioned for 14 days before the experiments and their vital signs (rectal temperature and respiratory rates) were monitored daily to observe whether they remained afebrile and free of any clinical signs of diseases. Wheat bran and water were provided ad libitum daily. The nasal swabs of the animals were negative for MH by cultural isolation twice prior to the inoculations. The animals were also confirmed seronegative by the Agar gel precipitation technique for antibodies to peste des petit ruminants virus (PPRV) prior to inoculation.

Preparation of MH inoculum

MH A2 employed was supplied by Professor A.I. Adetosoye of the Department of Veterinary Microbiology and Parasitology of the University of Ibadan. The MH isolate (from the pneumonic lungs of a goat) was subcultured onto blood agar and incubated at 37.8 °C overnight. Thirty colonies of the same size were then inoculated into 50 ml brain-heart infusion broth and incubated at 37.8 °C for 18 h before the number of Colony Forming Units (CFU) was estimated using the total plate count method. The live inoculum was prepared by diluting the prepared broth to give an end concentration of 1×10^9 CFU.ml^{-1}.

Challenge infection

The details of the challenge infection has been previously described (6). Group A with fifteen goats were infected intratracheally, while five uninfected control goats were inoculated intratracheally with 1 ml of sterile brain infusion broth. The infection was done with 1ml of pure culture (10^9 CFU) of a 4 hour log phase culture of MH A2 in brain infusion broth.

Pathology

The details of the timing of euthanisia and the selection of goats for euthanasia has been previously described (6). Briefly, the animals that died, and two goats that were euthanized at predetermined periods, were necropsied. The necropsies were carried out on 8, 12, 19, 28, 45 and 48 day post inoculation (dpi). Samples from the lungs, liver, heart, spleen, messenteric lymph nodes and intestine were collected in 10 % buffered formalin, routinely processed and stained with haematoxylin and eosin for histological examination using the light microscope.

Immunization of rabbits

The preparation of antigen was as described by Effendy *et al.* (5) and the immunization of rabbits were carried out by the method of Diker *et al.* (4). The antigen was prepared from MH (serotype A2) strain obtained from the culture collection of the Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University Putra, Malaysia. MH was grown in brain-heart infusion (BHI) agar (pH 7.2, Oxoid, Basingstoke, England) at 37 °C for 24 h. Thirty colonies of the same size were inoculated into 50 ml brain-heart infusion broth and incubated at 37 °C for 18 h before the number of colony forming units (CFU) was estimated using the total plate count method. The broth was diluted to give an end concentration of 1×10^6 CFU.ml^{-1} and formalin was added to give a final concentration of 0.5 %. Two New Zealand white rabbits were administered one ml of antigen via/by intramuscularly twice with a 15 day interval. Two weeks after that the last injection, sera were obtained from rabbits and stored at –20 °C until it was utilized. Preimmune sera of the rabbits were used as a control and negative labelling was observed in known positive samples.

Immunohistochemistry

Immunohistochemistry IHC labelling was performed with the avidin-biotin-peroxidase complex (ABC) procedure (17), using commercially available immunoperoxidase kits (LSAM™ 2 System Dako Corporation, Carpinteria, USA). For immunohistochemistry, deparafinized tissue sections were incubated with 3 % H_2O_2 in 70 % methanol to inhibit endogenous peroxidase activity, and then washed three times with phosphate-buffered saline (PBS). The sections were blocked with a solution of normal goat serum. After draining the blocking serum, the sections were incubated for an hour at 37 °C with the primary antibody, diluted to 1 : 150. After three washes with PBS, the slides were incubated with biotinylated anti-rabbit secondary antibody for 30 minutes at 37 °C. Then the sections were washed three times with PBS and treated with the peroxidase-conjugated streptavidin. After another PBS bath, the sections were counterstained with Mayer's haematoxylin. As a negative control, a non-pneumonic goat lung tissue, was processed in an identical manner to the pneumonic lung tissues. For serum control, replicate sections of the pneumonic lung tissues were processed, substituting the rabbit anti-MH serum with the normal rabbit serum.

RESULTS

Pathology

Histopathological changes were that of acute, subacute, and chronic bronchopneumonia. The acute case occured in the goats that died at 8 dpi, the lungs showed extensive areas of necrosis, oedema, hemorrhage, numerous oats cell accu-

mulations in the alveoli spaces and fibrin deposits (Fig. 1A, B). There were also purulent exudates in the bronchi and bronchioles. The bronchial associated lymphoid tissue (BALT) during this phase were poorly developed with few aggregates of only a few lymphocytes. The less purulent form was observed in the goats euthanized at 12 dpi, the lungs of these goats were characterized by mild pulmonary oedema and congestion. There was severe fibrinopurulent exudate in the alveoli (Fig. 1 C), the interlobular septa and pleura. The pleura and the interlobular septa were markedly thickened with fibrin. There was also numerous alveolar macrophages with hemosiderin within the alveoli. At this stage, the BALT were markedly prominent with more nodular forms. The BALT hyperplasia were more evident in areas of less inflammatory response.

The proliferative lesions were observed in goats euthanised between day 28 and 45 with the lungs characterized by alveoli epithelial hyperplasia and macrophage proliferation (Fig. 1 D). There were peribronchial and perivascular lymphoreticular hyperplasia, and obliterative bronchiolitis. There were multifocal necrotic bronchitis and bronchiolitis. There were bacteria accumulations in the alveoli while the lymphatic vessel, interlobular septa and pleura were mostly distended with fibrosis and moderate fibrinocellular exudates. The inflammatory cells were mostly the macrophages, lymphocytes and neutrophils. There were numerous necrotic foci walled off by fibrous connective tissue. The lungs of the control goats were normal.

Immunohistochemistry (IHC)

The specific IHC reaction was characterised by the presence of light to brown, fine to coarse granules or larger areas in the cell or tissues. The antigens were demonstrated in: pneumocytes; macrophages (Fig. 2 A, B); fibrin and leukocytes present in the lumen; blood vessels on the pleura (Fig. 2 C, D, E); and the desquamated bronchial and bronchiolar epithelial cells (Fig. 2 D). The intensity varied as the disease progressed with very slight immunostaining in the pneumocytes, interstitial macrophages, in blood vessels in 8 pi and moderate intensity in these cells and lymphatics in the less purulent phase 12 and 19 dpi; while the bronchial, bronchiolar epithelium and the luminal exudates with macrophages in the bronchial associated lymphoid tissue stained strongly 28–45 dpi (Fig. 2 F).

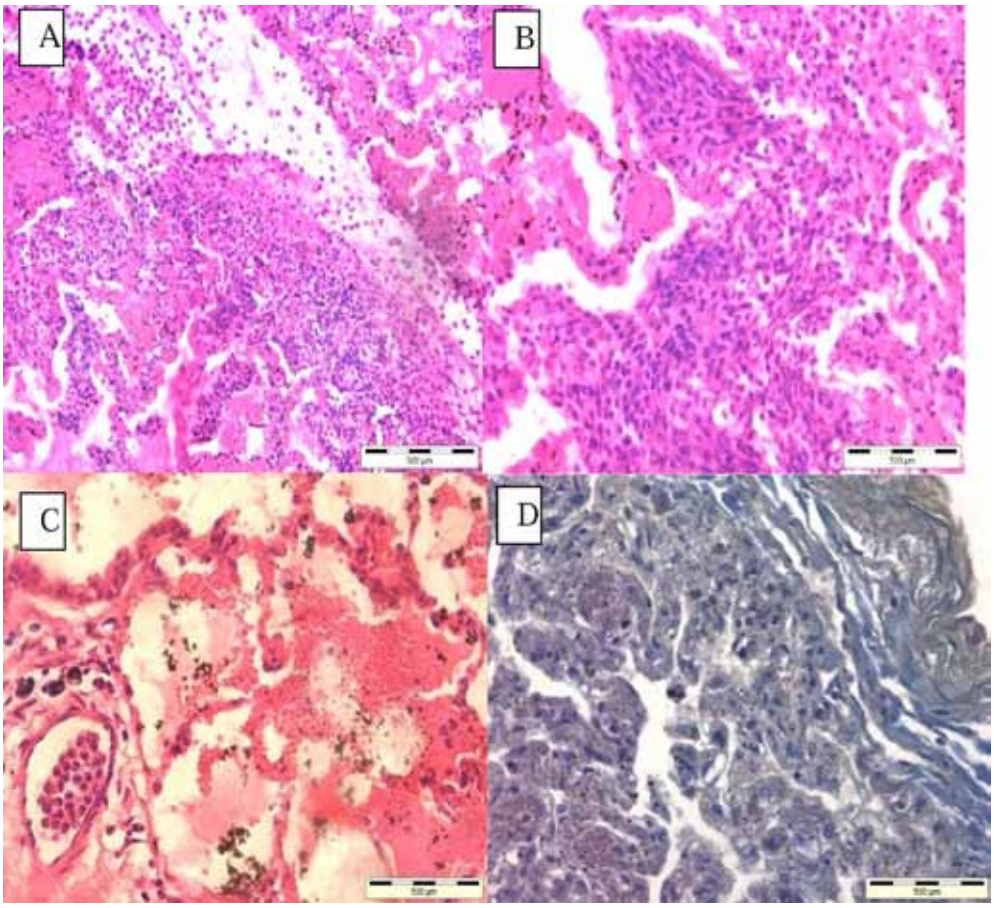

Fig. 1. A, B: The alveoli spaces and interlobular septae were flooded with fibrinopurulent exudates (oat cells) while the alveolar capillaries were dilated.
C: The lung is congested with dilated alveolar capillaries with oedema in the alveoli and numerous neutrophils in one of the blood vessel.
D: The hyperplasia of the pneumocytes with thickening of the pleura by fibrosis. H&E

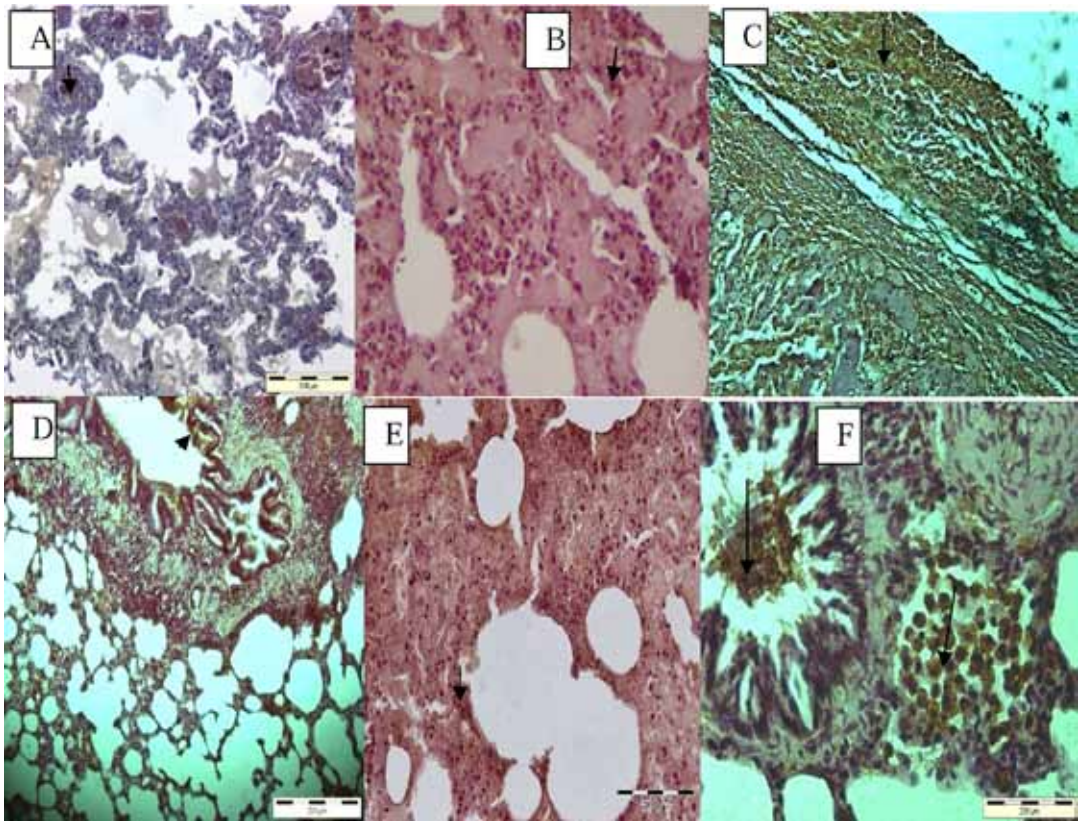

Fig. 2. A, B: The intra luminar cells of blood vessels showing MH antigens, the alveolar macrophages and the pneumocytes showing intracytoplasmic MH antigens in goats 8 pi. Note immunohistochemical labelling demonstrating slight specific reactions.
C: The pleura, alveolar macrophages, the pneumocytes showing intracytoplasmic MH antigens in goats 12 pi.
D: The epithelial cells of bronchial mucosae, bronchial glands and intra luminar exudates showing MH antigens in goats 19 pi. Note immunohistochemical labelling demonstrating strong specific reactions.
E: The alveolar macrophages, the pneumocytes and interalveolar septae showing moderate immunostaining in goats 28 pi.
F: The bronchial intra luminar exudates and macrophages in bronchial associated lymphoid tissue showing MH antigens in goats 42 pi.
ABC method counterstained with haematoxylin-eosin. Note immunohistochemical labelling demonstrating strong specific reactions

DISCUSSION

The results of this study describe the histopathological features and immunohistochemical detection of MH antigens in the experimental MH A2 infection in goats; the most predominant serotype in Nigeria (13). The severity of the lesions observed may be attributed to the effect of the cytotoxin produced by MH as the challenged bacterium was in the log phase before being used (15). This log phase helps the bacterium to circumvent the host defences (contrary to the reproduction of experimental pneumonic pasteurellosis) by combined viral and MH infection (8). It also showed that the use of dexamethasone and transport stress may not be absolutely neccesary for the induction of the experimental disease has described by Zamri-Saad *et al.* (18).

The histopathological features observed were not uniform in the affected goats. One of the goats showed an acute response while seven showed the less purulent form and seven others showed the chronic form of the diseases. The acute form of the infection which was characterised by the presence of large numbers of spindle-shaped cells with intensely basophilic nuclei (oat cells) in one of the goats, while the less severe fibrinopurulent bronchopneumonia type of lesion without oat cells as described by Zamri-Saad *et al.* (18) was also observed. However, the variation in the form of the disease in this study may not be associated with the virulence of the MH strain used (4), but may be due to the individual level of protective immune levels and the efficiency of the alveolar macrophages in this goats to phagocytose MH especially during the first week post challenge as this period has been described as the critical period for establishment of MH infection in goats (19). There is also a possibility that the animal that died 8 dpi with the characteristic histopathological features of acute pneumonic pasteurellosis with oat cell infiltration, may have failed to efficiently phagocytose this organism. This observation was also corroborated by the intensity of the immunostaining and the spread of the antigen. These findings are similar to reports by other workers in calves (12, 16) and also in naturally occurring pneumonia in goats (17).

The intensity of the immunostaining and the presence of the antigens in the exudates of the alveolar spaces, on the

pneumocytes, alveolar macrophages, bronchial and bronchiolar epithelia and in the exudates in these respiratory airways especially in the less purulent and chronic form, further confirm the transmission pattern of most respiratory infections.

The presence of large numbers of oat cells in one of the infected goats in this study has not been previously described in this breed. The possible reason for this observation as against that of Zamri-Saad *et al.* (18), may be due to the size of goats used in this study, as the Malaysian goats are 3–4 times the size of West African dwarf goats, hence the dose of MH used may be overwhelming.

The less purulent type outcome observed in this study was as reported in the stressed and dexamethasone treated goats (18) with acute fibrinopurulent bronchopneumonia. The chronic inflammatory response was characterised by a necrotic center and the walling off with thin fibrous connective tissue. Of note, was the massive peribronchial and peribronchiolar infiltration with lymphoplasmatic cells; there were proliferative changes with marked hyperplastic changes in the alveoli and bronchial and bronchiolar epithelia. This effect culmulated into tortuous respiratory airways and there were areas of bronchiolar obliterans.

The immunostaining reactions were more in the necrotic center, intraluminal exudates and epithelial cells of bronchioles and bronchus (12). In a previous investigation, MH antigens could be detected in the formalin-fixed paraffin embedded specimens, even after 4 years which further buttress the fact that immunohistochemical technique could be very useful for retrospective studies (7).

One of the significant findings in this study was the relationship of the level of inflammation and the development of the BALT. It was observed that the development of the BALT was more marked between day 12 and 19 pi which corroborated the findings of Zamri-Saad *et al.* (18). This hyperplastic BALT was inversely related to the level of the inflammatory response in the lungs of the affected goats. The BALT has been reported to be more organized, larger and nodular in antigen challenged goats (18) and in chronic pneumonia (2). This observation further showed that the mucosal immunity in the respiratory tract is very important to obtain protection as the development or increase in the size of BALT has been related to a decrease in the level of lung colonisation of MH (5). The strong immunostaining on the bronchial, bronchiolar epithelia and BALT coupled with the macrophage hyperplasia in the BALT 5–6 week pi further showed that the cells in the BALT are essential for antigen processing and production of IgA for prevention of further bacterial colonisation in the lungs. The occurrence of the strong immunostaining in cells in the BALT has not been previously described in the literature. This explanation was supported by the relative lower inflammatory response observed in the lungs of animals that elicit this hyperplastic BALT. The occurrence and development of the BALT has been reported to be antigen dependent and it may be markedly enlarged indicating the potential for local inflammation to induce lymphoid tissue in airways. This phenomenon can be explored in the development of intranasal vaccines in Africa to curtail pneumonia associated with respiratory viruses and bacteria.

From a diagnostician's point of view, although a definitive diagnosis of MH is by bacterial isolation, a diagnosis based on immunohistochemical detection of the MH antigens in formalinised samples might be helpful in retrospective studies and where microbiological examination is relatively difficult because antibiotic treatment of the animal may have precluded isolation of microorganisms.

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REFERENCES

1. Ademosun, A.A., 1985: Contributions of research to small ruminants production in Nigeria. In *Small Ruminant Production in Nigeria.* **Adu, I.F., Osinowo, O.A., Taiwo, B.B.A., Alhassan, W.S.** (Eds.), Nigeria, Zaria, 6–10th October, NAPRI Shika, 18–34.

2. Barman, N.N., Bhattacharyya, R., Upadhyaya, T.N., Baishya, G., 1996: Development of bronchus associated lymphoid tissue in goats. *Lung,* 174, 127–131.

3. Davies, D.H., Herceg, M., Thurley, D.C., 1982: Experimental infection of lambs with an Adenovirus followed by *Pasteurella hemolytica*. *Vet. Microbiol.,* 7, 369–381.

4. Diker, K.S., Akan, M., Kaya, O., 2000: Evaluation of immunogenicity of *Pasteurella hemolytica* serotypes in experimental models. *Turkish Journal of Veterinary Animal Science,* 24, 139–143.

5. Effendy, A.W.M., Zamri-Saad, M., Maswati, M.A, Ismail, M.S., Jamil, S.M., 1998: Stimulation of the bronchus associated lymphoid tissue of goats and its effect on *in vitro* colonisation by *Pasteurella haemolytica*. *Vet. Res. Comm.,* 22, 147–153.

6. Emikpe, B.O., Akpavie, S.O., 2010: The clinical and pathological features of experimental *Mannheimia hemolytica* A2 in West African Dwarf Goats (WAD). *Bulletin of Animal Health and Production in Africa,* 58, 261–270.

7. Emikpe, B.O., Akpavie, S.O., Sabri, Y.M., Zamri-Saad, M., 2011: The effect of type of incubation and two different antigen retrieval methods on the immunodetection of *Mannheimia hemolytica* antigens in archived caprine lung tissues. *J. Commonwealth Vet. Ass.,* 27, 18–21.

8. Emikpe, B.O., Sabri, Y.M., Akpavie, S.O., Zamri-Saad, M., 2010: Experimental infection of *Peste des petits ruminants* virus and *Mannheimia haemolytica* A2 in goats: Immuno-localisation of *Mannheimia haemolytica* antigens (DOI: 10.1007/s11259-010-9425-y), *Vet. Res. Comm.,* 34, 569–578.

9. Gourlay, R.N., Barber, L., 1960: A strain of *Pasteurella haemolytica* isolated from goats in Uganda. *J. Comp. Path.,* 70, 211–216.

10. Haritani, M., Nakazawa, M., Hashimoto, K., Narita, M., Tagawa, Y., Nakagawa, M., 1990: Immunoperoxidase evaluation of

the relationship between necrotic lesions and causative bacteria in lungs of calves with naturally acquired pneumonia. *Am. J. Vet. Res.,* 51, 1975–1979.

11. Kumar, S., Vihan, V.S., Deoghare, P.R., 2003: Economic implication of diseases in goats in India with references to implementation of a health plan calendar. *Small Ruminant Res.,* 47, 159–164.

12. Narita, M., Kimura, K., Tanimura, N., Arai, S., Katsuda, T.K., 2000: Immunohistochemical characterization of calf pneumonia produced by the combined endobronchial administration of bovine herpesvirus 1 and *Pasteurella haemolytica*. *J. Comp. Path.,* 123, 126–34. doi:10.1053/jcpa.2000.0402.

13. Odugbo, M.O., Odama, L.E., Umoh, J.U., Makinde A.A., 2003: Serotypes of *Pasteurella hemolytica* from pneumonic lungs of sheep in Northern Nigeria. *Small Ruminant Res.,* 48, 239–243.

14. Odugbo, M.O., Odama, L.E., Umoh, J.U., Lombin, L.H., 2004: The comparative pathogenicity of strains of eight serovars and untypeable strains of *Mannheimia hemolytica* in experimental pneumonia of sheep. *Vet. Res.,* 35, 661–669.

15. Shewen, P.E., Wilkie, B.N., 1985: Evidence for the *Pasteurella haemolytica* cytotoxin as a product of activity growing bacteria. *Am. J. Vet. Res.,* 46, 1212–1214.

16. Whiteley, L.O., Maheswaran, S.K., Weiss, D.J., 1990: Immunohistochemical localization of *P. haemolytica* A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine *Pasteurella pneumonia. Vet. Path.,* 27, 150–61.

17. Yener, Z., Ilhan, F., Ilhan, Z., Saglam, Y.S., 2009: Immunohistochemical detection of *Mannheimia (Pasteurella) haemolytica* antigens in goats with natural pneumonia. *Vet. Res. Comm.,* 33, 305–313.

18. Zamri-Saad, M., Jasni, S., Nurida, A.B., Sheikh-Omar, A.R., 1991: Experimental infection of dexamethasone treated goats with *Pasteurella haemolytica* A2. *Br. Vet. J.,* 147, 565–568.

19. Zamri-Saad, M., Maswati, M.A., Effendy, A.W.M., Salim, N.B., Sheikh-Omar, A.R., 1996: Establishment of *Pasteurella haemolytica* A2 in the lungs of goats following intratracheal exposure. *J. Vet. Malaysia.,* 8, 15–19.

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THE CRANIAL AND CAUDAL MESENTERIC ARTERIES IN THE RAT *(RATTUS NORVEGICUS F. DOMESTICA)*

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ABSTRACT

The experiments were carried out on 50 Wistar rats (*Rattus norvegicus f. domestica*); males and females, weighing 200–250 g. The architecture of the vessels in the abdominal cavity was studied using corrosion casts prepared with Duracryl Dental® and PUR SP (polyurethane material). Our observations focused on the *a. mesenterica cranialis et caudalis*. The *a. mesenterica cranialis* originated from the *aorta abdominalis* cranial to the *a. renalis dextra, or* as a common trunk with the *a. renalis dextra,* or after branching off of the *a. renalis dextra*. The *a. colica media, the a. pancreaticoduodenalis caudalis, the a. colica dextra, the aa. jejunales,* and the *a. ileocecocolica all* originated from *the a. mesenterica cranialis*. We observed a common origin of the *a. colica media et dextra,* or the *a. colica dextra* after the emanation of the *a. colica media*. The third branch was the *a. pancreaticoduodenalis caudalis*. The *Aa. jejunales* formed a jejunal arcade, or we observed the presence of a jejunal trunk. The *a. mesenterica caudalis* originated from: the zone of the aortic bifurcation; the aorta cranial to the branching off of the *a. sacralis mediana;* caudal to the *a. sacralis mediana;* or the vessel branched off from the *a. iliaca communis dextra*.

Key words: abdominal cavity; blood supply of the intestine; circulatory system; mesenteric arteries; rat anatomy

INTRODUCTION

There is presently more information on the *a. mesenterica cranialis et caudalis* in cats (7), dogs (6), and other laboratory animals (4, 14) than in rats. Many authors have used the laboratory rat for the study of structures in the animal body (1, 9), but there is a lack of information on *a. mesenterica cranialis et caudalis* in laboratory rats. The Wistar rat (*Rattus norvegicus f. domestica)* is one of the laboratory animals most frequently used in medical research. Currently, in both human and veterinary medical research endeavours, there is an increased focus of attention on the therapeutic interventions which may contribute toward the improvement of the treatment of human diseases. The rat model appears ideal for the study of various methods of intestinal transplantation that may serve in the future as suitable approaches to the treatment of human digestive tract diseases. Intestinal transplantation techniques have been the subject of investigations by many researchers. Some authors described techniques for transplanting a portion of the small intestine by conventional vasoligature (10, 15). Other authors have applied an experimental intestinal transplantation method based on a "cuff" anastomosis (11). A group of Japanese scientists investigated both methods using various combinations of donors and recipients organs (9). Intestinal transplantations were investigated recently by a team of scientists at the Institute of Clinical and Experimental Medicine in Prague (2). None of these studies described in detail the blood supply to the individual segments of the intestine. There-

fore, we focused our study on the origin, course and the individual variations of the *a. mesenterica cranialis et caudalis.*

MATERIAL AND METHODS

The experiments were carried out on 50 Wistar rats *(Rattus norvegicus f. domestica);* males and females, weighing 200–250 g. They were euthanized with ether. The vessels of the euthanized rats were perfused with saline (0.9 % NaCl) by means of the cannulation of the left ventricle. An improved method for the washing out of the clotted blood from the vessels was achieved by the addition of 0.05 % NaOH to the perfusion medium (5). The perfusion pressure was approximately 200–250 mm Hg (2.6–3.25 m H_2O). A portion of the venous system must be opened to ensure a good distribution of the perfusion medium. The right auricular appendage served this purpose. The success of the perfusion was indicated by the uniform fading of the tissues seen during the procedure. The injection media of Duracryl Dental®, based on polymethylmethacrylate, and PUR SP (polyurethane material), was used in the study. The maceration of the soft tissues was carried out in a 2–4 % solution of KOH at 60–70 °C. The maceration took approx. 2–3 days (3). The results were expressed in percentages. The latest edition of the Veterinary Anatomic Nomenclature was consulted throughout this study (13).

RESULTS

The arteria mesenterica cranialis et caudalis supplies blood to the individual parts of the intestine. The *arteria mesenterica cranialis* is the thickest branch of the *aorta abdominalis*. It is an azygos emanating from the ventral aorta wall caudal to the origin of the the *a. coeliaca.* It irrigates the small intestine and the cranial portion of the large intestine up to the transverse colon. The following variations were observed in the origin of the cranial mesenteric artery: in 15 % of the corrosion cast specimens, the *a. mesenterica cranialis* originated from the abdominal aorta cranial to the origin of the *a. renalis dextra* (Fig. 3); and in 42 % a common trunk originated from the *aorta abdominalis* for the *a. mesenterica cranialis* and the *a. renalis dextra* (Fig. 2). The third variation was the origination of the artery from the abdominal aorta caudal to that of the *a. renalis dextra* (Fig. 1), which was observed in 43 % of the cases. After the origin of the *a. mesenterica cranialis,* it provided branches to the *a. colica media,* the *a. pancreaticoduodenalis caudalis,* the *a. colica dextra,* the *aa. jejunales* and the *a. ileocecocolica.*

The first branch was the *a. colica media*, running caudally from its origin. It supplied blood to the transverse colon and cranial portion of descending colon. In 46 %, it branched off individually from *a. mesenterica cranialis* and in 37 % as a common trunk with the *a. colica dextra*.

The *a. pancreaticoduodenalis caudalis* originated as a second branch and was directed cranially. It divided into *rr. pancreatici* for *pancreas* and *rr. duodenales* for *duodenum*. In 46 % the caudal pancreaticoduodenal artery originated cranial to the *a. colica dextra* and in 37 % after the *a. colica dextra.* In 7 % the *a. pancreaticoduodenalis caudalis* anastomosed with the *a. pancreaticoduodenalis cranialis*.

The third branch of the cranial mesenteric artery was the *a. colica dextra,* which was directed caudally and supplied blood to the ascending colon. In 37 % it originated from the the *a. mesenterica cranialis* as a common trunk with the *a. colica media* before the origin of the *a. pancreaticoduodenalis caudalis.* In 50 % the artery branched off independently after the origin of *a. pancreaticoduodenalis caudalis.*

The principal continuation of the *a. mesenterica cranialis* was the *a. ileocecocolica* which divided into: the *r. colicus* for the *colon ascendens;* the *a. ilealis* for the *ileum;* and the *a. cecalis* for the *cecum.* The presence of the *a. ileocecocolica* (Fig. 4) together with its branches was observed in 45 %. In 35 % the *a. mesenterica cranialis* continued as the *a. ileocecalis* which bifurcated into the *a. ilealis* and the *a. cecalis.*

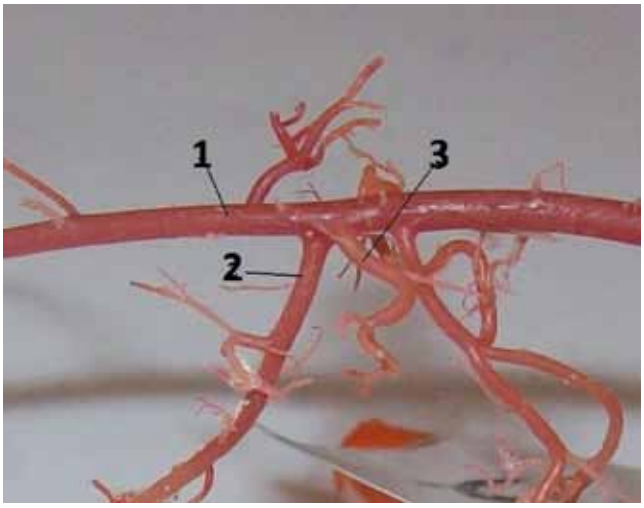

Fig. 1. The origin of *a. mesenterica cranialis* caudal to the *a. renalis dextra*
1 – *aorta abdominalis;* 2 – *a. mesenterica cranialis;* 3 – *a. renalis dextra*

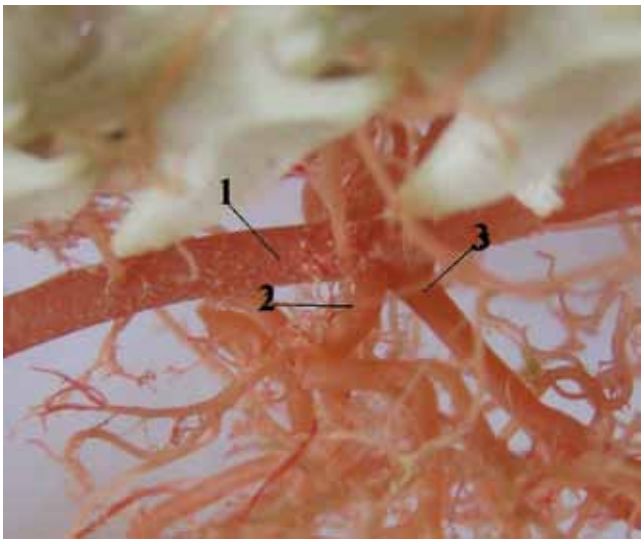

Fig. 2. The common trunk of the *a. mesenterica cranialis* with the *a. renalis dextra*
1 – *aorta abdominalis;* 2 – *a. renalis dextra;* 3 – *a. mesenterica cranialis*

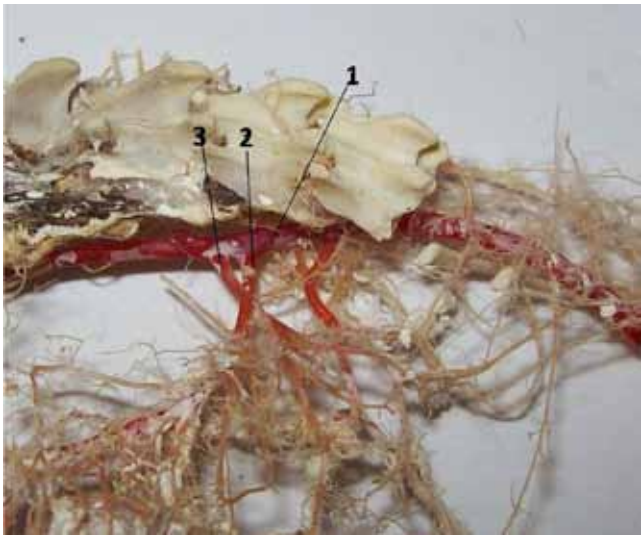

Fig. 3. The origin of the *a. mesenterica cranialis* cranial to the *a. renalis dextra*
1 – *aorta abdominalis;* 2 – *a. mesenterica cranialis;* 3 – *a. renalis dextra*

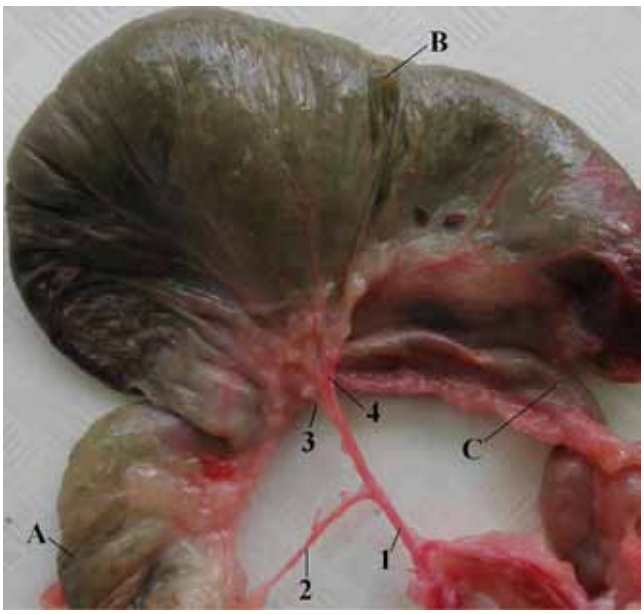

Fig. 4. The presence of the *a. ileocecocolica* and its division
1 – *a. ileocecocolica;* 2 – *r. colicus;* 3 – *a. ilealis;* 4 – *a. cecalis*
A – *colon;* B – *cecum;* C – *ileum*

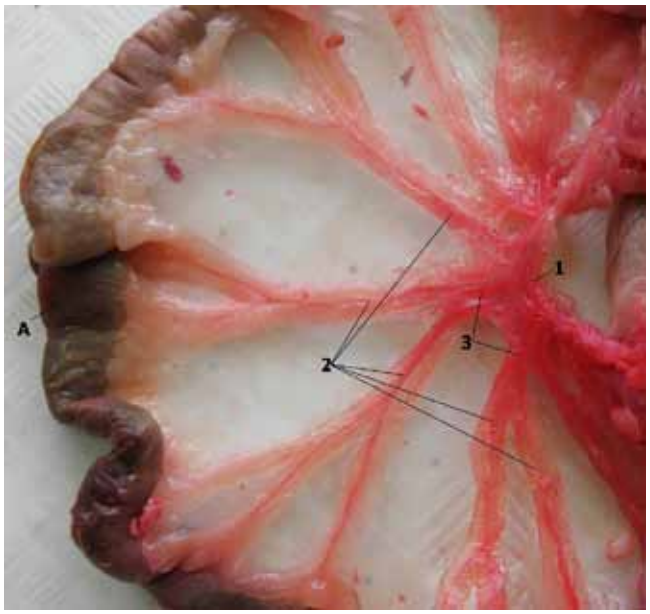

Fig. 5. The presence the *truncus jejunalis* and *aa. jejunales*
1 – *a. mesenterica cranialis;* 2 – *a. jednalis;* 3 – *truncus jednalis*
A – *jejunum*

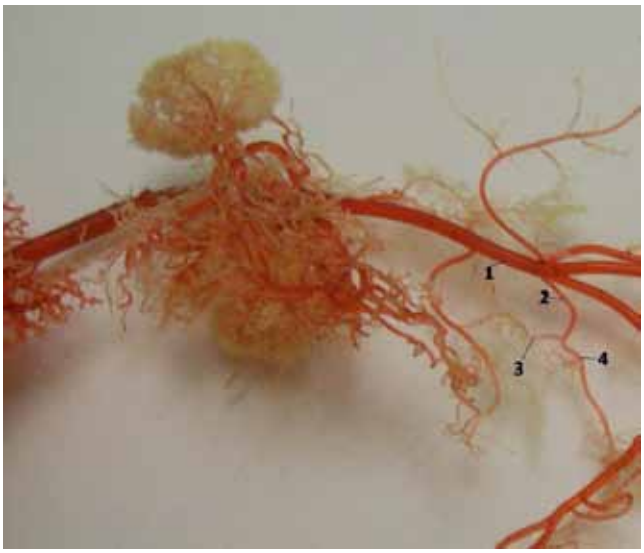

Fig. 6. The division of the *a. mesenterica caudalis*
1 – *aorta abdominalis;* 2 – *a. mesenterica caudalis*
3 – *a. colica sinistra;* 4 – *a. rectalis cranialis*

Additional branches from the cranial mesenteric artery were the *aa. jejunales* (Fig. 5). In 16 to 20 of the rats, they irrigated the *jejunum* and the cranial portion of the *ileum.* In 4% of the corrosion casts, we observed a jejunal arcade and in 7% a jejunal trunk (Fig. 5).

The *a. mesenterica caudalis* was the last azygos branch of the abdominal aorta which supplied blood to the caudal part of the descending *colon* and the *rectum*. The caudal mesenteric artery originated in 52% of the cases in the region of the aorta abdominalis bifurcation; in 2% it branched off from the aorta, cranially before the origin of the *a. sacralis mediana* and in the same number of casts originated directly from *a. sacralis mediana.* In 2% the *a. mesenterica caudalis* originated from the *a. iliaca communis dextra.* The *a. mesente-*

rica caudalis bifurcated to the *a. colica sinistra* and the *a. rectalis cranialis* (Fig. 6).

The a. colica sinistra (Fig. 6) supplied blood to the caudal portion of the descending colon and in 44 % anastomosed with the *a. colica media*.

The *a. rectalis cranialis* (Fig. 6) supplied blood to the *rectum.*

DISCUSSION

Some authors described the origin of the *a. mesenterica cranialis* 3–5 mm caudal to the origin of *a. coeliaca* (2, 8). *A. mesenterica cranialis* was the thickest branch of the abdominal aorta with basic branching identical to that described in our study (12). Our results agreed with other authors (8, 12) as well. Our results indicated that the *a. colica media* was consistent. It branched off independently or as a common trunk with *a. colica dextra.* According to Hebel and Stromberg (8), this artery was frequently dual. The *a. pancreaticoduodenalis caudalis* originated from the abdominal aorta as a second or third branch. According to these authors the artery always formed the second branch. The *a. colica dextra* branched off from the *aorta abdominalis* either as a second or third branch. The *a. colica dextra* originated as a common trunk with *a. colica media*. In this case *a. colica dextra* emerged as the second branch. It formed the third branch when originating independently after the origin of the *a. pancreaticoduodenalis caudalis.* According to our results the *aa. jejunales* always originated from the *a. mesenterica cranialis.* Other authors described the origin of the *aa. jejunales* from the *a. colica dextra* (2, 8). The corrosion casts prepared in our study allowed us to record two variations of the terminal branching of the *a. mesenterica cranialis.* The artery terminated either as the *a. ileocecocolica* or the *a. ileocecalis.* The *a. ileocecocolica* divided to supply the *r. colicus, or* the *a. ilealis et cecalis.* The *a. ileocecalis* divided to the *a. cecalis et ilealis.* The principal continuation of the *a. mesenterica cranialis* was the *a. ileocolica* which supplied blood to the ascending colon, *ileum* and *cecum* (2).

The *a. mesenterica caudalis* was the branch supplying the caudal parts of the colon and the rectum. We observed variations in the origin of the artery in comparison with other authors (2, 8, 12) who described its origin in the region of the bifurcation of the abdominal aorta or immediately cranial to it (8). In addition to these two origins, our study described the origin of this artery from the *a. sacralis mediana* or the *a. iliaca communis dextra.* After its origin, *a. mesenterica caudalis* bifurcated into *a. colica sinistra* and *a. rectalis cranialis.* A similar branching of the artery was described by others (2, 8, 12).

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REFERENCES

1. Ahmed, S. H., Rakhawy, M. T., Abdalla, A., Assaad, E. I., 1978: The comparative anatomy of the blood supply of cardiac ventricles in the albino rat and guinea-pig. *J. Anat*., 126, 51–57.

2. Baláž, P., Mergental, H., 2006: *Transplantation in Experiment*. Galén. Prague, 25–29.

3. Barta, A., Demčišák, L., 2000: Technique and methods of preparation of corrosive casts. In *Proceedings Current trends in morphology*, University of Veterinary Medicine in Košice, Slovak Anatomical Society, 179–181.

4. Craigie, E. H., 1948. *Practical Anatomy of the Rabbit. An Elementary Laboratory Textbook in Mammalian Anatomy*. 8th. edn., Toronto, University of Toronto Press., 123–133.

5. Flešárová, S., Tesfaye, A., Danko, J., Bátor, R., Dorko, F., Barta, A., 2003: Morphological variations of *arcus aortae* in rabbits. *Acta Vet. (Brno)*, 72, Supplementum, 116.

6. Gomercic, H., Babic, K., 1972: A contribution to the knowledge of the variations of the arterial supply of the duodenum and the pancreas in the dog *(Canis familiaris). Anat. Anz. Bd.,* 132, 281–288.

7. Gomercic, H., Babic, K., 1975: Variations of the arterial supply of the duodenum and the pancreas in the domestic cats *(Felis domestica Brisson). Acta. Anat.,* 91, 213–221.

8. Hebel, R., Stromberg, M. W., 1989: *Anatomy and Embryology of the Laboratory Rat*. Wőrthsee, BioMed Verlag, 97–112.

9. Kobayashi, E. Kamada, N., Enosawa, S., Toyama, N. *et al.,* 1993: Comparison of potentiality to induce graft-versus-host reaction with small bowel, pancreas/spleen, and liver transplantation in the rat. *Clin. Exp. Immunol*., 92, 527–531.

10. Monchik, G. J., Russel, P. S., 1971: Transplantation of small bowel in the rat, technical and immunological considerations. *Surgery*, 70, 693–702.

11. Nakao, A., Mitsuoka, N., Shen, S. D., Tanaka, N., Kobayashi, E., 2000: Rat small intestinal transplantation: a comparison of the cuff and hand-suture methods, *Acta Okayama*, 54, 259–264.

12. Nejedlý, K., 1965: *Biology and Anatomy of Laboratory Animals.*, SPN, Prague, 332–333.

13. Nomina Anatomica Veterinaria, 2005: 5th edn, Hannover, Columbia, Gent, Sapporo, 77–109.

14. Perneczky, V. A., 1969: Die Äste der Aorta abdominalis beim Meerschweinchen. *Anat. Anz*., 125, 443–453.

15. Wallender, J., Hollz, A., Larsson, E., Gerdin, B., Lackgren, G., Tufverson, G., 1988: Small-bowel transplantation in the rat with nonsuture cuff technique. Technical and immunological considerations. *Transpl. Int*., 1, 135–139.

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CHLAMYDOPHILA PSITTACI IN PIGEONS – ARE THE FERAL PIGEONS *(Columba livia domestica)* A DANGEROUS SOURCE OF CHLAMYDIAL INFECTIONS?

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ABSTRACT

Pigeons have been reported to be commonly infected by a zoonotic bacterium, *Chlamydophila psittaci,* that is the causative agent of psittacosis in human. We examined the seroprevalence of *Chlamydophila psittaci* in feral pigeons *(Columba livia domestica)* in the Košice region of Slovakia. Certain areas (industry halls) of this city harbor a high concentration of feral pigeons. The study was conducted between February and June of 2009. The blood samples were examined by a complement fixation reaction (CFR). Out of 102 samples tested for *Chlamydophila psittaci,* 4 samples were positive (3.92%). The anti-chlamydial antibodies titres ranged from 1 : 32 to 1 : 256. Our results suggest that pigeons in the Košice region are not such a dangerous source of chlamydiosis for humans, as reported by other authors.

Key words: *Chlamydophila psittaci*; complement fixation reaction; pigeons; seroprevalence; Slovakia; zoonosis

INTRODUCTION

Chlamydophila psittaci (7), an obligate intracellular bacterium, which is the causative agent of avian chlamydiosis in birds and psittacosis in humans, has been reported to be one of the most common pathogens found in feral pigeons worldwide and thus, constitutes a zoonotic risk (8, 14). Exposure to *Chlamydophila psittaci* by contaminated dust, direct contact with pigeons through handling and feeding have been identified as the cause in more than half of the human cases. Infections by indirect contact with feral pigeons have been reported in about 40 % of the cases (16). The disease in birds is characterized by fever, watery-green diarrhea, anorexia, respiratory distress and conjunctivitis (1). The incubation period is from 5 to 14 days. Human infections vary from inapparent to severe systemic disease. Infected humans typically develop headache, chills, malaise and myalgia, and pulmonary involvement is quite common (10).

The first case of the transmission of *Chlamydophila psittaci* from feral pigeons to humans was described in 1941 by Meyer (17). Since this first description, 101 case reports have documented the successful transmission of chlamydiosis from pigeons to humans (9). In Slovakia, chlamydiosis in pigeons was confirmed by Řeháček and Brezina (20), Řeháček *et al.* (21), Pospíšil *et al.* (19), Kocianová *et al.* (13) and Čisláková *et al.* (5).

Chlamydial infections are widespread in the feral pigeon populations of several European towns and cities. A high seroprevalence to *Chlamydophila psittaci* has been detected for several years in most of them. Therefore, we decided to determine the seroprevalence of *Chlamydophila psittaci* in the feral pigeons in the Košice region of Slovakia. This work summarises the results of the serological examination of the feral pigeons from this area during a 5 month period in 2009.

MATERIALS AND METHODS

Blood samples from 102 feral pigeons were collected during a pigeon elimination programme from certain areas (industry halls) in the Košice region of Slovakia. The blood samples were obtained between February and June of 2009. The pigeons were caught by

traps and were euthanized by cervical dislocation, which is a simple and humane method of dispatching small to medium-sized birds and small mammals. Blood samples taken from the hearts of the pigeons were examined after centrifugation with the micromethod of the complement fixation reaction (CFR) (15, 23) according to the manual of the OIE (2). CFR is the standard test for the detection of chlamydial antibodies in birds. Serum samples with antibodies titre of 1:32 and higher were considered to be positive.

RESULTS

During the 5 months (February–June 2009) period of research, out of the total number of 102 samples, 4 were positive, which represented 3.92%. The anti-chlamydial antibodies titres ranged from 1:32–1:256. Out of the total number of 4 positive samples, 2 samples (50%) had lower titres (1:32 and 1:64) and 2 (50%) had higher titres (1:128 and 1:256).

DISCUSSION

Feral pigeons play an important role in the transmission of chlamydiosis to humans. From 1988 to 2003, the Centers for Disease Control (CDC) received reports of 935 cases of chlamydial infections in humans (4), which does not represents all of the actual number of cases. Considering data from 11 European countries, the seroprevalence of *Chlamydophila psittaci* in feral pigeons ranged from 19.4% to 95.6%. In most studies, the complement fixation test was used, and antibodies were detected in 19.4 to 66.3% of the samples (16).

In our study, the seroprevalence was low when compared with the results of other authors. This discrepancy could be due to differences in the methodology, seasonality, type of sample and/or whether the pigeons came from urban or rural populations. The prevalence of anti-chlamydial antibodies in our study was 3.92%, while other authors, who investigated blood serum of pigeons by CFR reported seroprevalences in the range of 28.6% to 61.5% (6, 18, 22, 27).

Regarding the seasonality; our study was conducted between February and June of 2009 (spring–summer), and we detected a low seroprevalence. Fifty four of 102 of our samples were obtained in the period from February to April (spring) 2009 and only one of these samples was positive with a titre of 1:64. During the period from May to July (summer) the remaining 48 samples were harvested and three of the samples were positive with the respective titres of 1:32, 1:128 and 1:256. Trávniček *et al.* (27) repeatedly confirmed higher seropositivity (77.1% and 85.1%) in April as compared with summer-June (41.0% and 33.3%). In this study, the maximal antibody titre was also higher in April (1:1024) as compared with the maximal titres in June (1:512 and 1:256). Interestingly, Janiga (12) noted that significantly more pigeons were found with antibodies against *Chlamydophila psittaci* in the autumn and winter than in the spring and summer. In their study, from November to December, 172 of 332 (seroprevalence of 51.8%) birds had antibodies against *Chlamydophila psittaci*, while only 25 of 148 (seroprevalence of 16.9%) birds had detectable antibodies in the spring and summer (March to August).

According to the results of Vlahović *et al.* (28) and Schwarzová *et al.* (25), the positivity of free-living birds seems to be much lower than the positivity of city pigeons. Vlahović *et al.* (28) investigated the prevalence of *Chlamydophila* spp. in 107 free-living birds (including feral pigeons) from Croatia. *Chlamydophila* spp. was detected with a prevalence of 2.8% (3/107) of the tested free-living birds examined. Schwarzová *et al.,* reported a prevalence of 6.6% (6/91) using the polymerase chain reaction (PCR) from cloacal swabs in migrating birds in Slovakia (25). While Vlahović *et al.* (28) and Schwarzová *et al.* (25) reported a low positivity, Schettler *et al.* (24) detected *Chlamydophila psittaci* in free-living birds with a high positivity (74%) using the same method (PCR) as Schwarzová *et al.* (25). PCR was used also by Heddema *et al.* (11) to detected chlamydiosis in fresh fecal samples from city pigeons in Amstredam, with an overall positivity of 7.9%. There was a significant difference between positivity in the low breeding season (5% in February and March) and the heigth of the breeding season (10% in May). Tanaka *et al.* (26) in Japan found *Chlamydophila psittaci* in 106 of 463 (prevalence 22.9%) fecal samples examined from feral pigeons by using the nested PCR.

CFR seems to be an equally sensitive method for the detection of *Chlamydophila psittaci* in pigeons. Feral pigeons as an important source of chlamydiosis for people, were confirmed in Košice, Slovakia by Pospíšil *et al.* in 1988 (19). Also at present, in the Košice region, chlamydiosis represent a relatively serious problem. In the years between 2001 and 2005, of the 27 confirmed cases of chlamydiosis in Slovakia, 24 of the people came from the Košice region (3). Therefore, the control of pigeon populations and their correct sanitary management are essential for the prevention and control of chlamydiosis.

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REFERENCES

1. Acha, P., Szyfres, B., 1994: *Zoonoses and Communicable Diseases Common to Man and Animals.* Pan American Health Organization, Washington, 963 pp.

2. Andersen, A. A., 2004: Avian chlamydiosis. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual).* 5th edn., Office International des Epizooties, Paris, France, 856–867.

3. Balogová, L., Jarčuška, P., Seligová, J., 2006: Psittacosis – clinical manifestations and possibilities of treatment. Incidence in SR in 2001–2005 (In Slovak). *Slov. Vet. Čas.,* 4, 239–241.

4. Centres for Disease Control and Prevention (CDC), 2003: Summary of notifiable diseases – United States 2001. Morb. Mortal. Wkly. Rep. (MMWR), 50, I–XXIV, 1–8.

5. Čisláková, L., Dietzová, Z., Prokopčáková, H., 1998: Ornthiosis in urban wild pigeons *Columba livia* f. *domestica* in Košice (In Slovak). *Vet. Med. Czech.,* 43, 361–363.

6. Dottori, M., Nobili, L., Caneva, F. D., Paterlini, F., Perini, S., 2000: Evaluation of two different serological methods for determining the presence of antibodies to *Chlamydia psittaci* in the sera pigeons *(Columba livia). Selez. Vet.,* 6, 381–385.

7. Everett, K. D., Bush, R. M., Andersen, A. A., 1999: Amended description of the order *Chlamydiales,* proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae,* including a new genus and five new species, and standards for the identification of organisms. *Int. J. Syst. Bacteriol.,* 49, 415–440.

8. Haag-Wackernagel, D., 2005: Feral pigeons *(Columba livia)* as potential source for human ornithosis. In *Proceedings of the 3rd Workshop for Diagnosis and Pathogenesis of Animal Chlamydioses,* **Cevenini, R., Sambri, V.** (eds.), Bolonia University Press, Siena, Italy, 15–16.

9. Haag-Wackernagel, D., Moch, H., 2004: Health hazards posed by feral pigeons. *J. Infect.,* 48, 307–313.

10. Harkinezhad, T., Verminnen, K., Buyzere, M. D., Rietzschel, E., Bekaert, S., Vanrompay, D., 2009: Prevalence of *Chlamydophila psittaci* infections in a human population in contact with domestic and companion birds. *J. Med. Microbiol.,* 58, 1207–1212.

11. Heddema, E. R., Sluis, S., Buys, J. A., Vandenbroucke-Grauls, C. M. J. E., Wijnen, J. H., Visser, C. E., 2006: Prevalence of *Chlamydophila psittaci* in fecal droppings from feral pigeons in Amsterdam. *The Netherlands, Appl. Environ. Microbiol.,* 72, 4423–4425.

12. Janiga, M., 1989: The study of plumage polymorphism and ornithosis in feral pigeons *(Columba livia).* In *Proceedings of International Symposium of the Working Group on Granivorous Birds,* **Cevenini, R., Sambri, V.** (eds.), INTERCOL, Poland, 33–44.

13. Kociánová, E., Řeháček, J., Lisák, V., 1993: Transmission of antibodies to *Chlamydia psittaci* and *Coxiella burnetii* through eggs and "Crop milk " in pigeons. *Eur. J. Epidemiol.,* 9, 209–212.

14. Laroucau, K., Mahe, A. M., Bouillin, C., Deville, M., Gandouin, C., Touati, F. *et al.,* 2005: Health status of free-living pigeons in Paris. In *Proceedings of the 3rd Workshop for Diagnosis and Pathogenesis of Animal Chlamydiosis,* **Cevenini, R., Sambri, V.** (eds.), Bolonia University Press, Siena, Italy, 17–18.

15. Lennette, A. S., Schmid, N. J., 1974: *Laboratory examining methods of viral and ricketsiae infections* (In Czech). Avicenum, Prague, 375 pp.

16. Magnino, S., Haag-Wackernagel, D., Geigenfeind, I., Helmecke, S., Dovč, A., Prukner-Radovčić, E. *et al.,* 2009: Chlamydial infections in feral pigeons in Europe: Review of data and focus on public health implications. *Vet. Microbiol.,* 135, 54–67.

17. Meyer, K. F., 1941: Pigeons and barn yard fowls as possible sources of human psittacosis or ornithosis. *Schweiz. Med. Wochenschr.,* 44, 1377–1379.

18. Pavlak, M., Vlahovič, K., Gregurič, J., Županičič, Z., Jerčič, J., Božikov, J., 2000: An epidemiologic study of Chlamydia sp. in feral pigeons *(Columba livia* var. *Domestica). Zeitschr. Jagd.,* 46, 84–95.

19. Pospíšil, R., Čisláková, L., Baloghová, D., Čarnická, A., 1988: Ornithosis in restorers of St. Elisabeth Cathedral in Košice. *Prac. Lék.,* 40, 246–248.

20. Řeháček, J., Brezina, R., 1976: Ornithosis in domestic pigeons gone wild in Bratislava. *J. Hyg. Epidemiol.,* 20, 252–253.

21. Řeháček, J., Kocianová, E., Brezina, R., 1984: Potential importance of urban populations of pigeons *Columba livia* f. *domestica* in spreading *C. burnetii* and *Ch. psittaci* in Bratislava (In Slovak). *Biol.,* 39, 293–300.

22. Salinas, J., Caro, M. R., Cuello, F., 1993: Antibody prevalence and isolation of *Chlamydia psittaci* from pigeons *(Columba livia). Avian Dis.,* 37,523–527.

23. Sever, J. L., 1962: Application to microtechnique to viral serological investigations. *J. Immunol.,* 88, 320–328.

24. Schettler, E., Fickel, J., Hotzel, H., Sachse, K., Streich, W. J., Wittstatt, U. *et al.,* 2003: Newcastle disease virus and *Chlamydia psittaci* in free-living raptors from eastern Germany. *J. Wildl. Dis.,* 39, 57–63.

25. Schwarzová, K., Betáková, T., Neméth, J., Mizáková, A., 2006: Detection of Borrelia burgdorferi sensu lato and *Chlamydia psittaci* in throat and cloacal swabs from birds migrating through Slovakia. *Folia. Microbiol.,* 51, 653–658.

26. Tanaka, C., Miyazawa, T., Watarai, M., Ishiguro, N., 2005: Bacteriological survey of feces from feral pigeons in Japan. *J. Vet. Med. Sci.,* 67, 951–953.

27. Trávniček, M., Čisláková, L., Deptula, W., Stosik, M., Bhide, M., 2002: Wild pigeons and pheasants – a source of *Chlamydophila psittaci* for humans and animals. *Ann. Agric. Environ. Med.,* 9, 253–255.

28. Vlahović, K., Matica, B., Bata, I., Pavlak, M., Pavičić, Ž., Popović, M. *et al.,* 2004: Campylobacter, salmonella and chlamydia in free-living birds of Croatia. *Eur. J. Wildl. Res.,* 50, 127–132.

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AGE RELATED CHANGES IN THE CYTOMORPHOLOGY OF THE SEMINIFEROUS EPITHELIUM IN ASSAM GOAT (*CAPRA HIRCUS*) FROM BIRTH TO 10 MONTHS OF AGE

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ABSTRACT

This work was conducted on eighteen male Assam goats of various ages from birth to 10 months of age. The goats were divided into six age groups based on their postnatal ages; *viz.* group I (day 0), group II (2 months), group III (4 months), group IV (6 months), group V (8 months) and group VI (10 months).The seminiferous tubules did not have lumina up to the age of 2 month s (group II), hence they were called the sex cords and these contained centrally placed gonocytes and peripherally located sustentacular cells. The initiation of spermatogenesis began in 4 month old kids (group III) with the appearance of spermatogonia in the seminiferous tubules. The luminization process was completed by 6 month of age (group IV) with all the seminiferous tubules having well developed lumina at this age. These seminiferous tubules contained all the spermatogenic cells of the adult testis; *viz*. type A and B spermatogonia, primary spermtocytes at various stages of meiotic development (leptotene, zygotene, pachytene and diplotene), as well as round and elongated spermatids. The onset of puberty was observed to be established at 6 month of age in Assam goats as evidenced by the presence of spermatozoa adhering to the adluminal border of the Sertoli cells as well as in the tubular lumen. The histological characteristics of the spermatogenic and Sertoli cells were identical to other domestic animals.

Key words: age related changes; Assam goat; cytomorphology; seminiferous epithelium

INTRODUCTION

India possesses 122.92 million goats of which almost 3 million are found in Assam (3). Goats plays an important role in the socio-economic condition of the rural people of the North-Eastern region of India as they are endowed with short generation intervals, high rates of prolificacy and ability to be sustained on sparse vegetation and under extreme climatic conditions. Goat rearing has tremendous potential in the North-eastern states particularly among the small and marginal farmers and landless laborers because of the very low initial investment and adequate financial returns. This region has abundant natural grasses, pastures, shrubs and forests due to widespread rainfall. More than 85 per cent of the population in this region is non-vegetarian and chevon (goat meat) is preferred by all as it has no religious taboo. Therefore, it is important to know the normal anatomical growth and development characteristics of the male genital system at various ages, particularly the testis and its tubular system.

A few anatomical studies on testes have been reported in goats: *viz.* post natal development of testes in Malabari goat from 4 to 11 months of age (6); biometry of the testes in Sirohi goats (19); testicular measurements in Assam local X Beetal goats (21); and testicular growth in British Saanen, Alpine and Toggenburg breeds of bucks (1). Some works were also conducted in other ruminants, elucidating the morphology and biometry of the testes such as buffalos (20) and rams (24).

The postnatal development of the testis is associated with variation in the morphology in different cell types of the seminiferous epithelium (5). No work has been conducted to study the cytomorphology of the seminiferous epithelium at various ages from birth to 10 months of age in Assam goats, which prompted this present investigation. This work is the first of its kind in the native goat of Assam.

MATERIALS AND METHODS

A total of 18 male Assam goats varying in age from day 0 to 10 months were used in this study. The animals were divided into six age groups; *viz.* group I (day 0), group II (2 months), group III (4 months), group IV (6 months), group V (8 months) and group VI (10 months) consisting of three animals in each group having the same age. There is no particular breeding season in Assam goats, however, these goats show a tendency of relatively increased breeding behavior during the autumn season. The experimental goats used in this study were born in the same season. The ages of the goats were known from their birth records. Each animal was weighed using a spring balance to record the body weight. The animals were sedated by giving an intramuscular injection of Siquil (triflupromazine hydrochloride) at a dose of 1 $mg.kg^{-1}$ body weight and subsequently anaesthetized by administering an intravenous injection of Intravel sodium (pentobarbital sodium) at a dose of 15 $mg.kg^{-1}$ body weight (12). After induction of the proper level of anesthesia, the animals were sacrificed.

Tissue pieces were collected from three different regions of the testis; *viz*., upper, middle and lower and subsequently fixed in Bouin's solution prepared as per (18). All the tissues were processed for paraffin sections (18) by the alcohol-xylene method using cedar wood oil. Sections were cut at 5 μ thickness using a rotary microtome (Thermo, Germany) and stained by Haematoxylin and Eosin (8) and PAS-Haematoxylin method for nuclear staining (13).

RESULTS

In the animals of groups I and II, no lumen was found in the seminiferous tubules, hence these were called sex cords (Fig. 1). These solid sex cords contained peripheral primitive Sertoli cells and large centrally placed germ cells located in an eosinophilic material (Fig. 2). The gonocytes were large cells with a spherical nucleus of homogenous nucleoplasm. They showed variable degrees of degeneration in the form of vacuolated cytoplasm and different stages of karyolysis. The primitive Sertoli cells were polygonal or cone shaped cells with light and relatively homogenous cytoplasm. The nuclei of these primitive Sertoli cells showed heterochromatin material with infoldings of the nucleolemma. Nucleoli were not distinct. At this stage, these cells outnumber the gonocytes.

The type A spermatogonia (Fig. 3) were large oval or elliptical shaped cells located close to the basement membranes of the seminiferous tubules. They had large oval nuclei containing uniform chromatin material. The nucleoli were located at random within the nucleus and their numbers were usually more than one. The cytoplasmic periphery of the cell was weakly eosinophilic and the long axis of the cell was placed parallel to the basal lamina. The type B spermatogonia were proportionately rounded cells with distinct spherical nuclei. The chromatin substance was observed as distinct clumps which usually adhered to the nuclear membrane. The nucleoli were located at the centre of the nucleus but deviations were also noted. The cytoplasm was lightly eosinophilic (Fig. 3).

The meiotic phases of the primary spermatocytes were recognized in the kids from 4 month of age (group III) onwards. The leptotene primary spermatocytes were uniformly rounded cells with spherical nuclei. The chromatin substance was organized to form a dense filamentous network which filled the nucleus completely. The cytoplasm was lightly stained. The nuclei were in a distinct spheroid shaped (Fig. 4).

The zygotene primary spermatocytes were marked by their deeply stained nuclei, which were usually notched, but occasionally rounded in shape. The chromatin substance ap-

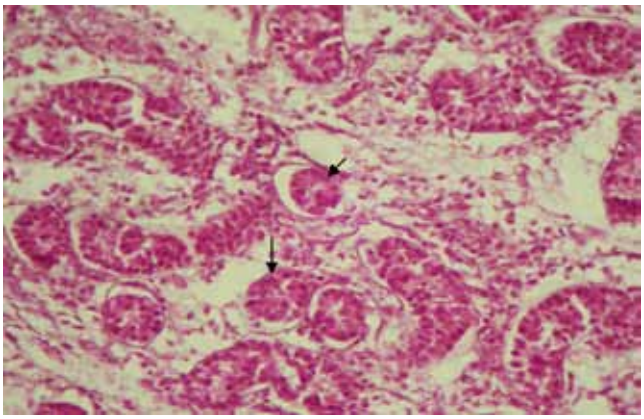

Fig. 1. Photomicrograph of the testis of a day-old kid (group I) showing the sex cords (arrows). H & E. Magn. × 400

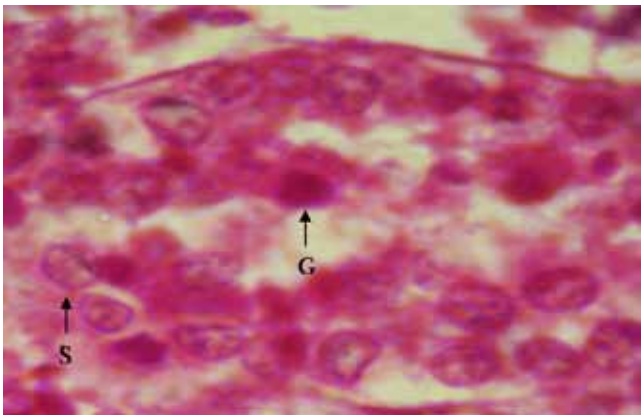

Fig. 2. Photomicrograph showing the gonocytes (G), sustentacular or Sertoli cells (S) and the basement membrane of the sex cords in the testis of a day-old (group I) male kid. H & E. Magn × 1000

peared coarse. The nuclei were ill-defined and the nuclear envelope was indistinct. The cytoplasm of these cells was pale stained (Fig. 4).

The pachytene primary spermatocytes were characterized by large spherical cells and their nuclei were uniformly spheroid. The chromatin material appeared in a coarse filamentous arrangement leaving irregular interstices in the nucleoplasm. The nuclear envelope was indistinct and nucleoli were small and faintly stained. The cytoplasmic rim was comparatively broad and faintly stained (Fig. 4).

The diplotene primary spermatocytes were characterized by the largest size of the spermatogenic series. They were large, rounded cells with spherical nuclei. The chromatin material was more loosely arranged. They were stained lighter than the pachytene nuclei. The nuclear membrane was indistinct and the nucleoli were rarely perceptible. The cytoplasmic rim surrounding the nucleus was weakly eosinophilic (Fig 5).

The secondary spermatocytes were smaller in size than the primary spermatocytes but larger than the round spermatids. They were round in shape and their nuclei were spherical with centrally placed small dot like clumps of chromatin interconnected with a fine filamentous network. The nuclear membrane was distinct with indistinct nucleoli. The cytoplasm was scant and eosinophilic. These cells were seen in the goats from 6 month of age (group IV) onwards.

The round spermatids (Fig. 5) were the smallest cells of the spermatogenic series. They possessed spherical nuclei and had a thin peripheral cytoplasmic rim. The chromatin substance appeared granular with 2 to 3 larger irregular aggregates which stained more intensely than the surrounding nuclear material. The nuclear membrane was thin and prominent.

The elongated spermatids (Fig. 3) were characterized by elongated nuclei with condensed chromatin. They stained more deeply and either remained attached to the Sertoli cell cytoplasm or migrating centripetally towards the lumina of the seminiferous tubules.

The spermatozoa (Fig. 4) were usually found attached to the Sertoli cells at their ad luminal border and in the lumen

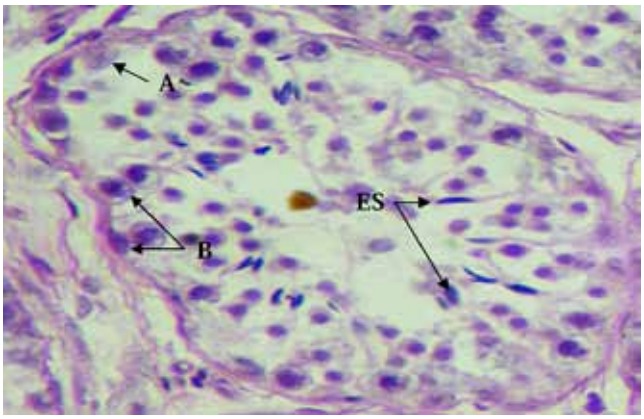

Fig. 3. Photomicrograph showing type-A spermatogonia (A), type-B spermatogonia (B) and elongated spermatids (ES) in the seminiferous epithelium of the testis of an eight months old (group V) goat. H & E. Magn × 400

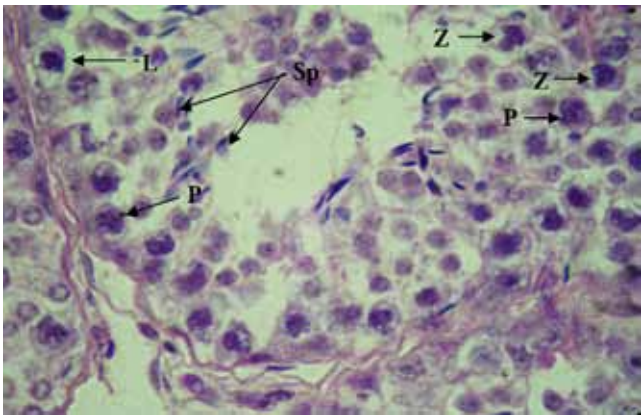

Fig. 4. Photomicrograph showing leptotene (L), zygotene (Z), pachytene (P) primary spermatocytes and spermatozoa (Sp) in the seminiferous epithelium of the testis of a six months old (group IV) goat. H & E. Magn. × 1000

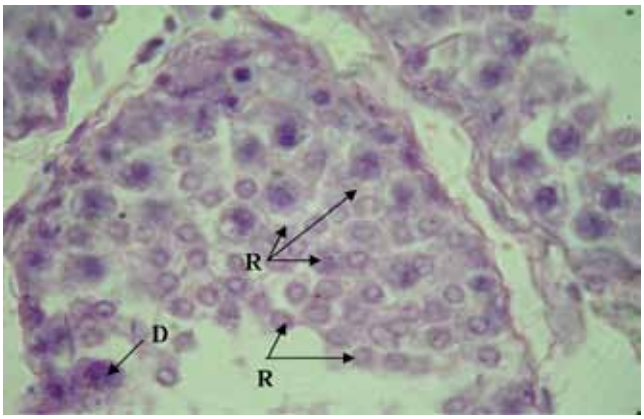

Fig. 5. Photomicrograph of the testis in a six months old (group V) goat showing deplotene (D) primary spermatocytes and round spermatids (R) in the seminiferous epithelium. H & E. Magn. × 1000

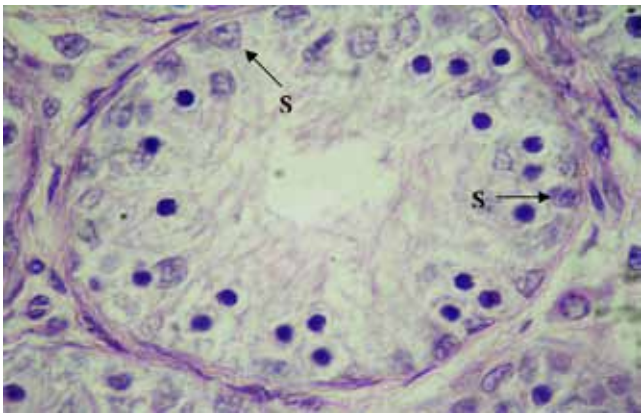

Fig. 6. Photomicrograph of the testis in a four months old (group III) male goat showing Sertoli cell nuclei (S) in the seminiferous epithelium. H & E. Magn. × 1000

of the seminiferous tubules after their release from the Sertoli cells. The head was elongated-oval in shape. The spermatozoa were first seen in the seminiferous tubules in the male Assam goats at 6 months of age (group IV) in this study.

The sustentacular cells (Sertoli cells) (Fig. 6) were elongated pyramidal cells whose broad base rested on the basement membrane. The nuclei were spherical or pear shaped and were located near the basement membrane of the seminiferous tubules. The centrally placed nucleolus was large and intensely stained. The chromatin material of the nucleus was fine, evenly distributed and lightly stained. The cellular outlines of the Sertoli cells were not distinct. As the process of spermatogenesis was fully established in the 6 months old bucks (group IV) onwards, the morphological maturation of the Sertoli cells was noticed which included: the movement of nucleus to the basal portion of the cell; the nuclear membrane became folded with the development of a prominent nucleolus; and proliferation of organelles as evidenced from darkly stained masses in the basal region of the Sertoli cells.

DISCUSSION

Based on the distribution pattern of the chromatin and the size of the spermatogonia, type-A and type-B spermatogonia were recognized. Similar types of spermatogonia were also reported in the rat (9), Dwarf Nigerian ram (2), stallion (25), bulls (11) and goat (7). The intermediate type of spermatogonia were not classified in the present study as the criteria of these cells having irregular distribution of numerous Fuelgen positive chromatin granules in ovoid nucleus and their attachment to the nucleoli and nuclear membrane did not hold good in goat as also observed by Bordooi and Dhingra (7).

Prior to the transformation of gonocytes into spermatogonia, an intermediate pre-spermatogonial stage occurred which had been ascertained as "quiescent" (16). The transformation of the gonocytes into spermatogonia was considered as the initiation of spermatogenesis (14). The first appearance of spermatogonia in the seminiferous tubules was an important factor. In this study, the transformation of gonocytes to spermatogonia was observed in the seminiferous epithelium at 4 month of age (group III) indicating the initiation of spermatogenesis at this age in the Assam goat. However, Baishya *et al.* (4) recorded the initiation of spermatogenesis at 69 days of age in Assam goats, which was much earlier as compared to the 4 month of age (group III) observed in our study. At 2 and 4 months of age, the possibity of the initiation of spermatogenesis after 2 months and prior to attaining 4 months of age could not be assertained in the present work. The age of the initiation of spermatogenesis was reported to be variable in different animals, which was recorded as 70 days in Dwarf Nigerian lambs (2), 150 days in rams (24), 120 days in Brown Swiss crossbred bulls (25) and 350 days in buffalos (15).

Our work confirmed the first occurrence of spermatozoa attached to the ad luminal border of the Sertoli cells or in the lumina of the seminiferous tubules at 6 month of age (group IV), indicating the onset of puberty at this age in male Assam goats. The first occurrence of spermatozoa had been reported at 18 weeks of age in Dwarf Nigerian rams (2) and 165 days in native rams of Iran (22). The initiation of puberty as evidenced by the presence of sperm cells in the ejaculated semen was found to be at 29 weeks in Beetal X Assam local male kids (17).

The histological characteristics of all the spermatogenic cells recorded in the present study in Assam goats at various ages were in agreement to the earlier findings reported in Dwarf Nigerian rams (2), domestic animals (10), goats (8) and native rams of Iran (22).

The Sertoli cells were elongated pyramidal cells whose broad base rested on the basement membrane of the seminiferous tubules. The nucleoli were absent in the nuclei of these cells at birth (group I), which might be due to their immaturity. The nucleoli were present in the Sertoli cells from 2 month of age (group II) onwards. Again, the morphological maturation of the Sertoli cells was noticed after full establishment of the spermatogenesis process, as also reported in buffalo calves (23).

REFERENCES

1. Ahmad, N., Noakes, D. E., 1996: Sexual maturity in British breeds of goat kids. *Br. Vet. J.*, 152, 93–103.

2. Aire, T. A., 1973: The development of the testis in the dwarf Nigerian ram lamb. *Res. Vet. Sci.*, 14,104–106.

3. Anonymus, 2003: *Statistical Handbook*, Assam, 57–61.

4. Baishya, G., Bhattacharya, M., Roy, T. C., 1987: A correlative note on testis of local kids of Assam. *Indian Vet. J.,* 64, 989–990.

5. Banks, W. J., 1981: *Applied Veterinary Histology*. Williams and Wilkins, Baltimore, 447–483.

6. Bilaspuri, G. S., Singh, K., 1992: Developmental changes in the body weight and testicular characteristics in Malabari goat kids. *Theriogenology*. 37, 507–520.

7. Bordooi, C. C., Dhingra, L. D., 1990: Seasonal studies on the histomorphological status of the seminiferous epithelium of Indian Black Beatle goats. *Indian Journal of Veterinary Anatomy*, 2, 1–7.

8. Bordoloi, C. C., 1979: *Seasonal Variations on the Cytomorphological and Cytochemical Architecture of the Testes in Sexually Mature Male Goats* (*Capra hircus*). PhD thesis, Haryana Agricultural University, Hissar, India, 82–97.

9. Clermont, Y., Perey, B., 1957: Quantitative study of the cell population in the seminiferous tubules in immature rats. *American Journal of Anatomy*, 100, 241–267.

10. Dellmann, H. D., Wrobel, K. H., 1976: Male reproductive system. In *Textbook of Veterinary Histology*. **Dellmann, H. D., Brown, E. M.** (eds.), 2nd edn., Lea and Febiger, Philadelphia, 147–159.

11. Dhingra, L. D., 1977: *The Testis*. **Johnson, A. D., Gomes, W. R., Vandemark, N. L.** (Eds.), Academic Press, New York., 4, 451–458.

12. Hall, L. W., Clarke, K. W., Trim, C. M., 2000: *Wright's Veterinary Anesthesia and Analgesia*. 10th edn., ELBS and Bailliere Tindall, London, 221–237.

13. Humason, G. L., 1967: *Animal Tissue Techniques*. W. H. Freeman and Company, San Francisco, Calif., 304–312.

14. Gondos, B., Rensten, R. M., Goldstein, D. A., 1977: Postnatal differentiation of Leydig cells in the rabbit testis. *American Journal of Anatomy*, 145, 167–174.

15. Goyal, H. O., Dhingra, L. D., 1973: A study on the postnatal histology of the testis in buffalo *(Bubalus bubalis)* from birth to one year of age. *Acta Anat.*, 84, 237–250.

16. Guraya, S. S., Bilaspuri, G. S., 1976: Spermatogenic cells of the buffalo *(Bubalus bubalis). Indian J. Anim. Sci.*, 46, 388–395.

17. Kakoti, D., 1999: *Studies on Body Measurements, Testicular Biometry, Age at Puberty, Sexual Behaviour and Semen Characteristics in Beetal X Assam Local Male Kids*. M.V.Sc. thesis, Assam Agril. University, Khanapara, Guwahati, India, 57–68.

18. Luna, L. G., 1968: *Manual of Histological Staining Methods of Armed Forces Institute of Pathology*. 3rd edn., McGraw Hill Book Co., New York., 153–173.

19. Mishra, R. K., Jain, R. P., Basuthakur, A. K., 1984. Testis biometry as a basis of selection of body weights in kids. *Indian J. Anim. Sci.* 54, 263–266.

20. Pal, C., Bhardwaj, M. M. L., 1983: Morphology and biometry of the testis in Indian buffalo. *Philippine Journal of Veterinary Medicine*., 22, 7–12.

21. Sarmah, B. K., Dutta, A., Dutta, D. J., Goswami, R. N., Sarmah, B. C., 1998: Testicular development during postnatal life of crossbred buck. *Indian J. Anim. Sci*., 68, 1173–1174.

22. Schahidi, R., Smidt, D., 1980: Investigation on the onset of sexual maturity in native rams in the province of Khuzestan in Iran. *Deutsche Tieraztliche Wochenschrift*, 87, 130–132.

23. Singh, P., 1996: *Histological and Histoenzymic Studies on the Postnatal Development and Differentiation of the Seminiferous Epithelium and Leydig Cell in Buffalo Testis*. M.V.Sc. thesis, Punjab Agricultural University, Ludhiana, 112–139.

24. Sergeev, N. I., Zaboloskii, V. A., 1976: Age changes in the development of the rete testis in rams. *Doklady Vsesoyuznoi Akademii Sel'skokhozyaistvennykh Nauk*, 12, 26–28.

25. Swierstra, E. E., Gebauer, M. R., Pickett, B. W., 1974: Reproductive Physiology of the bull. 1. Spermatogenesis and testis composition. *J. Reprod. Fertil.*, 40, 113–123.

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BIOCHEMICAL PARAMETERS OF LIVER AND KIDNEY FUNCTIONS IN RATS EXPERIMENTALLY EXPOSED TO LEAD

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ABSTRACT

In order to evaluate the liver and kidney functions of rats exposed to lead as lead acetate, twenty four male Wistar rats were divided into four different groups. The rats of group A received only deionised water while those of groups B, C and D were given 200 ppm, 300 ppm and 400 ppm lead as lead acetate in the drinking deionised water respectively for four weeks. There were significant increases in the liver biomarker enzymes activities such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), while there were significant decreases in the protein, albumin and globulin of rats exposed to lead. Also there were significant increases in the kidney biomarkers such as creatinine and blood urea nitrogen (BUN) ($P < 0.05$). Therefore it is concluded that exposure to lead could result in the impairment of both the kidney and liver functions.

Key words: ALT; AST; BUN; creatinine; liver and kidney functions; GGT

INTRODUCTION

Lead is widely recognized as a long-lived and persistent environmental toxicant. It is a persistent and toxic xenobiotic, as are other xenobiotics which can induce different health risks from the foetal stage until senescence (2). Several reports have indicated that lead can cause neurological, haematological, gastrointestinal, reproductive, circulatory and immunological pathologies in which all of them are related to the dose and the amount of time of lead exposure (1, 11, 12).

The liver plays a major role in the metabolism of lead. The liver is particularly subjected to special risks, due to the oxidative action of this xenobiotic. This is because there is unquestionable evidence that lead-induced lipid peroxi dation of cellular membranes plays a crucial role in the mechanisms of the hepatotoxic action by this xenobiotic (15). On the other hand, lead is also known to adversely affect the kidney, which is another important target organ of lead (6). Lead produces oxidative damage in the kidney as evidenced by enhancing lipid peroxidation (4, 5).

Heavy metal poisoning, like lead cause adverse effects to hepatic cells because after lead exposure, the liver is one of the organs involved in the storage, biotransformation and detoxication. Lead also affects the kidney, which is another important organ that participates in the detoxication.

The aim of the current study was to evaluate the effect of different dose level of lead acetate on the liver and the kidney using some biomarkers as indications of lead toxicity.

MATERIALS AND METHODS

The study was carried out on twenty four (24) male Wistar rats. The average weight of the rats was 120 ± 3.50 g. The rats were randomly divided into four (4) groups designated A, B, C and D with six (6) rats per group. The animals were maintained under standard laboratory conditions and were fed dry ration ad libitum (fat/oil 6 %, crude fibre 5 %, calcium 1 %, available phosphorus 0.4 %, lysine 0.85 %, methionine 0.35 %, salt 0.3 %, crude protein 18 %, metabolisable energy 2 900 Kcal.kg^{-1}, manufactured by TOPFEEDS®, Lagos, Nigeria). Animals of groups B, C and D were given lead as lead acetate ($(CH_3.COO)_2Pb.3H_2O$, assay (ex Pb) 99–103 %, maximum

limits of impurities, chloride (Cl) 0.005 %, copper (Cu) 0.002 %, a product of Cartivalues, England at 200 ppm, 300 ppm and 400 ppm in deionised drinking water respectively while group A (Control) received only deionised water for four (4) weeks.

After four weeks of lead exposure, blood samples were collected from the eye vein into heparinised capillary tubes.

Biochemical assessment

The following biochemical parameters were evaluated colorimetrically using biochemical kits supplied by Randox® Laboratory Limited, United Kingdom. These kits were as follows: aspartate aminotransferase (AST); alanine aminotransferase (AST); alkaline phosphatase (ALP); gamma glutamyl transpeptidase (GGT); creatinine; blood urea nitrogen (BUN); total protein; albumin; and globulin.

Statistical analysis

The values are expressed as the mean ± standard error. The mean values were compared by one way analysis of variance (ANOVA) and Duncan's range test. The significance of the differences among treatment groups to all statements of significance were based on the probability of $P < 0.05$.

RESULTS

The effects of lead toxicity on AST, ALT, ALP, GGT, creatinine, BUN, total protein, albumin and globulin are shown in Table 1.

Evaluation of liver function indicators: there were significant increases ($P < 0.05$) in the activities of ALT, AST, ALP and GGT of the treated groups compared to the control group, while there were significant decreases in the total protein, albumin and the globulin of the treated groups compared to the control group.

Evaluation of the kidney function indicators: there were significant increases in the creatinine and blood urea nitrogen (BUN) of the treated group compared to the control group.

DISCUSSION

The liver performs multiple diverse functions essential to life, exemplified by activities such as synthesis, excretion and detoxification among others. From our results we noticed an increase in the activities of the liver enzymes such as ALT, AST, ALP and GGT; this is an indication of the hepatotoxic effects of lead. This could be due to the increased cell membrane permeability or cell membrane damage of hepatocytes caused by lead. These findings are in accordance with the literature (14, 18). The increase in plasma activities of ALT, AST, ALP and GGT as marker of hepatocellular damage indicate some impairment of liver function. It has been reported that the serum activity of ALT was elevated significantly more than AST on lead exposure which indicated liver damage and the development of fibrosis (10, 14). The increase in alkaline phosphatase might be due to the damage of liver, kidney and bone resulting in the liberation of alkaline phosphatase (8). This observation is in agreement with the findings of Shalan *et al.* (14). The increase in GGT is an indication of hepatotoxicity and oxidative damage in the hepatocyte (18). The increase in AST and ALT with a significant decrease in the total protein concentration, is in agreement with Swarup *et al.* (17). The elevation in transaminases activities and the decrease in total protein concentration may be attributed to the liver injury. Therefore, the exposure to

Table 1. The effects of lead toxicity on aspartate aminotransferase (AST), alanine aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), creatinine, blood urea nitrogen (BUN), total protein, albumin and globulin

Parameters	Group A	Group B	Group C	Group D
ALT ($IU.l^{-1}$)	60.15 ± 1.34^{a}	65.21 ± 1.10^{b}	79.10 ± 1.24^{c}	85.11 ± 1.29^{d}
AST ($IU.l^{-1}$)	97.18 ± 1.13^{a}	99.79 ± 1.34^{a}	105.03 ± 1.03^{b}	112.01 ± 1.22^{c}
ALP ($IU.l^{-1}$)	69.54 ± 1.12^{a}	72.27 ± 1.04^{a}	83.12 ± 1.10^{b}	97.13 ± 1.12^{c}
GGT ($IU.l^{-1}$)	7.16 ± 0.13^{a}	9.11 ± 0.21^{b}	12.56 ± 0.33^{c}	16.13 ± 0.14^{d}
Creatinine ($mg.dl^{-1}$)	0.65 ± 0.034^{a}	0.97 ± 0.02^{b}	1.04 ± 0.13^{c}	1.13 ± 0.06^{c}
BUN ($mg.dl^{-1}$)	16.13 ± 1.34^{a}	17.42 ± 1.0^{4a}	19.57 ± 1.21^{b}	22.35 ± 1.28^{c}
Total protein ($g.dl^{-1}$)	8.66 ± 0.02^{a}	8.52 ± 0.04^{a}	7.23 ± 0.01^{b}	6.52 ± 0.03^{c}
Albumin ($g.dl^{-1}$)	5.24 ± 0.03^{a}	4.59 ± 0.02^{a}	3.68 ± 0.02^{b}	3.19 ± 0.04^{b}
Globulin ($g.dl^{-1}$)	3.42 ± 0.01^{a}	3.93 ± 0.02^{a}	3.55 ± 0.01^{b}	3.33 ± 0.02^{b}

Values in the same column with different superscript differ significantly ($P < 0.05$)

lead results in the alteration of serum biochemical parameters indicative of liver functions.

The observed decrease in the protein, albumin and globulin content of the plasma of the rats exposed to lead acetate may be due to a decrease in the hepatic DNA and RNA (14). This is similar to the observations of Stone and Soares (16) who reported a decrease in the total protein which may be due to the binding of lead to albumin. A decrease in the hepatic total protein content in response to lead intoxication was also reported by Goswani *et al.* (7). The observation of a lower mean plasma levels of total protein and albumin in the exposed than in the control is consistent with the trend observed in liver diseases involving the impaired synthetic function of the liver (9). The liver is the primary site of the synthesis of plasma proteins; hence disturbances of protein synthesis therefore occur as a consequence of impaired hepatic function which will lead to a decrease in their plasma protein concentrations. The lower plasma albumin level of the exposed rats in this study therefore may indicate a reduction in the synthetic function of the liver.

Blood urea nitrogen (BUN) and creatinine was observed to be increased in the treated groups. This might be due to the loss of kidney function and considered as functional evidence of lead induced nephrotoxicity (13). The increase serum urea and creatinine have been used as important indices for the elevation of the effects of chemicals on the kidney (3). The presence of increasing urea and creatinine concentration in the blood suggest the inability of the kidney to excrete these products, which further suggest a decrease in glomerular filteration rate. Significant increases in the urea and creatinine which are indicative of abnormal kidney functions agreed with the results of Goswani and Gachhui (7).

In conclusion, the exposure to lead posses the potential to induce hazardous biological effects in rats. The changes in liver and kidney functions as shown by the altered biochemical markers illustrate the hepatotoxic and nephrotoxic effects of lead on biological tissues.

REFERENCES

1. Abam, E., Okediran, B.S., Odukoya, O.O., Adamson, I., Ademuyiwa, O., 2008: Reversal of ionoregulatory disruptions in occupational lead exposure by vitamin C. *Environ. Toxicol. Pharmacol.,* 26, 297–304.

2. Annabi, B.A., Nehdi, A., Hajjaji, N., Gharbi, N., El-Fazaa, S., 2007: Antioxidant enzymes activities and bilirubin level in adult rat treated with lead. *Comp. Ren. Biol.,* 330, 581–588.

3. Davis, M.E., Berndt, W.D., 1994: Renal methods for toxicology. In **Hayes, A.W.** (Eds.): *Principles and Methods of Toxicology,* 3rd edn., New York, Raven, 871–894.

4. El-Nekeety, A. A., El-Kaddy, A. A., Soliman, M. S., Hassan, N. S., Abdel-Wahhab, M. A., 2009: Protective effect of *Aquilegia vulgaris* (L) against lead acetate induced oxidative stress in rats. *Food Chem. Toxicol.,* 47, 2209–2215.

5. Farrag, A.R., Mahdy, K.A., Abdel Rahman, G.H., Osfor, M.M., 2007: Protective effect of Nigella sativa seeds against lead-induced hepatorenal damage in male rats. *Pak. J. Biol. Sci.,* 10, 2809–2816.

6. Garcon, G., Leleu, B., Marez, T., Zerimech, F., Haguenover, J.M., Furan, D., Shirali, P., 2007: Biomonitoring of the adverse effects induced by the chronic exposure to lead and cadmium on kidney function: usefulness of alpha gluthathione-S-transferase. *Sci. Total Environ.,* 377, 165-172.

7. Goswani, K., Gachhui, R., Bandopadhyay, A., 2005: Hepatorenal dysfunction in lead pollution. *J. Environ. Sci. Eng.,* 47, 75–80.

8. Kaplan, M.M., Reghetti, A., 1970: Induction for rat liver alkaline phosphatase: the mechanism of serum elevation in bile obstruction. *J. Clin. Invest.,* 49, 508–516.

9. Keith, G., Tolman, M.D., Robert, R., 1999: Liver function. In **Burtis, C.A., Ashwood, E.R.** (Eds.): *Tietz Textbook of Clinical Chemistry,* 3rd edn., W. B. Saunders Company, Philadelphia, 1125–1177.

10. Latner, A.L., 1975: In **Contarow, T.,** (Ed): *Clinical Biochemistry.* Sunders, Philadelphia, 10–12.

11. Okediran, B.S., Ajibola, E.S., Thomas, F.C., Abam, O.E., Biobaku, K.T., Olaniyi, M.O., Rahman, S.A., 2010: Investigation on subchronic lead intoxication on blood indices of male rats. *Global Veterinaria,* 4, 532–535.

12. Patrick, L., 2006: Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. *Altern. Med. Rev.* 28, 33–39.

13. Qu, W., 2002: The metallothionein-null phenotype is associated with heightened sensitivity to lead toxicity and an inability to form inclusion bodies. *Am. J. Pathol.,* 160, 1047–1057.

14. Shalan, M.G., Mostafa, M.S., Hasouna, M.M., Nabi, S.E., Refaeie, A., 2005: Amelioration of lead toxicity on rat liver with vitamin C and silymarin supplements. *Toxicology,* 206, 1–15.

15. Sivaprad, R., Nagraj, M., Vanalakshm, P., 2004: Combined efficacies of lipoic acid and 2, 3-dimercaptosuccinic acid against lead induced lipid peroxidation in rat liver. *J. Nutr. Biochem.,* 15, 1823.

16. Stone, C.L., Soares, J.H., 1976: The effect of dietary selenium level on lead toxicity in the Japanese quail. *Poultry Sci.,* 55, 341–349.

17. Swarup, D., Patral, R.C., Naresh, R., Kumar, P., Shekhar, P., 2005: Blood lead levels in lactating cows reared around polluted localities, transfer of lead into milk. *Sci. Total Environ.,* 347, 106–110.

18. Tatjana, J., Gordana, K., Dusica, P., Ivana, S., 2003: Effects of captopril on membrane associated enzymes in lead induced hepatotoxicity in rats. *Acta. Fac. Med. Naiss,* 20, 183–183.

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2. Black, H., Duganzich, D., 1995: A field evaluation of two vaccines against ovine pneumonic pasteurellosis. *New Zeal. Vet. J.,* 43, 60–63.

3. Brown, L. W., Johnson, E. M., 1989: Enzymatic evidence of alkaline phosphatase. In **Caster, A. R.:** *Enzymology*. Plenum Press, New York, 99–101.

4. Ikuta, K., Shibata, N., Blake, J. S., Dahl, M. V., Nelson, R. D., Hisamichi, K. *et al.*, 1997: NMR study of the galac-tomannaus of *Trichophyton mentagrophytes* and *Trichophyton rubrum. Biochem. J.,* 323, 297–305.

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ACKNOWLEDGEMENT

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