FOLIA VETERINARIA

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

ISSN 0015-5748

2

XLVIII • 2004

UNIVERSITY
OF VETERINARY MEDICINE
IN KOŠICE

EDITORIAL BOARD

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

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

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

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

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

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

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

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

Tlač: **EMILENA**, Čermeľská 3, 040 01 Košice
Sadzba: **Aprilla,** s.r.o., Hlavná 40, 040 01 Košice

Registr. zn. 787/93

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

Indexed and abstracted
in AGRIS, CAB Abstracts

FOLIA VETERINARIA, 48, 2, 2004

CONTENTS

TESFAYE, A.: External anatomical body structures in practical use (A Review) **53**

SOBEKOVÁ, A., HOLOVSKÁ, K., jr., LENÁRTOVÁ, V., HOLOVSKÁ, K., PRISTAŠ, P., JAVORSKÝ, P.: The Effect of plant antioxidants on the elimination of environmental stress stimulated by mercury in rumen bacterium *Selenomonas ruminantium* 62

FIALKOVIČOVÁ, M., BENKOVÁ, M., KOZÁK, M., ŠKARDOVÁ, I., VALOCKÝ, I., SESZTÁKOVÁ, E., BOLDIŽÁR, M.: An additional method of distinguishing between hypothyroidism and euthyroid sick syndrome **66**

HROMADA, R., MOJŽIŠOVÁ, J., POŠIVÁKOVÁ, S., POŠIVÁK, J., MICHALKOVÁ, Z., BAJOVÁ, V., PAULÍK, Š.: The monitoring of selected parameters of non-specific immunity in dogs with pyoderma (A Preliminary Report) 73

ŠULLA, I., MARŠALA, J., LUKÁČ, I.: A light-microscopis study of a delayed phase of paraplegia induced by ischemia and reperfusion in dogs 76

BULECA, J., jr., URBANČÍKOVÁ, N., BUGARSKÝ, A., ŠVICKÝ, E.: The fundamentals of modern marketing and their influence on veterinary services performance 81

FILKO, J., TAKÁČOVÁ, D., BUGARSKÝ, A.: Honey adulteration 87

DOCIC, A., BILKEI, G.: *Chlamydophila*-infections, a potential cause of abortion in outdoor sows (A Short Communication) 90

ČURLÍK, J., LETKOVÁ, V., CIBEREJ, J., LAZAR, P., GOLDOVÁ, M., KOČIŠOVÁ, A., KOŠUTHOVÁ, L., TRÁVNIČEK, M., BHIDE, M., LAZAR. G., POŠIVAK, J., KONJEVIČ, D.: The occurrence of the genera *Hypoderma*, *Cephenemyia* and *Pharyngomyia in deer in the Slovak Republic (A Short Communication)* *92*

MITIKU, E., ASEFA ASMARE, A.: The prevalence and cause of camel mastitis (*Camelus dromedarius)* in Errer valley, Eastern Ethiopia 95

MARCINČÁK, S., BYSTRICKÝ, P., POPELKA, P., NAGY, J., SOKOL, J.: The determination of malondialdehyde in egg yolk by solid phase extraction and high-performance liquid Cchromatography 100

GLIGIĆ, L. J. , PALIĆ, T., NIKOLOVSKI, J.: The beneficial effects of iron-enriched yeast *Saccharomyces cerevisiae* biomass in poultry breeding 104

KOŽÁROVÁ, I., HUSSEIN, K., MÁTÉ, D., JEVINOVÁ, P.: The high-performance liquid chromatographic determination of sulphadimidine residues in eggs 108

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 53—61, 2004

EXTERNAL ANATOMICAL BODY STRUCTURES IN PRACTICAL USE (A Review)

Tesfaye, A.

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

tesfaye@uvm.sk

ABSTRACT

***Regio presternalis, regio hypochondriaca, processus costarius, regio tuberis coxae, regio articulationis coxae, tuber ischiadicum, regio clunis, regio sacralis, regio glutea, fossa paralumbalis* and *regio analis* are used to evaluate the body condition scores of cattle. Condition scores range from 1, a very thin cow with no fat reserves, to 5, a severely overconditioned cow in a 1—5 scale system for dairy and to 9 in a 1—9 scale system for beef cattle. Ideal condition scores fall in the range of 3.5—4.0 and 5.5—7.0 respectively at dry off and calving and 2.5—3.0 and 5.0—6.0 respectively at peak lactation. The age, the reproduction cycle and the influence of feeding principles on body condition scoring are discussed.**

Key words: anatomical feature; body condition scoring; cattle; fat reserve

INTRODUCTION

Perhaps one of the most important management skills of livestock producers is the ability to score the body condition of their animals and track progress towards meeting a desired degree of fatness to meet a given reproductive goal in a herd.

Body condition scoring (BCS) is an index of the degree of fatness expressed in the anatomical features of an animal that can be viewed with the naked eye (Fig. 1). Essentially, BCS is a systematic process of attempting to visualize the degree of underlying skeletal features that can be detected by observing the animals (11, 40).

Every dairy producer has cattle that are too fat or too thin for their stage of lactation. Failure to recognize these cows and take action costs dearly in disease treatment, lost milk production and decreased fertility (17).

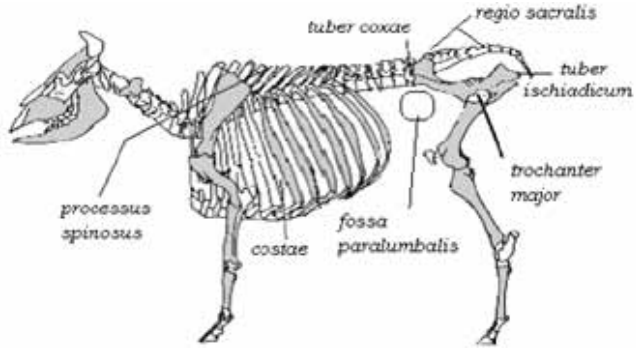

Fig. 1. Important anatomical structures, which indicate the body condition in cattle

Body condition is a reflection of the body fat reserves carried by the animal (Tab. 1). These reserves can be used by the cow in periods when it is unable to eat enough to satisfy its energy needs. In high-producing cows this normally happens during early lactation, but it may also happen when cows get sick, are fed poor quality feeds, or feed intake is restricted (19, 26). After a period of weight loss, cows should be fed more than their requirements to restore normal body condition.

Cows should be scored both visually (Fig. 2) and by palpating the backbone, loin and rump areas (28, 29, 40) (Fig. 3).

Since the pin bone (*tuber coxae*), hipbone (*os ischiadicum*), the top of the backbone (*processus spinosus)* and the ends of the short ribs do not have muscle tissue covering them, any covering you see or feel is a combination of skin and fat deposits (Fig. 3).

Assessing condition by handling is quite easy. Press the fingertips against the backbone, pin bone and hipbone. Grip the loin of the cow where the short ribs project from the

Table 1. Relationship between body condition score and fat storage

Body condition score	Total body fat in %	Subcutaneous fat cover in cm
1	0.7	0
2	5.0	0.01016
3	9.3	0.0127
4	13.7	0.2794
5	18.0	0.4826
6	22.3	0.7366
7	26.7	1.0414
8	31.0	1.3716
9	35.3	1.7272

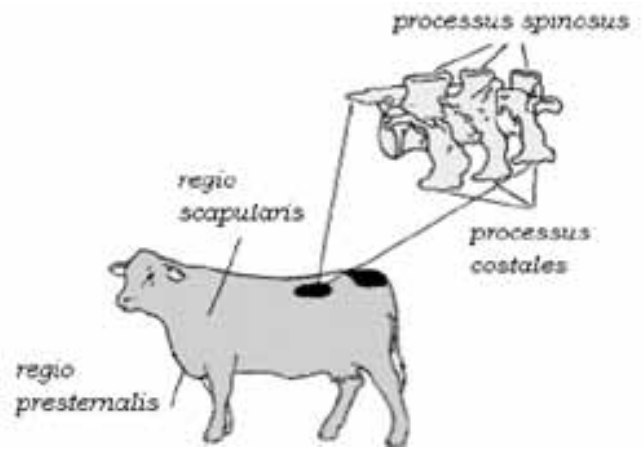

Fig. 2. Some of the anatomical features used for evaluating the BCS in cattle in general

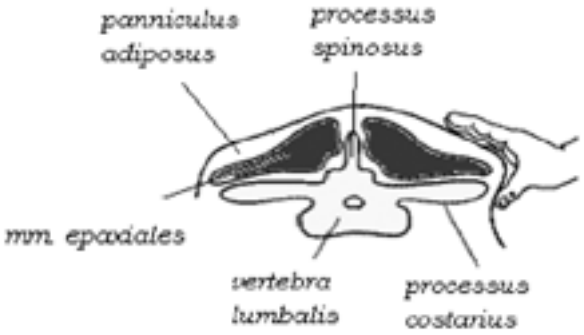

Fig. 3. Only skin and fat cover the backbone and ends of the short ribs, making these ideal locations to assess body condition

backbone, just ahead of the hips, with your fingers on top of the loin and the thumb curved around the ends of the short ribs. Fingertip pressure will provide a good indication of the amount of fat cover (38, 40).

Body condition scoring has to be approached in a systematic manner. There are different systems using different scoring scales. The most frequently used are the 1—5 for dairy and the 1—9 scale system for fattening cattle (6). A 1—9 system is used to describe animals that are extremely emaciated to animals that are so fat they have difficulty walking. The threshold body condition score is put at 5. Note that the deposition of the subcutaneous fat is also evaluated with ultrasound measurement (12, 18, 34, 38).

The key anatomical feature that distinguishes an animal below average fatness (<5) is the visible expression of the 12th and 13th ribs (last two ribs in the rib cage).

In standard European beef breeds *(Bos taurus),* if you are able to observe the 12th and 13th rib they are classified as 4 score or lower (41).

Zebu or *Bos indicus* breeds store more internal fat and are scored below 5 if more than the upper crest of the 12th and 13th rib are showing, that means about 0.5 score higher than *B. taurus* (6). If the short ribs or transverse processes are not showing, the animal is classified as 4. If the short ribs are showing and a moderate "V" has formed between the hooks and pins down to the *trochanter major*, the animal is a solid 3. However, if the fore ribs are distinct, the transverse processes showing, the vertebrae in the tail head are showing and a strong "V" is evident between the hook and pins, the animal is classified as 2. An animal with score 1, has a distinct hipbone showing with a strong "V" effect between the hooks and pins. The ribs are distinct, no tissue can support the tail head and the animal appears near death.

Animals with body condition score 5 (in 1—9 scale) have no 12th and 13th rib showing and exhibit a slight inverted "V" across the back when viewed from the rear. There is a slight "U" effect between the hooks and pins. If the back appears smooth to near level without any indention along the spine and there is a very shallow "U" effect between the hooks and pins, the animal is classified as 6. If the "U" effect is not evident between the hooks and pin and anatomical features express themselves with a slight indention along the spine, the animal is scored at 7. If the indention along the spine is deep and there is evidence of pockets of excess fat expressed across the body, the animal is scored at 8. An animal appearing excessively fat and walking in an awkward manner is scored as 9 (18, 41).

All producers are urged to develop the skill of body conditioning their animals and conducting periodic sampling of their herds using a simple whole number scoring system. Individuals who have improved their scoring skills can assign partial scores such as 5+, 5- or just a 5 (middle value) (18, 41).

The most critical element to a successful BCS is approaching the animal in a systematic manner. Identifying the 12th and 13th rib, locating the short ribs (*processus transversus),* recognizing the "U"/"V" effect, observing the degree of fat deposition at the tail head and distinctness of the ribs in the ribcage are major points of interest that affect the scoring process.

To attain high pregnancy rates, one must manage for a 5+ to 6 score to insure sufficient fatness for reproductive fitness. Scores >7 do not provide any meaningful levels of improvement in pregnancy rates, as fertility rates begin to limit pregnancy rates more than nutrition at this degree of fatness. As an animal's condition falls below 5 so will pregnancy rates and calving intervals. However, the timing can result in different assessments of potential pregnancy rates (43, 46).

Generally, scoring of cows is most convenient at weaning

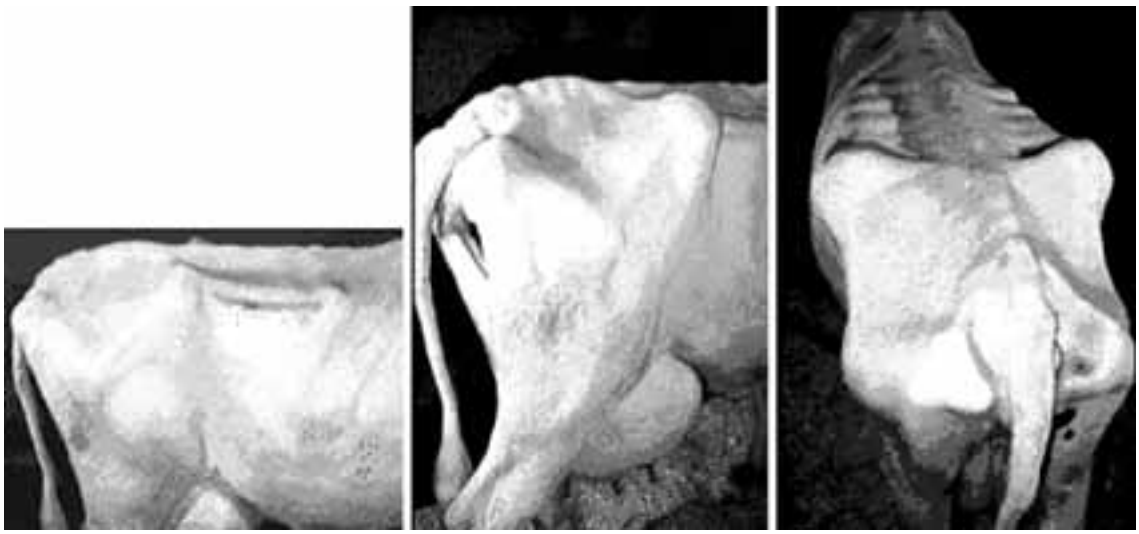

Fig. 4. A cow evaluated as body condition score 1 (58)

since the cows are being gathered and calves separated from the cows. However, this production phase is the least sensitive to predicting the likely pregnancy rate of a cow. For instance, cows that have a body condition score of 5 (in the 1—9 scale), which is average fatness at weaning, have time to recover, posing a problem for predicting their likely reproductive success. The most sensitive time to condition score animals is at calving followed by scoring at breeding. However, both stages give similar assessments of potential pregnancy rates (42, 50).

Once an animal falls to a score of 3, nutritional management to attain good levels of pregnancy in a herd becomes difficult and probably uneconomical to recover. Scores below a 3 at breeding can lead to uneconomical levels of herd performance (51, 54).

Animals, which slip from 5 to 4 at the start of the breeding season, will result in a delayed cycling of the animals, resulting in fewer percentages of animals born in the first oestrus cycle. However, if they decline in condition below 4 at breeding, the delivery time can be delayed by 50—65 days in those reduced number of animals that do become pregnant (52).

For dairy cows the crucial periods are at calving and during early lactation. Achieving correct body condition at calving is important in order to avoid calving difficulties and losses. While in early lactation it is important to prevent excessive weight loss when meeting the extra nutritional demands of high yielding cows.

As mentioned above BCS is a technique for assessing the condition of livestock at regular intervals (25). The purpose of condition scoring is to achieve a balance between economic feeding, good production and good body condition. The evaluation is done: according to the following guide (13, 28, 29, 55, 56):

Body condition score 1

This cow is emaciated. The ends of the short ribs are sharp to the touch and together give a prominent shelf-like appearance to the loin. *Processus spinosus of* the individual vertebrae of the backbone are prominent. The hook and pin bones are sharply defined. The thurl region and thighs are sunken and in-curving. The anal area has receded and the vulva appears prominent (Fig. 4).

Body condition score 2

This cow is thin. The ends of the short ribs (*processus costales)* can be felt but they and the individual vertebrae are less visibly prominent. The short ribs do not form as obvious an overhang or shelf effect. The hook and pin bones are prominent but the depression of the thurl region between them is less severe. The area around the anus is less sunken and the vulva less prominent (Fig. 5).

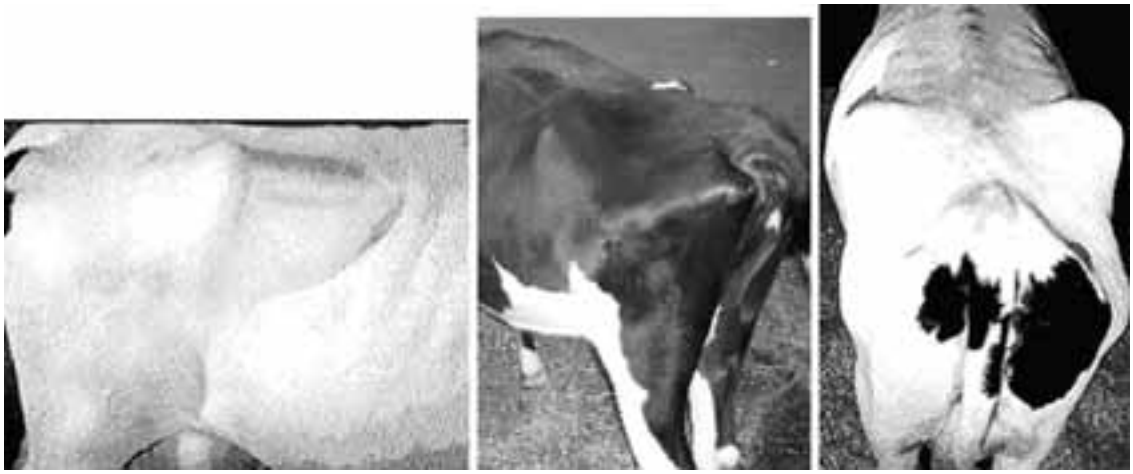

Fig. 5. A cow evaluated as body condition score 2 (58)

Fig. 6. A cow evaluated as body condition score 3 (original photo)

Body condition score 3

The cow has an average body condition. The short ribs can be felt by applying slight pressure. The overhanging shelf like appearance of these bones is gone. The backbone is a rounded ridge and the hook and pin bones are round and smoothed over. The anal area is filled out but there is no evidence of fat deposit (Fig. 6).

Body condition score 4

The cow is heavy. The individual short ribs can be felt only when firm pressure is applied. Together they are rounded over with no shelf effect. The ridge of the backbone is flattened over the loin and rump areas and rounded over the chine. The hook bones are smoothed over and the span between the hook bones over the backbone is flat. The area around the pin bones is beginning to show patches of fat deposit (Fig. 7).

Body condition score 5

The cow is fat. The bone structure of the top line, hook and pin bones and the short ribs is not visible. Fat deposits around the tailbone and over the ribs are obvious. The thighs curve out, the brisket and flanks are heavy and the chine is very round (Fig. 8).

Cows should be scored regularly to reflect changes in fat reserves at each stage of lactation. Ideally all cows should be scored at the beginning and end of their dry period and at least 4 or 5 times during lactation (4). BCS should be evaluated based on the stage of lactation (30).

One convenient way to do this is to record condition scores in each animal farm and record it on a fact sheet (14). This provides a single reference for cow identification, days in milk, production level and condition score, thereby including all the information needed to set feeding levels for individual cows (4).

To analyse condition scores for a herd or for a cow throughout the lactation period, the individual scores can be plotted on the chart on the back page of this fact sheet. Remarks such as lactation number, production level or health problems can be added above plotted points to improve interpretation of the chart.

There are six key times during the yearly cycle when each cow should have its condition evaluated. These occur: midway through the dry period, at calving and at about 45th, 90th, 180th and 270th days of the lactation phase (25, 33, 43). The timing of the checks coincides with the time for making important decisions about the future feeding, breeding and health condition of the cow.

The following describes specific goals regarding body condition for each stage of the lactation cycle:

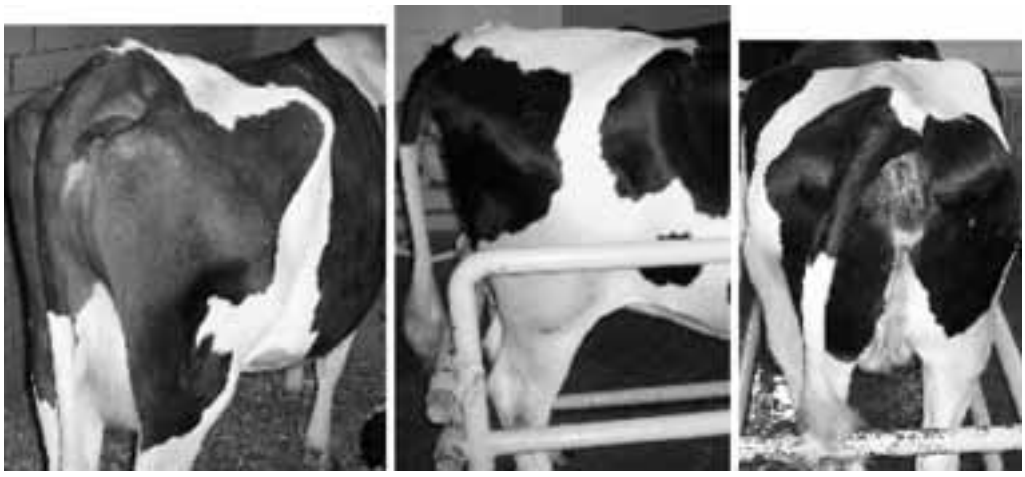

Fig. 7. A cow evaluated as body condition score 4 (original photo)

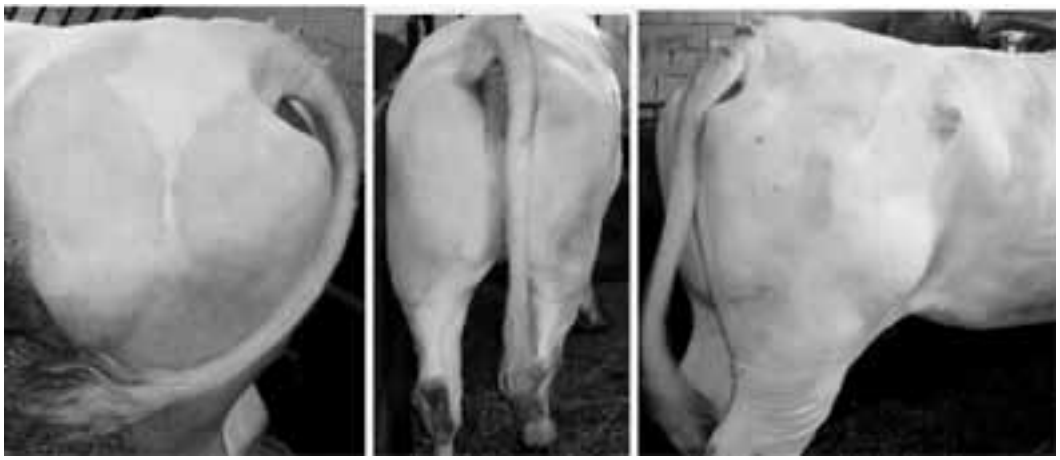

Fig. 8. A cow evaluated as body condition score 5 (original photo)

Dry period: The goal for ideal body condition score for the dry cow is 3.5. To achieve satisfactory health and performance early in the subsequent lactation condition score has to be between a minimum of 3 and a maximum of 4 (33, 58).

It is a well-accepted fact that cattle, replenish body fat reserves more efficiently while lactating than during the dry period. Occasionally a cow must be dried off before an acceptable condition score is reached (33, 58). It will pay manager to continue to feed under conditioned dry cows for gain, to achieve a desirable BCS. Obviously, a well-managed feeding program combined with frequent observation is required to achieve condition gain without over fattening the dry cow.

Average quality, long stemmed grass hay has proven to be the ideal forage for the dry cow. Higher quality (energy and protein) forages, such as corn silage and alfalfa haylage, must be limit fed to prevent excessive condition gain (3). With correct forage quality and quantity, a low-energy high-fibre supplement, containing appropriate protein, mineral and vitamin levels, could be fed in controlled amounts to achieve the desired amount of gain.

Removing excess fat from over-conditioned cows by limiting energy intake during the dry period does not appear to impair subsequent performance seriously (46, 48).

Early lactation: The cow should be evaluated frequently during early lactation. It is the BCS that reflects the energy reserve and has the greatest impact on the health, production and fertility of the dairy cattle.

The cow newly overweight, with a condition score of more than 4, is at greater risk of fat cow syndrome problems such as difficult calving, retained placenta, *metritis, mastitis*, displaced abomasum, ketosis and milk fever (34, 35, 36). Its immune response is usually inadequate to combat the stress of calving and its appetite is less than ready to meet the demands of early lactation.

Another situation occurs when the cow starts lactation without enough energy reserves, having a condition score of less than 3 (31).

This cow may experience fewer health problems at calving but its later productive and reproductive performance will be less than expected.

As shown in Fig. 9, the average cow commonly peaks in milk production at 4 to 6 weeks into lactation. Its feed (dry matter) intake lags behind, normally peaking at about 9 to 11 weeks (6, 20, 32). This situation puts the cow in a negative energy balance for several months in early lactation. This means that feed energy intake is less than milk energy output. The cow uses available body fat deposits to cover the deficit.

The cow starting lactation in a thin condition lacks adequate energy reserves and it will peak at a lower milk yield. Peak milk yield is directly related to total lactation yield with mature cows. For each additional kilogram of milk at peak there will be approximately 200 more kilograms of milk over the whole lactation (9, 20, 48). A below optimal condition at calving is also a cause of low milk fat test. In early lactation, a high proportion of milk butterfat precursors originate from body fat stores (24, 31, 45, 46).

The average mature cow calving at the desired body condition, with a score of 3.5 (maximum four) and in good health, can be expected to lose between one-half and one kilogram of body tissue per day during the first 60—80 days in milk (9, 48).

During the first two months in milk the average mature cow will drop between half and one full point in its condition score, stabilizing at a score near three by the 10th week and beginning to regain its lost condition by the 90th day. At this time, rising feed energy intake can satisfy the declining milk energy demand. This coincides with the optimum period for observation of regular oestrous activity, breeding and conception (4, 58).

Experience and research have shown that cows gaining weight at the time of service have a higher conception rate than cows losing weight (5, 7). A condition score between 2.5 and 3.5 would indicate an adequate condition for good reproductive efficiency.

Very high producing cows may drop to a score near to 2.5 before stabilizing, having lost up to 1.5 kilograms of tissue per day during the 3rd—4th month lactation phase. The expression of oestrus and fertility may be suppressed in these cattle, resulting in delayed conception. Cows with good production that demonstrate no or little condition loss in early lactation are most likely very efficient feed converters. Cows that gain condition at this stage are probably poor producers (58).

Low energy intake in early lactation can lead to excessively high rates of fat mobilization of greater than 1.5 to 2.0 kilograms per day. This increases the risk of the accumulation

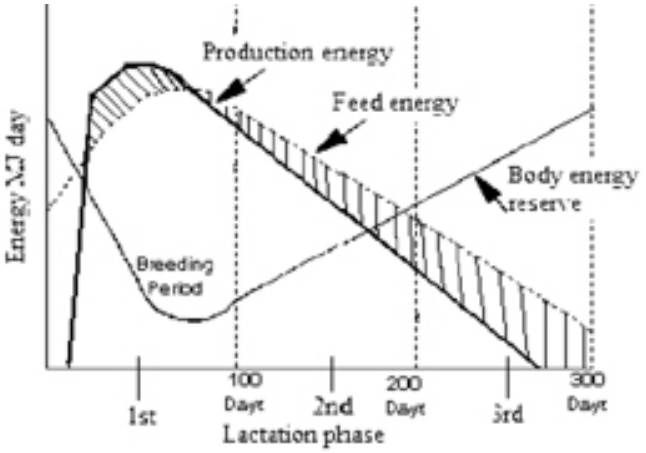

Fig. 9. A lactating dairy cow general energy curve (27)

of fat in the cow's liver and can lead to ketosis (34, 35, 36), increased susceptibi-lity to disease, a delayed return to oestrus and reduced fertility.

The feeding program for cows in early lactation must therefore be carefully managed to achieve maximum dry matter intake and ration digestibility. Adequate amounts of protein are critical to stimulate intake and provide nutrients especially amino acids and volatile fatty acids, VFA (acetic, propionic and butyric acids) for milk production. Due to the limited body protein reserves it is very difficult for the cow to fit the production (31).

Cows in early lactation will consume about 10 % less dry matter than cows at the same level of production in mid lactation (32). Therefore, providing enough protein to meet the requirement for peak milk means that the ration protein content will be in the range of 18 to 20 % of the dry matter. Ideally, 40 % of the protein should bypass rumen degradation and provide the amino acids that are limiting for milk production (10).

A compromise must be met between providing the fresh cow with large amounts of highly digestible and rapidly fermented grain starch for energy and providing adequate forage fibre to maintain rumen function and butter-fat synthesis.

The ration should be formulated to provide 72 to 75 % total digestible nutrients (TDN) or 6.74 to 6.99 mega joules (MJ) per kilogram of net energy for lactation (NEL). Total ration fibre levels should be between 19 and 21 % acid detergent fibre (ADF) and between 25 and 28 % neutral detergent fibre (NDF). A minimum of 21 % of the total ration dry matter should come from forage NDF. Ideally some of the forage should be in the form of hay to provide stimulation for optimum rumen function (53).

Mineral and vitamin levels in the ration should be balanced to currently recommended standards.

The following recommended feeding management practices would also help to maximize dry matter intake, eliminate the risk of cows going off-feed, and reduce the cow's dependence on body fat reserves.

These practices include (3, 53):

– Lead feeding grain to the dry cow for two weeks, increasing to a maximum of 1 % of body weight at calving.

– Challenge feeding grain and protein supplement to the fresh cow, increasing gradually to the recommended maximum advised by ration formulation, by three weeks into lactation.

– Feeding concentrates in meals of less than four kilograms, more frequently (i.e. four times) per day.

– Feeding the highest quality forages available.

– Following the feeding sequence of forage before grain and grain before protein supplement, ideally with some time delay between, for optimum ration digestibility.

– Feeding more often when rapid feed spoilage is a problem.

– Keeping mangers and water bowls clean and free of hazards.

– Chopping forages to maintain adequate particle size (greater than one cm) and processing concentrates as coarsely in texture as possible to stimulate rumen function and feed consumption.

– Using molasses to improve the intake of unpalatable or dusty feeds.

– Using buffers, such as sodium bicarbonate at 0.75 to 1.0 % of total dry matter intake, to improve the digestibility and intake of high concentrate rations.

– Adding 0.5 to 0.75 kilograms/day of rumen protected fat to the cow's ration to increase the energy density while reducing the need to rely on starch as the primary source of dietary energy. When adding fat to the ration, calcium and magnesium levels need to be raised to 1.0 % and 0.3 %, respectively, and attention must be given to providing adequate bypass protein and functional fibre in the ration.

– Adding six to 12 grams of niacin to the ration during the lead feeding and throughout the early lactation period will help high producing cows that are newly in the desired or heavy body condition to use dietary fat and body fat stores more efficiently.

Mid lactation: At about 180 days of the lactation period, a body condition appraisal should confirm that cows are replenishing body fat reserves that were lost at early lactation. By this stage of lactation, the BCS should be approaching 3 for the highest producing cows in the herd and between 3 and 3.5 for the average-producing cows. Below average cows may have already exceeded a condition score of 3.5 and will need to be fed carefully to prevent fattening. All cows being rebred should be confirmed pregnant by mid lactation.

Late lactation: The condition score check done at about 270 days of the lactation phase should show the average cow approaching a score of 3.5. During this period, low producing cows tend to become over-conditioned, showing scores at or above 4. This occurs more often where large amounts of corn silage are fed and where attention is not paid to limiting access to concentrates.

Cattle fed grain in milking parlours should be allowed sufficient time to clean their share, leaving none behind for the next cow that occupies the stall. In tie-stall barns, manger dividers may be needed to prevent cows from stealing unneeded grain from immediate neighbours.

Over-conditioning also happens in free-stall herds fed total mixed rations where the cattle are not adequately grouped according to production. At least four and perhaps five lactation groups; early, mid, late, first-calf, and dry may be needed to prevent over conditioning.

In herds where extended calving intervals prolong the period of low production and/or the dry period, many cows will become too fat. In this situation breeding management needs to improve.

Very high producing and persistent cows, like first-calf heifers, with normal calving intervals, may be difficult to get to the goal of 3.5 in condition score while still milking. With these cows, it may be necessary to continue to feed for gain during the dry period to recharge their energy reserves.

Note that growing cows need a little different observation and feeding care, therefore the following should be taken in to consideration (2).

The first lactation period of heifers: The ideal condition score for the heifer calving for the first time is about 3.0. Heifers with condition scores in excess of 3.5 have experienced more calving difficulty.

First-calf heifers need to be managed somewhat differently from their older herd mates. They will calve with 100 to 150 kilograms less body weight than older cows in the herd (45, 55).

Their daily concentrate amount must be adjusted accordingly to maintain correct forage-to-concentrate ratios to prevent problems related to digestive system malfunction.

The lactation curve of a first calved does not show the early high peak that higher lactation number cows demonstrate. Therefore, the negative energy balance occurring in early lactation will not be as demanding on body fat reserves as it can be for older cows (2, 10).

First-calf heifers do show greater persistency of lactation than older herd mates. The first calved will show an average drop of 4 % per month in mid-lactation compared to 8 % in older cows. In late lactation, the first calved will fall in milk at 6 to 8 % monthly while the higher lactation number cows decline at 10 to 14 %. This greater persistency means that the heifer cannot direct as high a proportion of energy intake as its older herd mates toward the replenishment of body fat stores (10).

First and second calf heifers also have a major additional need for energy and for growth, throughout mid- and late lactation and the dry period. These cattle must gain 50 to 75 kilograms during each of the first two lactations to reach mature body weights (1, 2, 8).

To ensure that the additional nutrients needed for growth, feed more concentrates to these cattle. During the mid- and late lactation phase the first calved should get 10 % and the second calved 5 % more concentrates than required for milk and body condition gain.

Today's genetically superior cattle can produce large volumes of milk, even during their first lactation. If special care is not provided, they will begin the second lactation stunted and/or lacking adequate energy reserve.

With the mature lactation curve, typical of second lactations, adequate tissue energy reserves are critical to achieving desirable peak milk yields as well as satisfactory butterfat synthesis. Body size is a major factor influencing dry matter intake. Lack of sufficient growth will limit the improvement in feed intake needed to support higher milk yields.

As a result of inadequate management the genetically superior heifer could demonstrate poor second lactation performance or burnout and may be wrongly culled. Correct management of energy balance throughout the lactation and reproduction cycles of the dairy cow can significantly improve its capacity to generate profit.

The last but not the least important consideration is the observation and evaluation of calves (1).

Newborn calf: The newborn calf will have a body condition score of about 2.0. Until it has been on feed for a few weeks, it will not have much flesh. Condition 2 has some flesh over the *processus spinosus*, but hooks; pins and the *processus spinosus* are prominent. A depression between hooks and pins and a prominent tail head can be observed. Its overall frame is obvious.

Six-month-old calf: At six months, a heifer will have more flesh over the vertebrae, ribs, hooks, and pins. Hooks and pins are rounded and smooth. A moderate depression can be observed between the hooks, and a depression between hooks and pins. The frame is well covered and well balanced.

12-month-old calf: At one year the heifer has adequate flesh over the *processus spinosus* and between the hooks and pins. At this stage, even though the heifer is growing rapidly, you do not want a score of more than 4, which would indicate too much energy in the diet. The optimal score is 3.25.

15-month-old calf: This breeding age heifer has flesh over the *processus spinosus* and a slight depression between the hooks and pins. Its vertebrae are not prominent and its hooks and pins are smooth. The optimal score is 3.5.

24-month-old heifer: This heifer will soon enter the milking herd. It has adequate flesh in all areas. Her hooks and pins are not prominent. A slight slope can be observed between hooks and pins. *Processus spinosus* is not evident. Frame is well covered, becoming less visible. This will translate into better milk production after it calves. The optimal score is 3.75.

The purpose of this article is to show how the external anatomical features, which are used as BCS can contribute significantly to good husbandry and management of dairy cows. This will help to ensure that the cow is in the correct condition for each stage of its annual cycle and that appropriate dietary changes can be made in order to correct any deficiencies.

CONCLUSION

Body condition scoring is an easy technique to learn and can easily be applied even to groups of animals in the field. It allows essential management decisions to be made to enable high standards of husbandry to be achieved – and ensures costly welfare problems are avoided.

Begin by a review of the general names for the various body locations, which will be referred to in the systematic approach to BCS. For dairy cows, ideal condition at drying off should be close to that for calving and ideally not more than half a score above. This "fit not fat" condition avoids having to slim cows off as this leads to metabolic disorders. Maiden dairy heifers require special attention, because they are "nutritionally" on a knife-edge. An example of this is that the loss of one condition score equals to 15 kg live weight in a heifer, *versus* 30 kg in an adult cow, and similarly when gaining condition in mid lactation, heifers need to gain 90 kg for one condition score *versus* 60 kg in an adult cow.

Keep in mind that this system is done from behind the animal. The person responsible for feeding the herd should do body condition scoring. To keep the scores "standardized", regularly refer back to the standards outlined in your record book, and discuss condition scores with your nutrition advisors and herd veterinarian.

ACKNOWLEDGEMENT

The author would like to thank Prof. Danko, J., DVM, PhD and Prof. Vajda, V., DVM, PhD for their professional guide.

REFERENCES

1. Arango, J. A., Cundiff, L. V., Van Vleck, L. D., 2002: Breed comparisons of Angus, Charolais, Hereford, Jersey, Limousin, Simmental, and South Devon for weight, weight adjusted for body condition score, height, and body condition score of cows. *J. Anim Sci*., 80, 3123—3132.

2. Arango, J. A., Cundiff, L. V., Van Vleck, L. D., 2004: Comparisons of Angus, Charolais, Galloway, Hereford, Longhorn, Nellore, Piedmontese, Salers, and Shorthorn breeds for weight, weight adjusted for condition score, height, and condition score of cows. *J. Anim Sci*., 82, 74—84.

3. Boyles, S., Flakoll T., Ringwall, K., 1992: *Cow Nutrition and Body Condition. NDSU Extension Publ. AS-1026.*

4. Boyles, S., 1998: *Beef Cow Nutrition*. The Ohio State University Extension Beef Team Library (http://ag.ohio.state.edu/~beef/library/cow nutr.html).

5. Butler, W. R., Smith, R. D., 1989: Interrelationships between energy balance and postpartum reproductive function in dairy cattle. *J. Dairy Sci*., 72, 767—783.

6. Ciccioli, N. H., Wettemann, R. P., Spicer, L. J., Lents, C. A., White, F. J., Keisler, D. H., 2003: Influence of body condition at calving and postpartum nutrition on endocrine function and reproductive performance of primiparous beef cows. *J. Anim. Sci*., 81, 3107—3120.

7. Curtis, C. R., Erb, H. N., Snifeen, C. J. *et al.,* 1985: Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders and mastitis in Holstein cows. *J. Dairy Sci*., 68, 2347—2360.

8. Dechow, C. D., Rogers, G. W., Klei, L., Lawlor, T. J., 2003: Heritabilities and correlations among body condition score, dairy form and selected linear type traits. *J. Dairy Sci.,* 86, 2236—2242.

9. Dechow, C. D., Rogers, G. W., Klei, L., Lawlor, T. J., 2004: Heritability and correlations for body condition score and dairy form within and across lactation and age. *J. Dairy Sci.*, 87, 717—728.

10. Dobos, R., Riley, K., Nandra, K., Kellaway, R., Fulkerson, W., Lean, I. J., 1997: The effect of rumen escape protein on carcass characteristics of rapidly growing prepubertal dairy heifers. In *Recent Advances in Animal Nutrition in Australia*. University of New England, Australia, 255 pp.

11. Domecq, J. J., Skidmore, A. L., Lloyd, J. W., Kaneene, J. B., 1997: Relationship between body condition scores and milk yield in a large dairy herd of high yielding Holstein cows. *J. Dairy Sci*., 80, 101—112.

12. Domecq, J. J., Skidmore, A. L., Loid, J. W. *et al*., 1997: Validation of body condition scores with ultrasound measurements of subcutaneous fat of dairy cows. *J. Dairy Sci*., 78, 2308—2313.

13. Dunn, R. T. Jr., Smith, M. F. Gall, T. J., Massey, J. W., 1982: Survey of *Reproductive Performance in Missouri Beef Herds*. MO Beef Cattle Research Report, 101, 56 pp.

14. Edmonson, A. J., Lean, I. J., Weaver, L. D. *et al.,* 1989: A body condition scoring chart for Holstein dairy cows. *J. Dairy Sci.*, 72, 68—78.

15. Encinias, A. M., Lardy, G., 2000: *Body Condition Scoring I: Managing Your Cow Herd Through Body Condition Scoring*. NDSU Extension Publ. AS-1026.

16. Ferguson, J. D., 1991: Nutrition and reproduction in dairy cows. Dairy nutrition management. *Veterinary Clinics of North America. Food Animal Practice*, 7, 483—507.

17. Ferguson, J. D., Byers, D., Ferry, J. *et al*., 1994: Round table discussion: body condition of lactating cows. *Agric. Practice*, 15, 17—21.

18. Ferguson, J. D., Galligan, D. T., Thomsen, N., 1994: Principal descriptors of body condition score in Holstein cows. *J. Dairy Sci*., 77, 2695—2703.

19. Forbes, G., 1985: Body composition as affected by physical activity and nutrition. *Fed. Proc*., 44, 343—347.

20. Garnsworthy, P. C., Topps, J. H., 1987: The effect of body condition of dairy cows at calving on their food intake and performance when given complete diets. *Anim. Prod.*, 35, 113—119.

21. Gearheart, M. A., Curtis, C. R., Erb, H. N. *et al.,* 1990: Relationship of change in condition score to cow health in Holsteins. *J. Dairy Sci*., 73, 3132—3140.

22. Gregory, N. G., Robins, J. K., 1998: A body condition scoring system for layer hens. *New Zealand J. Agric. Res.,* 41, 555—559.

23. Gregory, N. G., Robins, J. K., Thomas, D. G., Purchas, R. W., 1998: *Relationship between body condition score and body composition in dairy cows. New Zealand J. Agric. Res.,* 41, 527—532.

24. Grummer, R. R., 1993: Etiology of lipid-related metabolic disorders in periparturient dairy cows. *J. Dairy Sci*., 76, 3882—3896.

25. Hady, P. J., Domecq, J. J., Kaneene, J. B., 1994: Frequency and precision of body condition scoring in dairy cattle. *J. Dairy Sci*., 77, 1543—1547.

26. Herd, D. B., Sprott, L. R., 1986: Body condition, nutrition, and reproduction of beef cows. *Texas Agricultural Extension Service Bulletin*, B-1526 pp.

27. Hutjens, M. E., Jaster, E. H., 1982: *Relationships Between Dry Matter Intake, Milk Production and Body Weight Changes.* NFIA.

28. Kováč, G. Podmanický, D., Kováč, M., 2001: Clinical approach to diagnosis of nutritional diseases in dairy cows. (In Slovak). *Infovet*, 6, 9—10.

29. Kováč, G. *et al.,* 2001: *The Diseases of Cattle. (In Slovak). M & M vydavateľstvo, Prešov, 859 pp.*

30. Lago, Ernani Paulino do, Pires, Alexandre Vaz, Susin, Ivanete *et al*., 2001: Effect of body condition score at calving on energy metabolism, milk yield and disease

occurrence in postpartum of dairy cows. *Rev. Bras. Zootec.*, 30, 1544—1549.

31. Lassen, J., Hansen, M., Sorensen, M. K., Aamand, G. P., Christensen, L. G., Madsen, P., 2003: Genetic relationship between body condition score, dairy character, mastitis, and diseases other than mastitis in first-parity Danish Holstein cows. *J. Dairy Sci.,* 86, 3730—3735.

32. Lean, I. J., Galland, J. C., Scott, J. L., 1989: Relationship between fertility, peak milk yields and lactational persistency in dairy cows. *Theriogenology,* 31, 1093—1103.

33. Lean I. J, Edmondson, A. J., Versteeg, J., 1991: *Body Condition Scoring Dairy Cattle*. Published by The Upjohn Company, USA.

34. Lean, I. J., Bruss, M. L., Baldwin, R. L., Troutt, H. F., 1991: Bovine ketosis: a review. I. Epidemiology and pathogenesis. *Vet. Bull.* 61, 1209—1218.

35. Lean, I. J. Bruss, M. L. Baldwin, R. L. Troutt, H. F., 1992: Bovine ketosis: a Review II. Biochemistry and prevention. *Vet. Bull.,* 62, 1—14.

36. Lean, I. J., Bruss, M. L., Troutt, H. F., Galland, J. C., Farver, T.B., Rostami, J., Holmberg, C. A., Weaver, L. D., 1994: Bovine ketosis and somatotrophin: risk factors for ketosis and effects of ketosis on health and production. *Res. Vet. Sci.,* 57, 200—209.

37. León, H. V., Hernández-Cerón, J., Keisler, D. H., Gutierrez, C. G., 2004: Plasma concentrations of leptin, insulin-like growth factor-I, and insulin in relation to changes in body condition score in heifer. *J. Anim. Sci*., 82, 445—451.

38. MacDonald, K. A., Penno, J. W., Verkerk, G. A., 1999: Validation of body condition scoring by using ultrasound measurements of subcutaneous fat. *Proc. New Zealand Soc. Anim. Prod*., 59, 177—179.

39. Momont, P. A., Pruitt R. J., 1988: *Relationships Between Body Condition Scores and Live Animal Measurements of Beef Cows*. SDSU Beef Cattle Report. Cattle, 88—13.

40. Nelson, T. C., Short, R. E., Reynolds, W. L., Urick, J. J., 1985: Palpated and visually assigned condition scores compared with weight, height, and heart girth in Hereford and crossbred cows. *J. Animal Sci*., 60, 2.

41. Odde, K. G., 1997: Reproductive efficiency pre-calving nutrition and improving calf survival. *Proc. Bovine Connection*, 60, 86—92.

42. Paputungan, U., Makarechian, M., 2000: The influence of dam weight, body condition and udder scores on calf birth weight and preweaning growth rates in beef cattle. *Asian-Aus. J. Anim. Sci*., 13, 435—439.

43. Patton, R. A., Bucholthz, H. F., Schmidt, M. K. *et al*., 1988: Body condition scoring: a management tool. In *Dairy Guide*, East Lansing: Michigan.

44. Pedron, O., Chele, F., Senatore, E., Baroli, D., Rizzi, R., 1993: Effect of body condition score at calving on performance, some blood parameters, and milk fatty acid composition in dairy cows. *J. Dairy Sci*., 76, 2528—2535.

45. Pedron, O., Cheli, F., Senatore, E. *et al.,* 1993: Effect of body condition score at calving on performance, some blood parameters, and milk fatty acid composition in dairy cows*.** *J. Dairy Sci*., 76, 2528—2535.

46. Pethick, D. W., Dunshea, F. R., 1993: Fat metabolism and turnover. In: **Forbs, J. M., France, J.** (eds.): *Quantitative Aspects of Ruminant Digestion and Metabolism.* Cambridge: C.A.B. International (1st. edn.), 291—311.

47. Rae, D. O., Kunkle, W. E., Chenoweth, P. J., Sand, R. S., Tran, T., 1993: Relationship of parity and body condition score to pregnancy rates in Florida beef cattle. *Theriogenology,* 39, 1143—1152.

48. Reid, I. M., Roberts, C. J., Treacher, R. J. *et al.,* 1986: Effect of body condition at calving on tissue mobilization, development of fatty liver and blood chemistry of dairy cows. *Anim. Prod.*, 43, 7—15.

49. Ruegg, P., Milton, R. L., 1995: Body condition score of Holstein cows on Prince Edward Island, Canada: Relationships with milk yield, reproductive performance, and disease. *J. Dairy Sci*., 78, 552—564.

50. Selk, G. E., Wettemann, R. P., Lusby, K. S., Rusby, R. T., 1986: The importance of body condition at calving on reproduction in beef cows. In *Animal Science Research Report*, 316 pp. Oklahoma State Univ.

51. Selk, G. E., Wettemann, R. P., Lusby, K. S., Oltjen, J. W., Mobely, S. L., Rusby, R. J., Garmendia, J. C., 1988: Relationships among weight change, body condition and reproductive performance of range beef cows. *J. Animal Sci*., 66, 3153—3159.

52. Short, R. E., Bellows, R. A., Staigmiller, R. B., Berardinelli, J. G., Custer, E. E., 1990: Physiological mechanisms controlling anoestrus and infertility in postpartum beef cattle. *J. Anim. Sci.*, 68, 799—816.

53. Van Soest, P. J., Robertson, J. B., Lewis, B. A., 1991: Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci*., 74, 3583—3597.

54. Wagner, J. J., Lusby, K. S., Oltjen, J. W., Rakestraw, J., Wettemann, R. P., Walters, L. E., 1988: Carcass composition in mature Hereford cows: estimation and effect on daily metabolizable energy requirement during winter. *J. Animal Sci*., 66, 603—612.

55. Waltner, S. S., McNamara, J. P., Hillers, J. K., 1993: Relationships of body condition score to production variables in high producing Holstein dairy cattle. *J. Dairy Sci*., 76, 3410—3419.

56. Wettemann, R. P. Lusby, K. S., Rusby, R. T., Richards, M. W., 1987: Body condition at calving and post partum intake influence reproductive performance of range cows. In *Anim. Sci. Res. Rep.*, 70 pp. Oklahoma State Univ.

57. Wettemann, R. P., Lusby, K. S., 1987: Body condition at calving, calf survival and reproductive performance of first calf heifers. *Anim. Sci. Res. Rep.*, Oklahoma State Univ. 73 pp.

58. Wildman, E. E., Jones, G. M., Wagner, P. E. *et al.,* 1982: A dairy body condition scoring system and its relationship to selected production characteristics. *J. Dairy Sci*., 65, 495—501.

Received June 11, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 62—65, 2004

THE EFFECT OF PLANT ANTIOXIDANTS ON THE ELIMINATION OF ENVIRONMENTAL STRESS STIMULATED BY MERCURY IN RUMEN BACTERIUM *Selenomonas ruminantium*

Sobeková, A., Holovská, K., jr., Lenártová, V. Holovská, K., Pristaš, P*., Javorský, P*.

University of Veterinary Medicine, Komenského 73, 041 81 Košice
***Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice**
The Slovak Republic

asobekova@hotmail.com

ABSTRACT

The main aim of this paper was to investigate the activity response of the antioxidant enzyme GSHPx and TBARS content in rumen bacterium *Selenomonas ruminantium* following exposure to $HgCl_2$ in the presence of plant non-enzyme antioxidants substances (AOS). S. *ruminantium* was grown alone or together with α-toc, β-car or with 5 mg.ml^{-1} of $HgCl_2$ alone or together with tested AOS. The activity of GSHPx and TBARS content of *S. ruminantium* were estimated in supernatants of disrupted bacterial cells. A significant changes in the *S. ruminantium* GSHPx activity and TBARS content in the presence of $HgCl_2$ and tested AOS was observed. The potential role of plant antioxidants in elimination of the environmental stress of ruminal bacteria stimulated by heavy metals is discussed.

Key words: environmental stress; non-enzyme antioxidant; rumen bacteria; *Selenomonas ruminantium*

INTRODUCTION

Ruminants can be exposed to the toxic concentrations of different environmental pollutants, including heavy metal, by consumption of contaminated feed and water. The ingested toxic substances can be inhibitory to both the fermentative activity and growth of microbes, thereby changing the physiological steady-state of rumen fermentation (13, 15). The pollutants can also contribute to enhancing free oxyradicals content, which could generate oxidative and environmental stress for ruminal microbes.

Microorganisms have developed efficient enzymatic and non-enzymatic mechanisms to eliminate these toxic and mutagenic oxygen by-products (19). Superoxide is eliminated by dismutation to H_2O_2 by superoxide dismutase (15) and accumulation of H_2O_2 is prevented by the action of catalases and peroxidases (8). McCord *et al.* (17) have proposed that the absence of the enzyme superoxide dismutase accounts for the sensitivity of anaerobes to oxygen. However, evidence from other laboratories (10, 6) has indicated that this enzyme is present in various anaerobes. Wimpenny and Samah (22) have described the response of *S. ruminantium* to changes in oxygen tension in batch cultures and documented the presentation of inducible superoxide dismutase activities under these conditions.

In our previous studies we have observed significant changes in antioxidant enzymes activities of the ruminal bacteria *Strep. bovis* 4/1 and *S. ruminantium* E32 in the presence of various concentrations of mercury chloride (15) and the positive effect of a-toc and b-car on the elimination of environmental stress, evoked by mercury, in *Strep. bovis* (11).

In the present study we have documented the different effect of nonenzyme antioxidant substances (alpha tocopherol, beta carotene) on the elimination of the environmental stress stimulated by mercury in the ruminal anaerobe *S. ruminantium* E32 *in vitro*.

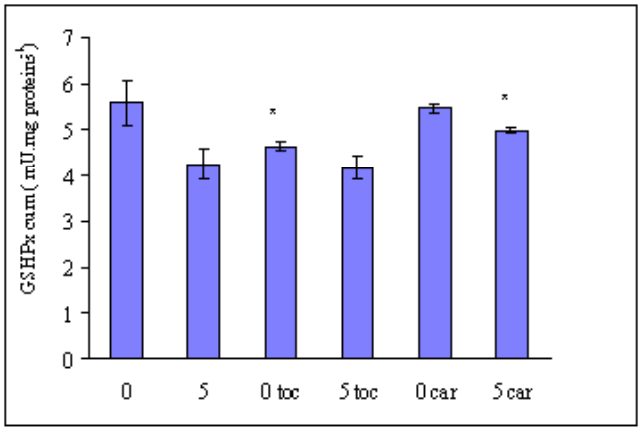

Fig. 1. Activity of GSHPx-cum enzyme in S. ruminantium E32 under anaerobic condition in the presence of 0 (0) and 5 mg ml^{-1} (5) of Hg^{2+} or in the presence of 0 and 5 mg ml^{-1} of Hg^{2+} and α-tocopherol (0toc, 5toc) and β-carotene (0car, 5car)

Note:
Asterisks represent significant differences between the culture with 5 mg.ml^{-1} of Hg^{2+} (5) and bacteria grown in the culture with 5 mg.ml^{-1} of Hg^{2+} and α-toc, β-car or significant differences between the free culture and bacteria grown in the culture with tested antioxidant (— $p < 0.05$)*

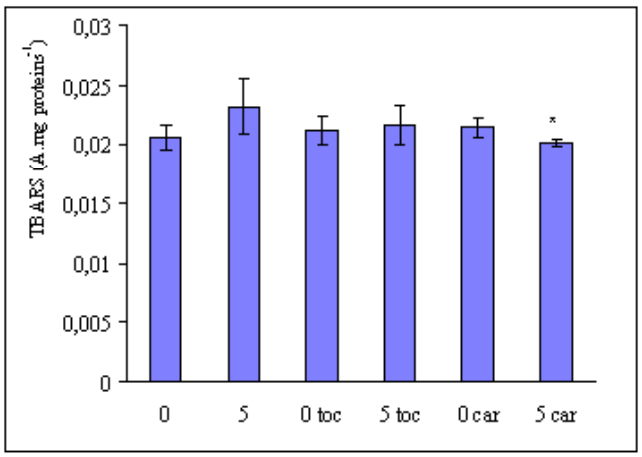

Fig. 2. The TBARS content in S. ruminantium E32 under anaerobic condition in the presence of 0 (0) and 5 mg ml^{-1} (5) of Hg^{2+}or in the presence of 0 and 5 mg ml^{-1} Hg^{2+} and α-tocopherol (0toc) 5toc) and β-carotene (0car, 5car)

Note:
Asterisks represent significant differences between the culture with 5 mg.ml^{-1} of Hg^{2+} (5) and bacteria grown in the culture with 5 mg.ml^{-1} of Hg^{2+} and α-toc, β-car or significant differences between free culture and bacteria grown in the culture with tested antioxidant (* – $p < 0.05$)

MATERIALS AND METHODS

Growth conditions and sample preparation

Selenomonas ruminantium E32 used in this study was isolated from the rumen of sheep and is maintained in our microbe collection (15). Strain E32 was grown anaerobically overnight at 37 °C in the selective M medium described by Tiwari *et al.* (20) in the presence of 0 or 5 mg.ml^{-1} of $HgCl_2$. The experimental cultures were grown under the same conditions and contained 0 or 5 mg.ml^{-1} of $HgCl_2$ and calculated amounts of nonenzyme antioxidant substances: α-tocopherol and β-carotene. The calculation was done for 50—60 kg sheep, daily intake of 1.5 kg of food dry matter and for a 10 l volume of sheep rumen according to the Slovak nutrition standard (ČSN 467006). For α-tocopherol this dose represents 20 mg.ml^{-1} and for β-carotene 45 mg.ml^{-1}. The cultures were harvested by centrifugation at 10 000 × *g* for 15 min at 4 °C and cells were washed in potassium phosphate buffer containing 0.1 mM EDTA, pH 7.4, pelleted by centrifugation as before and resuspended in the same buffer. The cells were disrupted by sonication using 30-s bursts for a total of three minutes with a one minute cooling period between bursts using a MSE Soniprep 150 ultrasonic disintegrator at 4 °C. Cellular debris was removed by centrifugation at 12 000 × *g* for 15 min, the supernatants were dialysed against a potassium phosphate buffer and used for the enzyme assays.

Enzyme assays

Glutathione peroxidase activity (GSHPx, EC 1.11.1.9) was measured by monitoring the oxidation of NADPH at 340 nm as described by Flohé *et al.* (4) in a coupled assay with glutathione reductase. Cumene hydroperoxide and hydroperoxide were used as a substrates. Specific activity was defined as the unit of the enzyme activity per mg of protein. Lipide peroxide formation was measured as malondialdehyde and other aldehydes appearance, by reaction with thiobarbituric acid which yielded the coloured products named thiobarbituric acid reactive substances (TBARS) that absorb at 535 nm (7). The content of TBARS was expressed as absorbance/mg of protein. Protein concentration was measured by the method of Bradford (2), using bovine serum albumin as a standard. All reagents of the highest purity were from Sigma, Merck and Boehringer.

Statistics

The results are given as means ± SEM at least of three independent determinations in three different batches. Data were analysed using the Student's *t*-test with a significance level of $p < 0.05$.

RESULTS

Incubation of *S. ruminantium* E32 with Hg^{2+} significantly decreased the GSHPx activity (from 5.58 ± 0.49 to 4.25 ± 0.32 mU.mg^{-1} of proteins). Incubation of *S. ruminantium* E32 with a-tocopherol without mercury chloride showed the possible direct effect of tested substances on the activities of GSHPx or on its expression by a route independent of the oxidative stress response. The addition of a-toc into the cultivation medium significantly decreased the GHPx activity in *S. ruminantium* (from 5.58 ± 0.49 to 4.63 ± 0.11 mU.mg^{-1} of proteins). After the addition of a-tocopherol into cultivation medium with mercury no significant changes were observed.

The activity of the GSHPx in the presence of mercury was significantly increased only when the b-carotene was added into cultivation medium (from 4.25 ± 0.32 to 4.98 ± 0.03 mU.mg^{-1} of proteins, Fig. 1). The TBARS content of *S. ruminantium* was effected in the presence of mercury similary only by b-car. The addition of b-carotene significantly decreased the lipide peroxide formation (Fig. 2).

DISCUSSION

The rumen provides an anaerobic environment where both the obligate and facultative anaerobic bacteria compete and survive. *Selenomonas ruminantium* is a well-characterized Gram-negative anaerobe that has been reported to represent up to 16 % of the total bacterial counts in the rumen (3).

Herbivores, including ruminants, can be exposed to mercury mainly by consumption of contaminated feed. It is known that the plant cells have evolved a complex series of both enzymatic and non-enzymatic antioxidant mechanisms having a direct effect on the reactive oxygen species formed by the environmental stress such as temperature, metal toxicity, air pollutants and xenobiotics (1, 14, 9). Among the main plant antioxidant substances (AOS) are enzymes such as superoxide dismutase, catalases, peroxidases and non-enzymatic substances mainly: vitamins (A, C, E), trace elements (zinc, copper, manganese, selenium) and plant phenolic compounds.

Mercury is a well-known pro-oxidant. Studies with $HgCl_2$ have demonstrated that it exerts an oxidative stress via H_2O_2 generation, GSH depletion, and reaction with membrane bound protein thiols and these may lead to lipid peroxidation (18, 16). The activities of antioxidant enzymes in rumen bacteria *S. ruminantium* are described in more detail in Holovská *et al.*(12). They have shown that *S. ruminantium* under strict anaerobic conditions did not exhibit any SOD, GSHPx-H_2O_2 and CAT activities. Only GSHPx-cum activity, which act mainly on organic hydroperoxides, was determined in tested strain *S. ruminantium* E32 under anaerobic cultivation. GSHPx plays a major role in protecting cells from oxidative damage especially lipid peroxidation of biological membranes (4). Our results have shown the negative effect of experimental concentrations of α-tocopherol on the actitivity of *S. ruminantium* GSHPx. The decrease in GSHPx activity stimulated by α-tocopherol alone, documented in our work, is evidence that some of the non-enzymatic plants AOS are able to limit the expression of enzymes by a route independent of oxidative stress response.

β-carotene is reactive toward peroxyd radicals as a moderate radical-scavenging antioxidant, especially in the lipophilic domain of the membrane at low oxygen concentration (21). In our study, only the plant antioxidant b-carotene showed a significant protective effect of tested GSHPx and TBARS content of *S. ruminantium* E32 in the presence of mercury, similary as described Holov-ská *et al.* (11) in experiments with *Strep. bovis*. *S. ruminantium*, as others ruminal bacteria e.g. *Megaspheare elsdenii* and *Desulfovibrio desulfuricans*, is a sulfide producer. This was evident from the black precipitates which formed during growth of this bacterium at an experimental concentration of mercury. At the same time *S. ruminantium* E32 also possesses the mercury reductase activity (15), enzyme that reduce Hg^{2+} to metalic mercury (Hg^0) and thereby decreases its toxicity.

Our presented results with the protective effect of plants antioxidant β-carotene on elimination of potential environmental stress evoked by mercury in ruminal anaerobic bacteria *S. ruminantium in vitro* and our previous results (11, 12) with *Strep. bovis* encouraged us to formulate a working hypothesis that some of the plant antioxidant substances may play an important role in the elimination of the negative effects of environmental contaminants within the rumen microbial ecosystem. To reveal the whole potentional role of the plant origin of antioxidant substances in the complexity of defence mechanisms of the rumen bacteria against environmental stress will require further more complete studies.

This investigation was supported by Grant Agency VEGA 2/3064/23 and APVT-51-012602.

REFERENCES

1. Bowler, C. C., van Montagu, M., Inzé, D., 1992: Superoxide dismutase and stress tolerance. *Ann. Rev. Plant Physiol. Plant Molec. Biol.,* 48, 83—116.

2. Bradford, M. M., 1976: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* 72, 248—254.

3. Bryant, M. P., 1984: Commentary on the Hungate techniques for culture of anaerobic bacteria. *Am. J. Cli. Nutr.,* 25, 1324—1328.

4. Flohé, L., Günzler, W. A., Ladenstein, R., 1976: Glutathione peroxidase, pp. 115—138. In **Arias, J. M., Jacoby, W. B**. (eds.): *Glutathione Metabolism and Function.* Raven Press, New York.

5. Fridovich, I., 1978: The biology of oxygen radicals. *Science,* 201, 875—880.

6. Fulghum, R. S., Worthington, J. M., 1984: Superoxide dismutase in ruminal bacteria. *Appl. Environ. Microbiol.,* 48, 675—677.

7. Gutteridge, J. M. C., 1984: Ferrous ion-EDTA — stimulated phospholipid peroxidation. *Biochem. J.,* 224, 697—701.

8. Hassan, H. M., Fridovich, I., 1978: Regulation of the synthesis of catalase and peroxidase in *Escherichia coli. J. Biol. Chem.,* 253, 6445—6450.

9. Hernández, J. A., Del Río, L.A., Sevilla, F., 1994: Salt stress-induced changes in superoxide dismutase isoenzymes in leaves and mesophyll protoplasts from *Vigna unguiculata* (L.) Walp. *New Phytol.,* 126, 37—44.

10. Hewitt, J., Morris, J. G., 1974: Superoxide dismutase in some obligately anaerobic bacteria. *FEBS Lett.*, 50, 315—318.

11. Holovská, K., jr., Lenártová, V., Holovská, K., Pristaš, P., Javorský, P., 2002a: Are ruminal bacteria protected against environmental stress by plant's antioxidant? *Reprod. Nutr. Develop.,* 42 (Suppl. 1) – P91.

12. Holovská, K., Holovská, K., jr., Lenártová, V., Javorský, P., 2002b: The activities of antioxidant enzymes in rumen bacteria *Streptococcus bovis* and *Selenomonas ruminantium. Biologia Bratislava,* 57, 299—304.

13. Javorský, P., Rybošová, E., Mlynarčíková, H., Mayerová, Z., Legáth, J., 1993: Resistance of ruminal microorganisms to supermethrin. *Vet. Hum. Toxicol.,* 35, 15—19.

14. Kröniger, W., Rennenberg, H., Polle, A., 1992: Purification of two superoxide dismutase isoenzymes and their subcellular localization in needles and roots of Norway spruce *(Picea abies* L.) trees. *Plant Physiol.,* 100, 334—340.

15. Lenártová, V., Holovská, K., Javorský, P., 1998: The influence of mercury on the antioxidant enzyme activity of rumen bacteria *Streptococcus bovis* and *Selenomonas ruminantium*. *FEMS Microbiol. Ecol.,* 27, 319—325.

16. Lund, B. O., Miller, D. M., Wood, J. S., 1991: Mercury-induced H_2O_2 production and lipid oxidation *in vitro* in rat kidney mitochondria. *Biochem. Pharmacol.,* 42, 181—187.

17. McCord, J. M., Keele, B. B., Fridovich, I., 1971: An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proc. Nat. Acad. Sci. U.S.A.,* 68, 1024—1027.

18. Ribarov, S. R., Benov, L. C., 1981: Relationship between the hemolytic action of heavy metals and lipid peroxidation. *Biochim. Biophys. Acta,* 640, 721—726.

19. Storz, G., Tartaglia, L. A., Farr, S. B., Ames, B. N., 1990: Bacterial defenses against oxidative stress. *Trends in Genetic,* 6, 363—368.

20. Tiwari, A. D., Bryant, M. P., Wolfe, R. S., 1969: Simple method for isolation of *Selenomonas ruminantium* and some nutritional characteristics of the species. *J. Dairy Sci.,* 52, 2054—2056.

21. Tsuchihashi, H., Kigoshi, M., Iwatsuki, M., Niki, E., 1995: Action of β-carotene as an antioxidant against lipid peroxidation. *Arch. Biochem. Biophys.,* 323, 137—147.

22. Wimpenny, J. W. T., Samah, O. A., 1978: Some effects of oxygen on the growth and physiology of *Selenomonas ruminantium*. *J. Gen. Microbiol.,* 108, 329—332.

Received May 17, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 66—72, 2004

AN ADDITIONAL METHOD OF DISTINGUISHING BETWEEN HYPOTHYROIDISM AND EUTHYROID SICK SYNDROME

**Fialkovičová, M., Benková, M.*, Kozák, M.
Škardová, I., Valocký, I., Sesztáková, E., Boldižár, M.**

**University of Veterinary Medicine, Komenského 73, 041 81 Košice
* Technical University, Letná 9, 041 01 Košice
The Slovak Republic**

fialkovicova@uvm.sk

ABSTRACT

Our study is focused on the deeper analysis of dermatological diseases, which we monitored for seven years (1992—1998) at the surgery of the First Internal Clinic at University of Veterinary Medicine in Košice. We have used the results of the examinations of 175 dogs from the monitored group with cutaneous changes and the results of 99 healthy dogs, which were of the different age and sex. All the dogs were divided according to their weight into big, medium and small breeds. Separations of the blood samples were carried out in regular intervals during the year, which has enabled us to determine the fluctuation of the thyroid levels during all the seasons. The results of endocrinological examinations confirmed the fact that assignment of concentrations of total thyroxine (tT4) in blood serum in dogs is more important for assessment of the thyroid gland function than the assignment of the total triiodothyronine levels (tT3). We have also discovered that the elevation of thyroxine levels is different in various breeds. Reduced concentrations of tT4 can in concert with those clinical symptoms indicate the hypofunction of thyroid gland in affected dogs, but also the other non-thyroid diseases, in which the level of thyroxine in blood serum can fall below the referential scale. The distinguishing of true hypothyroidism from euthyroid syndrome has a crucial importance for the patient, because the supplemental thyroid therapy used in the hypofunction of thyroid gland is contra-indicated in euthyroid dogs. The aim of this work was to offer a quick way of distinguishing euthyroid dogs from hypothyreoid ones by determination of tT4. We determined the ranges of tT4 levels in which, as expected, thyroxine concentrations of euthyroid, hypothyroid and healthy dogs would most probably occur in big, medium and small breeds.

Key words: dogs; endocrinopathy; euthyroid sick syndrome; hypothyroidism; intervals of reliability; skin

INTRODUCTION

This article presents a deeper analysis of dermatological diseases, which we monitored for seven years (1992—1998) at the surgery of the First Internal Clinic at the University of Veterinary Medicine in Košice. After division of dermatological patients into groups according to the character of the disease we have recorded the most represented group ofE endocrine dermatological disease, where low concentration levels of thyroid hormones were discovered. Hypothyroidism is the most common endocrinopathy in dogs that is manifested as a disorder of canine skin. The main causes of canine hypothyroidism are auto-immune lymphocytic thyroiditis and idiopathic thyroidal atrophy.

Dermatological disease, related directly to pathological damage to the thyroid gland, is called *true hypothyroidism*. The problem occurs when low levels of thyroid hormones in the blood are induced by diseases of other organs (adrenal gland, pancreas, liver, kidneys) that are not connected with the thyroid gland. This phenomenon is called *euthyroid sickness*

Table 1. The number of monitored dogs of individual breeds divided according to weight and health state

Weight	Healthy dogs	No.	Dogs with skin changes	No.	No.
	Great Dane	3	Mastiff	3	
	Barzoi	1	Barzoi	1	
	Old English Sheep Dog	1	Old English Sheep Dog	1	
	Briard	1	Fila Brasileiro	1	
	Newfoundland	1	Briard	3	
	Dobrman Pinscher	3	Doberman Pinscher	11	
over 25 kg	Siberian Husky	3	Labrador Retriever	7	
	Collie	1	Leonberger	1	
	Labrador Retriever	2	Mastino Neapolitano	1	
	German Shepherd	30	Great Dane	9	
	Kuvasz	4	Newfoundland	2	
	Staford Terrier	1	German Shepherd	44	
			Rottweiler	4	
			Siberian Husky	1	
			Kuvasz	1	
			Staford Terrier	2	
			Giant Snauzer	2	
	Total	**51**		**94**	**145**
	Boxer	5	Boxer	7	
	Dalmatian	7	Dalmatian	2	
	Bullterrier	2	Bullterrier	2	
10–25 kg	Cocker Spaniel	6	Cocker Spaniel	9	
	German Spitz	1	German Spitz	2	
	Crossbread	2	Crossbread	7	
	Springer Spaniel	1	Shar-pei	1	
			Pittbull	2	
			Chow-chow	4	
			Lhasa-apso	1	
			Puli	2	
	Total	**24**		**39**	**63**
	Dachshund	9	Dachshund	3	
	Poodle Toy	7	Poodle Toy	14	
	German Spitz	2	German Spitz	4	
to 10 kg	Pekinese	2	Miniatur Schnauzer	5	
	Cavalier King Ch. Spaniel	2	Cavalier King Ch. Spaniel	1	
	Miniature Pincher	1	Miniature Pincher	5	
	Maltese	1	Maltese	5	
			Carlin Pug	3	
			Foxterrier	2	
	Total	**24**		**42**	**66**

syndrome. In such euthyroid dogs, dermatological diseases are caused by non-thyroid diseases, but concentrations of tT3 and tT4 are as low as in hypothyroidism.

The differentiation of euthyroid sickness syndrome from true hypothyroidism plays a key role in the therapy of chronic dermatoses in dogs. Dermatological disease caused by hypothyroidism can be treated by a supplement of missing hormones in substitute hormonal therapy. With euthyroid sickness syndrome the administration of substitute hormonal therapy may be contraindicated, because the thyroid gland is not impaired.

In a proper treatment of the dermatological patient with reduced tT4 concentrations levels it would be more appropriate to monitor the dynamics of thyroid gland function and to evaluate tT4 levels more than at least twice a year. We should

not forget that the thyroid gland plays an important role in thermoregulation of the organism. Serum levels of tT4 may be low in the summer months, but they often increase to reference range in autumn. The veterinary practitioner is often urged to start the treatment of cutaneous disease at the first discovery of lowered tT4 serum concentration, when it need not be confirmed yet whether the dog is euthyroid or hypothyroid. This is why, we undertook to define the ranges of tT4 levels in which the resulting values of total thyroxine could be expected with the highest probability in euthyroid dogs and hypothyroid dogs right after the first measurement of their concentrations. These tT4 level ranges are also referred to as intervals of reliability and help to discern faster between euthyroid dogs and hypothyroid dogs in big, medium and small breeds.

If the serum level of tT4 concentration is lower than the physiological range, it is very desirable to use an additive method of the intervals of reliability, which determine the tT4 levels range where the results of the examinations of the absolutely healthy dogs, hypothyroid dogs and euthyroid dogs should be.

MATERIAL AND METHODS

Out of the group of dogs with potential endocrine dermatoses 175 dogs with skin changes were included in the study and the results obtained in them were compared with those of 99 healthy dogs. The selected dogs were of different age and of both sexes. Healthy dogs and those with skin changes were divided into three groups according to their weight: small breeds (up to 10 kg), medium breeds (10—25 kg), and large breeds (over 25 kg). Table 1 marks the number of monitored dogs of individual breeds divided according weight and health state.

The healthy dogs and patients with skin changes were subjected to a total clinical examination that included haematological and endocrinological examination of blood. The biochemical examination included determination of ALT, AST, ALP, alpha-amylase, glucose, urea, creatinine, cholesterol, total lipids and proteins. The patients were subjected to bacteriological, mycological and parasitological examination of their skin. TRH-stimulation tests were performed according to need.

The levels of thyroid hormones were determined in all weight groups of dogs at regular intervals throughout the year. The concentrations of thyroid hormones were determined by RIA tests total tri-iodothyronine and total thyroxine manufactured by HUMA- Lab Košice, SR. The accuracy of determination was based on intra (c.v.: 3.8 ± 0.4; 5.2 ± 0.93; 5.8 ± 0.81) and inter assay (c.v.: 8.45 ± 1.1; 7.63 ± 0.96; 9.29 ± 0.31). We used the readings of total thyroxine to show their average concentrations in diagrams and we also specified the decisive deviations. Biochemical examinations were carried out by means of Bio-La tests produced by Lachema Brno, CzR.

Dogs with skin changes (n = 175) were grouped according to verified diagnoses into hypothyroid and euthyroid dogs, and, on the basis of their tT4 concentrations, we calculated the corresponding reliability intervals.

These, in turn, determine the range of tT4 levels where, as assumed, the concentrations of total thyroxine of euthyroid, hypothyroid as well as healthy dogs of all weight groups may most probably occur.

RESULTS

Repeated examinations of sera obtained from 99 healthy dogs showed that the level of tT4 depends on size or weight of breed. The physiological tT4 levels are higher in small breeds of dogs than in healthy dogs of medium and large breeds (Fig. 1).

Average concentrations of thyroxine in healthy large

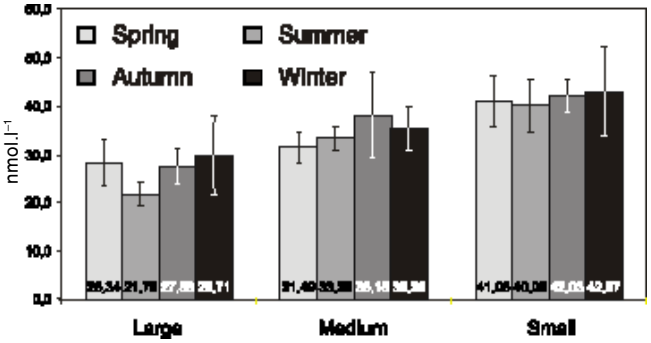

Fig. 1. tT4 concentration (nmol. l^{-1}) in the blood serum of healthy dogs

breeds show half values tT4 than in small dogs. This is mostly evident in comparing average concentrations tT4 in large dogs during summer (21, 75 nmol.l^{-1}) with an average concentration of tT4 in small breeds during winter (42, 87 nmol.l^{-1}). An evaluation of the results according to seasons confirmed that the level of tT4 changes over the year. Concentrations of thyroxine in blood serum are during spring and summer lower than during autumn and winter in all healthy dogs of all weight groups.

The mean values of tT4 in dogs with skin changes were highest in dogs of small breeds and lowest in dogs of large (Fig. 2).

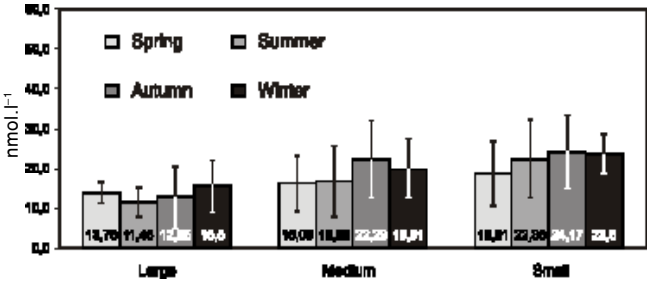

Fig. 2. tT4 concentration (nmol.l^{-1}) in the blood serum of dogs with skin changes

When comparing all weight groups of sick dogs, we observed the same seasonal dynamics of tT4 as those determined in clinically healthy dogs. We found out that the range of reference values for total thyroxine depends

Table 2. The range of references values for total thyroxin

small breeds	medium breeds	large breeds
30–60 nmol.l^{-1}	25–50 nmol.l^{-1}	20–40 nmol.l^{-1}

particularly on the size of breed and because of this the dogs were divided as follows (Tab. 2).

Biochemical examinations were carried out with the aim of finding in the dogs with skin changes signs of other non-thyroid diseases that could explain the decrease in total levels of T3 and T4.

On the basis of additional clinical findings accompanying skin changes in a total of 36 dogs, which exhibited corresponding impaired biochemical parameters, we were able to identify a group of euthyroid dogs. According to the dominant non-thyroid diseases we divided the dogs into several subgroups. The first included dogs suffering from gastrointestinal diseases, the second — dogs with urological problems and the third group consisted of three bitches after parturition. The fourth subgroup comprised individuals with other diseases in which multi-organ affection, polyendocrinopaties, and impairment of the immune system was suspected (Tab. 3).

The dogs suspected of hypothyreosis had increased levels of cholesterol and total lipids which approached the acceptable limits. The activity of ALP and alpha-amylase in these dogs was increased considerably in comparison with the reference levels. Our observations indicated that the tT4 concentrations in dogs suspected of hypothyreosis but also in euthyroid dogs had decreased compared to the reference range. The calculation of mean values of T4 in euthyroid dogs of all weight groups revealed that their concentrations were higher than the mean concentrations of T4 in hypothyroid dogs but noticerably lower than those in healthy dogs. (Figs. 3, 4, 5).

It follows from the Figs. 3, 4, 5 that the discovered low tT4 concentrations in hypothyroid dogs may overlap the decreased tT4 levels of euthyroid dogs. Due to this, as an objective, we set the determination of tT4 level ranges, in which we could expect the resulting tT4 of

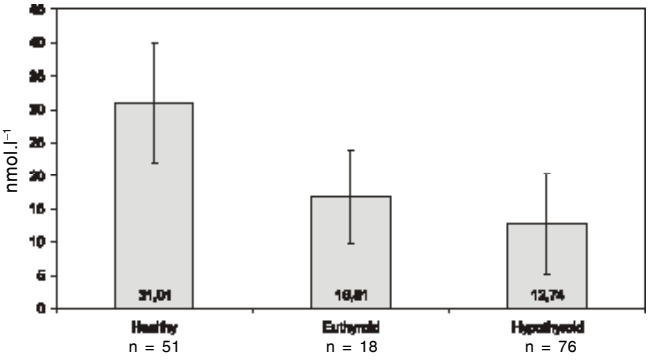

Fig. 3. Average levels of thyroxine in healthy, euthyroid and hypothyroid dogs of large breeds

Table 3. Average values of biochemical parameters in euthyroid, hypothyroid and healthy dogs

Parameter	Reference range	EUTHYROID DOGS Urological diseases n =10	Gastro-intestinal diseases n =16	After parturition n = 3	Another diseases n = 7	HYPOTHYROID DOGS n = 137	HEALTHY DOGS n = 99
tT4	20–60 nmol.l^{-1}	14.4	22.18	14.33	20.48	16.7	36.9
ALT	to 0.33 mkat.l^{-1}	0.55	1.09	0.42	0.76	0.6	0.6
AST	0.1–0.3 mkat.l^{-1}	0.75	1.25	0.39	0.99	0.6	0.4
ALP	0.08–0.4 mkat.l^{-1}	2.14	3.48	1.06	1.86	1.9	1.4
Urea	3.3–6.3 mmol.l^{-1}	38.65	5.44	5.41	7.46	7.6	5.2
Creat.	88–177 mmol.l^{-1}	520.4	105.2	94.3	120.7	138.6	103.3
Glu.	4.0–6.0 mmol.l^{-1}	3.78	5.48	3.49	5.61	5.4	4.7
pAMS	to 25 mkat.l^{-1}	28.92	37.28	39.13	29.56	38.7	24.0
Chol.	3.2–6.5 mmol.l^{-1}	6.48	6.77	6.55	5.86	6.07	4.6
Tot.lip.	4.7–7.25 g.l^{-1}	6.93	7.01	6.49	6.06	7.2	5.9
Tot. prot.	**57–75 g.l^{-1}**	**59.69**	**61.07**	**63.05**	**60.08**	**63.49**	**69.89**

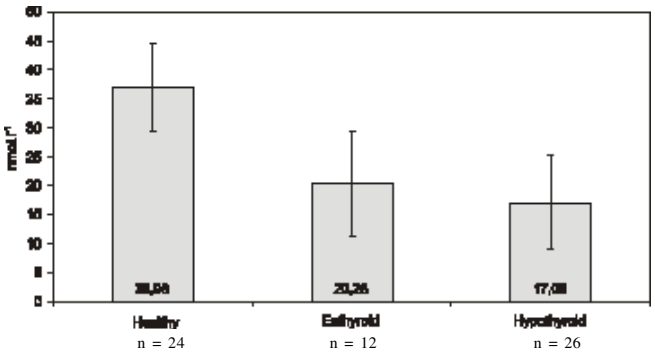

Fig. 4. Average levels of thyroxine in healthy, euthyroid and hypothyroid dogs of medium breeds

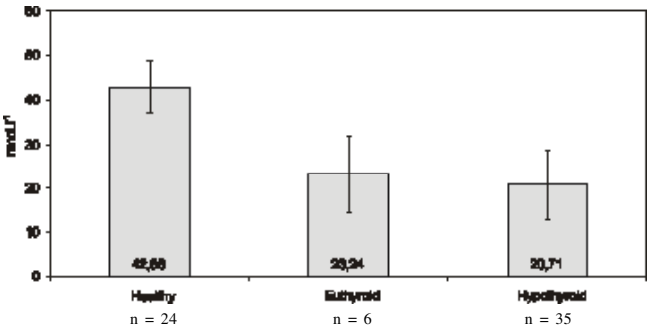

Fig. 5. Average levels of thyroxine in healthy, euthyroid and hypothyroid dogs of small breeds

both euthyroid and hypothyroid dogs with the highest probability when compared with healthy dogs in all weight groups. These ranges are referred to as reliability intervals and they are unique due to the fact that they were acquired from tT4 concentrations of relatively large groups of patients, in which both the diagnosis of hypothyreosis and euthyroid syndrome were verified by therapy applied over a couple of years.

Because it is very important to distinguish between euthyroid and hypothyroid dogs in skin disease therapy it is possible to use an orientating method for the quick evaluation of the results of T4 before TRH stimulation test (Tab. 4).

The reliability intervals for tT4 were obtained by statistical calculations using tT4 concentrations of individual euthyroid dogs and the same approach was used to calculate these intervals also for healthy and hypothyroid dogs. This calculation allowed us to determine the total range of T4 values that should include the results of healthy dogs and hypothyroid dogs and, at same time, the dispersion variance of tT4 values of most of euthyroid dogs of all weight groups. By comparing individual intervals we were able to observe that the ranges of tT4 concentrations for euthyroid dogs overlapped with thyroxine ranges, particularly those of hypothyroid dogs of small and medium breeds, and, to a small extent, with the reference level for healthy individuals of small breeds.

Table 4. Reliability intervals for tT4 in healthy, euthyroid and hypothyroid dogs

Breed	Healthy dogs	Euthyroid dogs	Hypothyroid dogs
Large	26.15–28.58	13.23–20.38	12.02–13.68
Medium	33.71–40.24	14.57–25.98	13.95–20.15
Small	40.36–45.35	14.07–32.40	18.17–23.25

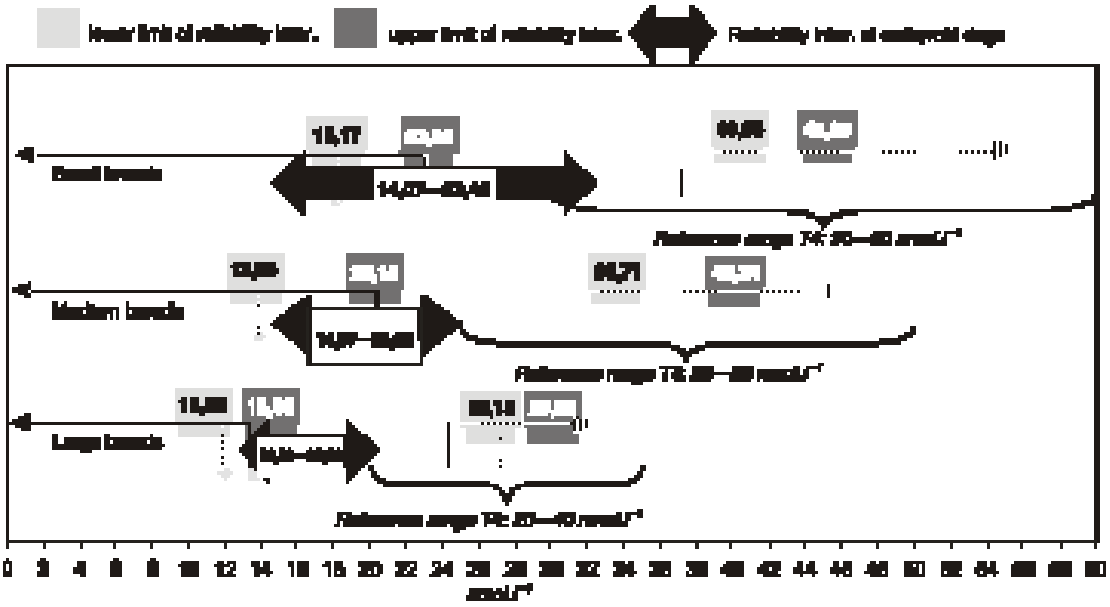

Fig. 6. Comparing of reliability intervals for tT4 in healthy, euthyroid and hypothyroid dogs

The upper limit of reliability intervals for euthyroid dogs allowed us to confirm the correctness of determination of the lower reference limit of tT4 for healthy dogs of all weight groups (Fig. 6).

DISCUSSION AND CONCLUSIONS

The measurement of the total level of thyroxine is one of the basic examinations in differential diagnosis of chronic skin diseases. Panciera (8) has stated that tT4 concentrations are a better measure of the thyroid gland function than the levels of tT3. Our results, however, show a large series of variations in tT4 level values, not only in sick, but even in healthy dogs. Several authors have tried to account for large differences in the physiological levels of total thyroxine between the breeds with wide ranges of reference limits of healthy dogs. Ramsey (12) considers reference ranges T4 = 25—80 nmol.l^{-1} as physiological results of examinations. Nelson (7) uses the following range for total thyroxine: tT4 = 20—60 nmol.l^{-1}.

The application of such a wide standard has not proved to be satisfactory for us for the reason that, though the results in the lower limit of standard, e.g. 22—25 nmol.l^{-1} found in small dogs, were in reference levels under our conditions they are insufficient for this weight category of dogs up to 10 kg of live weight. In contrast, the concentration results of tT4 above 40 nmol.l^{-1} are common physiological findings in small dog breeds and occur rather rarely in large breeds (17).

We tried to overcome the considerable interbreed differences in physiological values of tT4 between dog breeds by dividing the reference values of tT4 according to weight groups. According to Ferguson (3), the levels of tT4 in dogs of small breeds are generally higher than those in medium and large breeds. Panciera (9) also claims that tT4 concentration results obtained from Great Danes and Borzois reached only the half of what was found in small breeds.

The decreased tT4 levels may be ascribed to physiological factors, such as breed and age, but the literature has not paid too much attention to the influence played by the season of the year on its concentrations. Under our conditions, we recorded this fact in healthy dogs as well as in dogs with skin changes (17). We should not forget that the thyroid gland plays an important role in thermoregulation of the body. In summer months, its function decreases and tT4 levels physiologically decrease. However, in autumn when the weather changes and temperatures gradually decrease, its activity is stimulated and this, in turn, makes tT4 concentrations in blood increase, too. Similarly, other authors (9, 16), due to variations in the level of total thyroxine, recommend that its levels be monitored repeatedly in the course of the year.

The decreased tT4 may also occur in hypothyroidism. Insufficient production of thyroid hormones causes a slower metabolism and changes in the physiological functions of many organ systems (13, 5, 7, 14).

The thyroid gland takes part in most of the metabolic processes of the body – it influences protheosynthesis, metabolism of lipids and sugars, regulates intake and expenditure of energy, and oxygen consumption by tissues (4). Hypothyroidism therefore does not have to manifest itself only in various dermatological diseases, but it is connected with the occurrence of other symptoms (lethargy, obesity, infertility, cardiovascular diseases, neuromuscular diseases). The decreased T4 levels may reflect the response of an organism to internal diseases, which means that reduced concentrations of T4 occur in dogs with euthyroid syndrome suffering from non-thyroidal diseases.

The discrimination between euthyroid and hypothyroid dogs plays an important role in the therapy of skin diseases. Evaluation of the effect of individual factors on the level of T4 is rather demanding and because of that the final diagnosis depends on evaluation of anamnesis, clinical and biochemical examination, and evaluation of thyroid gland function and response to experimental therapeutic supplementation (11). Merchant and Taboada (6) have stated that the lack of tT4-decreasing factors together with very low serum tT4 means that the clinical symptoms indicating hypothyroidism are sufficient proof of the diagnosis.

Some authors (2, 9, 15) abide by the rule that the probability of hypothyreosis in dogs increases directly with the decrease in total level of T4 below the reference level of healthy dogs. The observation of total T4 remains still a valuable practical tool for assessment of thyroidal function in dogs despite the fact that the opinions about the usefulness in practice of some tests for testing the thyroid gland function vary. The majority of authors (11, 10, 1) use a combination of several methods for evaluation of the thyroid function in dogs. Besides total T4, the concentration of free T4, canine TSH, and thyroid stimulation tests are of particular importance. In our study we used also the TRH stimulation test, which contributed to discrimination between euthyroid and hypothyroid dogs.

For this differentiation, biochemical indices in blood are also commonly used. So as a routine, the occurrence of non-thyroid disease is looked for, because this might explain the decreased tT4 concentration and simultaneously skin lesions too (12). We also used these examinations for differentiatiing patients with gastrointestinal, urological and other diseases too and so we created a group of euthyrioid dogs. Though the biochemical examinations of blood in hypothyreoid dogs are not decisive establishing a diagnosis, however, they may support it. The most common abnormalities which occur are hyperlipidemia and hypercholesterolemia, which may be found in 60—80 % of patients with such diagnosis (9,12). Under our conditions, they occurred in 47 % of hypothyroid dogs and this manifested itself in average results of cholesterol and total lipids which were found on the upper limit of the standard.

Problems in establishing a diagnosis in patients with chronic skin disease and decreased concentration of tT4

are found mainly when the non-thyroid disease has not yet manifested itself at a biochemical level. If this is the case, it is very helpful to use the method of intervals of reliability which helps to distinguish euthyroid dogs from hypothyroid dogs.

REFERENCES

1. Dixon, M., Mooney, C. T., 1999: Evaluation of serum free thyroxine and thyrotropin concentrations in the diagnosis of canine hypothyroidism. *Journal of Small Animal Practice,* 40, 72—78.

2. Ferguson, D. C., 1997: Euthyroid sick syndrom. *Canine Practice*, 22, 49—51.

3. Ferguson, D. C., 1988: The effect of nonthyroidal factors on thyroid function tests in dogs. *Compendium on Continuing Education for the Practicing Veterinarian*, 10, 1365—137.

4. Freake, H. C., Oppenheimer, J. H., 1995: Thermogenesis and thyroid function. *Annual Review of Nutrition*, 15, 263—291.

5. Jaggy, A., Oliver, J. E., Ferguson, D. C., Mahyffey, E. A., Glaus, T. Jr., 1994: Neurological manifestations of hypothyroidism, a retrospective study of 29 dogs. *Journal of Veterinary Internal Medicine*, 8, 328—336.

6. Merchant, S. R. ; Taboada, J., 1997: Endocrinopathies — Thyroid and adrenal disorders. *Veterinary Clinics of North America – Small Animal Practice*, 27, 1285—1303.

7. Nelson, R. W., 1996: Diagnosis and treatment of canine hypothyroidism. *Veterinary Quarterly*, 18, 29—31.

8. Panciera, D., 1994: Hypothyroidism in dogs – 66 cases (1987—1992). *Journal of American Veterinary Medical Association*, 204, 761—767.

9. Panciera, D., 1999: It is possible to diagnose canine hypothyroidism? *Journal of Small Animal Practice*, 40, 152—157.

10. Peterson, M. E., Melian, C., Nichols, R., 1997: Measurement of total thyroxine, thriodothyroxine, free thyroxine and thyrotropin concentrations for diagnosis of hypothyroidism in dogs. *Journal of American Veterinary Medical Association*, 211, 1396—1402.

11. Ramsey, I., Herrtage, M., 1997: Distinquising normal, sick and hypothyroid dogs using total thyroxine and thyrotropin concentrations. *Canine Practice*, 22, 43—44.

12. Ramsey, I., 1997: Diagnosing canine hypothyroidism. *In Practice*, 19, 378—381.

13. Rios, A., Fernandez, T., Gomez L., 1990: Hypothyroidism in dog: Diagnosis and treatment. *Veterinary International,* 20, 25—34.

14. Rosychuk, R. A. W., 1998: Cutaneous manifestation of endocrine disease in dogs. *Compendium on Continuing Education for the Practicing Veterinarian*, 20, 287–303.

15. Scott-Moncrieff, J. C., 1997: Serum canine thyroptropin concentrations in experimental and spontaneous canine hypothyroidism. *Canine Practice*, 22, 41—42.

16. Thacker, E. L., 1997: Etiology of adult-onset canine autoimmune hypothyroidism. *Canine Practice,* 22, 12—13.

17. Tučková, M., Fialkovičová, M., Baranová, D., Bekeová, E., Kozák, M., Páleník, L., 1995: The influence of year dynamic of serum thyroid hormones on the health status of German Shepherd and Badler-dog (In Slovak). *Veterinární Medicína-Czech,* 40, 249—252.

Received May 13, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 73—75, 2004

THE MONITORING OF SELECTED PARAMETERS OF NON-SPECIFIC IMMUNITY IN DOGS WITH PYODERMA (A Preliminary Report)

Hromada, R., Mojžišová, J., Pošiváková, S., Pošivák, J. Michalková, Z., Bajová, V., Paulík, Š.

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

hromada@uvm.sk

ABSTRACT

The occurrence of immunodeficiencies in a group of ten dogs with pyoderma was evaluated in this study. The level of phagocytic activity of leukocytes, proliferation activity of lymphocytes and total leukocyte count were tested and compared with those in ten healthy dogs. In most cases *Staphylococcus intermedius* was isolated as a main causative agent. In dogs with pyoderma a low total leukocyte count, phagocytic activity of leukocytes, proliferation activity of lymphocytes after stimulation with Con A and PHA-P and higher ingestion capacity of phagocytes were found. We can conclude that pyoderma is associated with decreasing activity of cell-mediated immunity.

Key words: dogs; immune system; pyoderma

INTRODUCTION

A variable level of immunosuppression is associated with infectious and parasitic diseases (4). The occurrence of immunodeficiencies in the canine population is not yet completely known because of the lack of diagnosis in small animal practices. Signs of immunosuppression are variable and dependent on the primary disease and degree of the immune system alteration. Pyoderma belongs among diseases in which immunosuppression was reported (16, 20). The aim of our study was to evaluate some parameters of cell-mediated immunity in dogs with pyoderma as a primary disease caused by *Staphylococcus intermedius* in comparison with healthy dogs.

MATERIAL AND METHODS

Animals. Ten dogs of various, sex and breeds with an average age of 4.1 years were treated at the Infectious Disesase Department of UVM Košice.

Blood was taken from *v. cephalica*. Skin scrapings were collected for laboratory examination. Bacteriological infection was verified by the BBL Crystal identification system (Becton Dickinson, USA). Superficial pyoderma was a principal diagnosis in dogs examined, without signs of other diseases.

Ten uninfected dogs with an average age of 3.6 years, different breeds and sexes served as control animals.

Blastogenic response of blood lymphocytes to mitogens. Lymphocytes were separated from venous blood on the Ficoll density gradient (Pharmacia Biotech AB, Sweden). Concanavalin A (Con A, Sigma Chemical Co., USA) and Phytohaemagglutinin (PHA-P, Sigma Chemical Co., USA) were used for stimulation at a concentration of 25 $mg.ml^{-1}$ and 20 $mg.ml^{-1}$, respectively (18). The level of the blastogenic response of the lymphocytes was estimated using the EB fluorescence test (15) and expressed as the fluorescence intensity of stimulated (FISC) and unstimulated cells (FIUC), and the stimulation index (SI). The FI was measured by a spectrofluorometer (Jasco FP–550, Japan).

The phagocytic activity of blood leukocytes was examined as described by Větvička *et al.* (21) using phagocytosis of 2-hydroxyethylmetacrylate particles (MSHP, diameter 1.2 mm, ARTIM Prague, The Czech Republic). The phagocytic activity (PA) of neutrophils (Ne) was expressed as the percentage of the neutrophils phagocytizing 3 and more MSHP, and as the index of phagocytic activity (IPA) representing the ingestion

ability of neutrophils (the ratio of the number of phagocytized MSHP and the number of potentially phagocytizing Ne).

The total leukocyte count was determined using common haematological methods.

Statistical analyses. The data were characterized by the mean and standard deviation. The significance of differences was checked by Anova-Manova.

RESULTS

The results of the hematological and immunological tests are shown in Table 1. The pyoderma was diagnosed in ten dogs – clinical patients by the clinical examination and laboratory bacteriological tests that confirmed the presence of *Staphylococcus intermedius.* No signs of other disease were detected. Pyoderma was classified as superficial**.** Marked reduction of total leukocyte count was observed in the affected dogs in contrast with those of the healthy animals. The phagocytic activity of leukocytes was significantly suppressed in dogs with pyoderma in contrast with healthy dogs. On the other hand the ingestion capacity of phagocytes expressed as a phagocytic index was markedly increased. The stimulation index of lymphocytes expressing the degree of the proliferation activity of lymphocytes after stimulation with mitogens (ConA and PHA-P), was significantly reduced in all cases of the affected dogs. These results indicate that bacterial superficial pyoderma is accompanied by serious impairment of cellular immune system activity.

Tab. 1. Some haematological and immunological parameters in dogs with pyoderma and clinically healthy dogs

Dogs parameter	Healthy dogs	Dogs with pyoderma	Signi-ficance
Lc	9700 ± 1972	6980 ± 2263	*
PA	79.5 ± 12.841	45 ± 6.819	*
PI	6.3 ± 0.367	9.48 ± 0.148	*
SI(ConA)	2.8 ± 0.633	1.36 ± 0.401	*
SI(PHA-P)	2.82 ± 0.626	1.20 ± 0.494	*

Legends: Lc — leukocytes, PA — phagocytic activity, PI — phagocytic index, SI — stimulation index, * — $p < 0.05$

DISCUSSION

Staphylococcal infection of skin is a common clinical problem in the dog population. The disease can be characterized as a superficial or deep infection. Pyoderma is usually associated with flea bite hypersensitivity, superficial dermatitis, food hypersensitivity, endocrinological disorders, idiopathic diseases (16). Pyoderma can develop as a secondary complication (7) resulting from the suppression of cell-mediated immunity in demodicosis (1), dermatomycosis (13) or atopy (9).

The results reported in this study demonstrate a reduction in total leukocyte count, phagocytic activity, an increase of the ingestion capacity of phagocytes and marked suppression of the functional activity of lymphocytes.

The immunosuppression associated with pyoderma has been described in a previous study (20), where its severity depended on the stage and duration of the primary disease. In our findings pyoderma developed without signs of any other known disease thus indicating a direct relationship between reduced immune system reactivity and pyogenic skin infection.

In inflammatory skin disease, neutrophils play a crucial role in the defense mechanism against pathogenic bacteria (14). Defective neutrophil functions increase susceptibility to opportunistic pathogens (17). Neutrophil dysfunctions have been described in pyoderma affected dogs (10, 5, 19). In most studies the initial cause of reduced neutrophil reactivity is a primary disease that predisposes the development of a secondary infection. Neutrophil dysfunction was a secondary phenomenom to demodicosis (12).

Skin inflammatory process in our study stimulated the ingestion capacity of neutrophils, but phagocytic activity as well as total leukocyte count significantly decreased. Prolonged exposure of neutrophils to a high concentration of inflammatory mediators could have resulted in a generalized deactivation of cellular function due to receptor down-regulation (8). So, a longer lasting skin condition as in the dogs in our study can be accompanied by immunosuppression.

The alteration of the proliferation activity of lymphocytes in dogs with pyoderma reported in this paper is in agreement with the findings of other authors (2, 20). The occurrence of the suppression of functional activity of lymphocytes is clearly present in German Shepherds pyoderma (11, 3) in relation to the presence of a serum suppressive factor (2). GSP is described as a deep pyoderma, while in our study pyogenic process was limited to a superficial condition. Analyses of the alteration of lymphocyte population requires more detailed study. Based on the results of Chabane *et al.* (3) the operation of helper lymphocytes is the most altered.

The most common etiological agents of pyoderma are staphylococci. The immunomodulatory effects of staphylococcal antigens on canine leukocytes *in vitro* has been clearly presented in a study by DeBoer (7). The possible suppressive effect of staphylococci *in vivo* on the canine immune system is indicated in study by Toman *et al*. (20). Our findings contribute to this explanation of immunosuppression in dogs with pyoderma, where the main causative agent is *Staphylococcus intermedius*.

REFERENCES

1. Barriga, O. O., Al-Khalidi, N. W., Martin, S., Wyman, M., 1992: Evidence of immunosupression by *Demodex canis*. *Vet. Immunol. Immunopathol*., 32, 37—46.

2. Barta, O., Waltman, C., Shaffer, L. M., Oyekan, P., 1982: Effect of serum on lymphocyte blastogenesis. I. Basic characteristics of action by diseased dog serum. *Vet. Immunol. Immunopathol*., 3, 567—583.

3. Chabanne, L., Marchal, T., Denerolle, P., Magnol, J. P., Fournel, C., Monier, J. C., Rigal, D., 1995: Lymphocyte subset abnormalities in German shepherd dog pyoderma (GSP). *Vet. Immunol. Immunopathol*., 49, 189—198.

4. Cockerell, G. L., 1978: Naturally occurring acquired immunodeficiency diseases of the dog and cat. *Vet. Clin. North. Am. Small Anim. Pract*., 8, 613—628.

5. Couto, C. G., Krakowka, G., Johnson, P., 1989: *In vitro* immunologic features of Weimaraner dogs with neutrophil abnormalities and reccurent infections. *Vet. Immunol. Immunopathol*., 23, 103—112.

6. DeBoer, D. J., 1990: Strategies for management of recurrent pyoderma in dogs. *Vet. Clin. North Am. Small Anim. Pract.*, 20, 1509—1524.

7. DeBoer, D. J., 1994: Immunomodulatory effects of staphylococcal antigen and antigen-antibody complexes on canine mononuclear and polymorphonuclear leucocytes. *Amer. J. Vet. Res.,* 12, 1690—1696.

8. Donabedian, H., Gallisn, J. I., 1981.: Deactivation of human neutrophil chemotaxis by chemoattractants: effect on receptors for the chemotactic factor f-Met-Leu-Phe. *J. Immunol.,* 127, 839—844.

9. Hromada, R., Mojžišová, J., Paulík, Š., Chandoga, P., Čížek, M., 2001: Monitoring of non-specific immunity in dogs with skin atopy. *Scientific Proceedings Voorjaarsdagen 2001, World Congress, Amsterdam*, 20—22 April, 169.

10. Latimer, K. S., Prasse, K. W., Dawe, D. L., 1982: A transient deficit in neutrophilic chemotaxis in dog with recurrent staphylococcal pyoderma. *Vet. Pathol*., 19, 223—229.

11. Miller, W. H., Jr., 1991: Deep pyoderma in two German shepherd dogs associated with cell-mediated immunodeficiency. *J. Amer. Anim. Hosp. Assoc*., 27, 513—517.

12. Mojžišová, J., Paulík, Š., Baranová, D., 1999: Impairment of neutrophil and lymphocyte functions in dogs with uncomplicated and pyoderma complicated demodicosis. *Vet. Med.-Czech*, 44, 19—24.

13. Mojžišová, J., Hromada, R., Paulík, Š., Hipíková, V., Bajová, V., Čížek, M., 2002: Non-specific immune response in dogs with dermatomycosis. *Folia Veterinaria*, 46, 139—141.

14. Muller, G. H., Kirk, R. W., Scott, D. W., 1989: *Small Animal Dermatology* (4th edn.). WB Sauders Co, Philadelphia, 382—385, 428—436.

15. Nakanashi, A., Aimi, K., Ejima, H., Kurokawa, K., 1986: Measurement of blastogenesis of canine peripheral blood lymphocytes against PHA by glucose consuption test. *Jpn. J. Vet. Sci*., 48, 53—60.

16. Rosser, E. J., 1997: German shepherd dog pyoderma: A prospective study of 12 dogs. *J. Am. Anim. Hosp. Assoc*., 33, 355.

17. Rotrosen, D., Gallin, J. I., 1987: Disorders of phagocyte function. *Ann. Rev. Immunol*., 5, 127—150.

18. Tajima, M., Fujinaga, T., Okamoto, Y., Otomo, K., Koike, T., 1990: Relationship between mitogen receptors in peripheral blood lymphocytes and blastogenic responses to mitogen. *Res. Vet. Sci*., 48, 1—5.

19. Toman, M., Rybníček, J., Svoboda, M., Krejčí, J., 1995: Secondary immunodeficiency or immunosupression in dogs. In *Fourth International Veterinary Symposium*, Davis, 167.

20. Toman, M., Svoboda, M., Rybníček, J., Krejčí, J., Faldyna, M., Bárta, O., 1997: Immunosupression in dogs with pyoderma and/or demodicosis. *Vet. Med-Czech*, 42, 299—306.

21. Větvička, V., Fornusek, I., Kopeček, J., Kamínková, J., Kašpárek, I., Vránová, M., 1982: Phagocytosis of human blood leukocytes, a simple micromethod. *Immunol. Lett*., 5, 97—100.

Received April 19, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 76—80, 2004

A LIGHT-MICROSCOPIC STUDY OF A DELAYED PHASE OF PARAPLEGIA INDUCED BY ISCHEMIA AND REPERFUSION IN DOGS

[1]Šulla, I., [2]Maršala, J., [3]Lukáč, I.

[1]University of Veterinary Medicine, Komenského 73, 040 81 Košice
[2]Neurobiological Institute of the Slovak Academy of Sciences, Šoltésovej 4, 040 01 Košice
[3]Department of Neurosurgery, University Hospital, Tr. SNP 1, 040 66 Košice
The Slovak Republic

sulla@uvm.sl

ABSTRACT

Following the study of light-microscopic changes of spinal cord gray matter structures induced by thirty minutes of high thoracic aorta occlusion and thirty minutes of survival, the authors have continued their research with the mapping of the further development of neuronal damage during a subacute phase of ischemia-reperfusion paraplegia. Twelve adult mongrel dogs of both sexes, weighing between 18 and 25 kg, were used in the study. In a general anaesthesia induced by pentobarbital (30 mg.kg^{-1} i.v.), maintained endotracheally by a mixture of 1—2 % halothane with oxygen, the thoracic aorta was occluded for thirty minutes by a tourniquet applied on the vessel through a left-sided thoracotomy. After releasing the tourniquet, the thoracotomic wounds were closed by suturing tissues in anatomical layers. Operations were performed in sterile conditions, strictly observing basic surgical principles, antibiotics were administered during procedures. Complete paraplegia developed in all animals. They were let survive (reperfusion period) for 72 hours (1st experimental group, n = 6), or six days (2nd experimental group, n = 6), respectively. After elapsing the designated time period, the animals were killed by transcardial perfusion (3000 cc of saline) and fixation (3000 cc of 10 % neutral formaldehyde) in a deep intravenous anaesthesia (50 mg.kg^{-1} pentobarbital), their spinal cords were removed, postfixed, and 30 µm thick sections from L3—S1 segments were processed by the Nauta staining method. Light-microscopic changes in dogs of the 1st group were characterised by a massive occurrence of enlarged presynaptic boutons, encircled by clear halos, attached to thin fibres. In animals of the 2nd group all Rexed's laminae were filled with argyrophylic, polymorphic fragments of disintegrated spinal cord neurons. The results of the presented study explain the unfavourable prognosis of paraplegia induced by ischemia-reperfusion and principal significance of preventive measures during procedures requiring an interruption of spinal cord blood flow.

Key words: delayed phase; histopathology; ischemia-reperfusion; neuronal damage; paraplegia

INTRODUCTION

Noteworthy results of the study of early histopathologic changes of spinal cord neurons in dogs suffering from paraplegia induced by a temporary high thoracic aorta occlusion (an ischemic period) and a short-time survival (a reperfusional period), inspired the authors to continue their research with the mapping of a further development of the light-microscopic manifestation of this specific type of spinal cord damage (17).

MATERIALS AND METHODS

The experimental protocols were elaborated in compliance with the *Animal Protection Act of the Slovak Republic No. 15/1995* and approved by the *Ethical Commission of the Neurobiological Institute of the Slovak Academy of Sciences in Košice.*

Fig. 1. A thoracic aorta of a dog approached through a left-sided thoracotomy constricted by a tourniquet for 30 min. The proximal part of the vessel is considerably distended byincreased blood pressure, while the distal part is visibly narrowed and non-pulsating

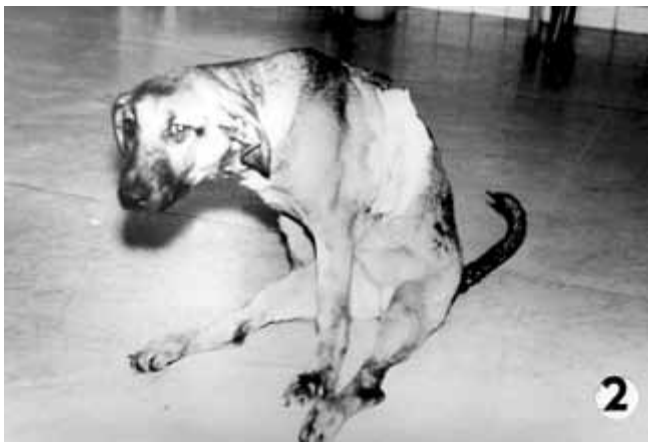

Fig. 2. The clinical manifestation of an irreversible spinal cord damage induced by 30 min of ischemia and 24 h reperfusion

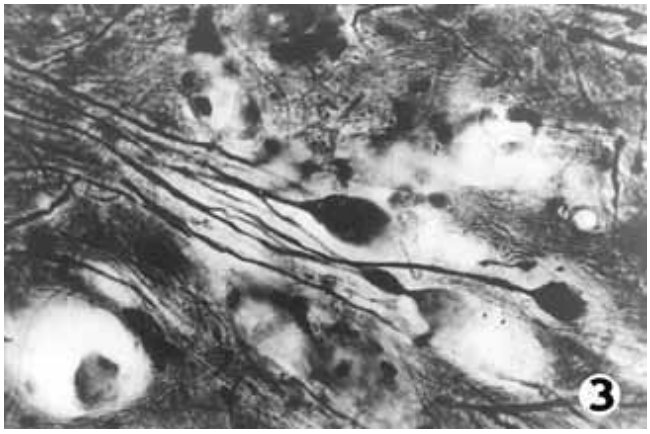

Fig. 3. Light-microphotograph of the L5 spinal cord segment after of 30 min ischemia followed by 72 h of reperfusion. Enlarged round axonal terminals (boutons) with periboutonal halos in the intrmediate zone (Rexed's lamina VII); magnification 280 ×

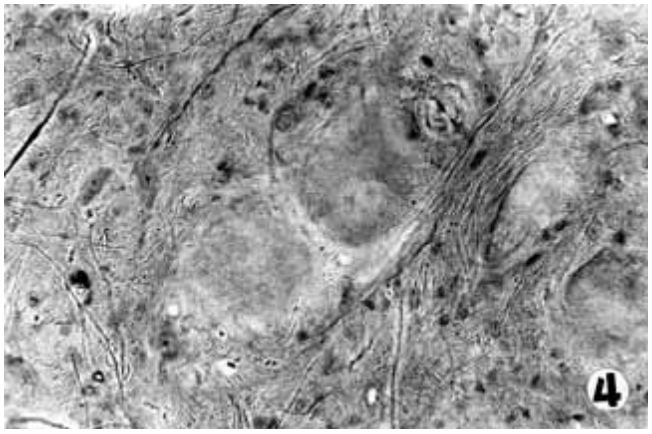

Fig. 4. Light-microphotograph of the L5 spinal cord segment damaged by 30 min of ischemia and 72 h of reperfusion. A group of dying large motoneurons from ventrolateral part of anterior spinal cord horn (Rexed's lamina IX) with dissolved nuclei and nucleoli; magnification 150 ×

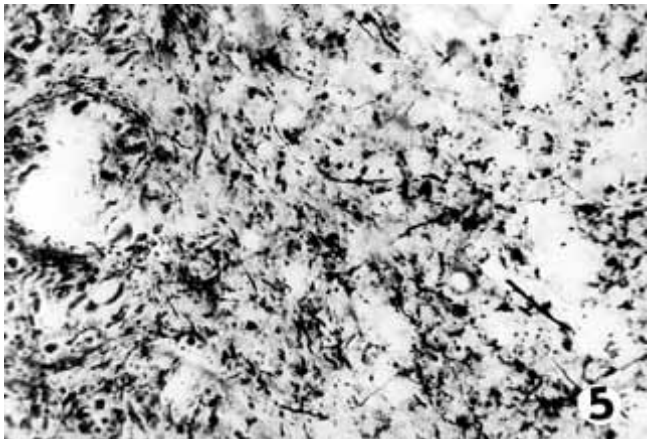

Fig. 5. Light-microphotograph of the L5 spinal cord segment after 30 min of high thoracic aorta occlusion followed by 6 d of reperfusion. All Rexed's laminae are packed with dark argyrophilic fragments of disintegrated spinal cord neural elements; magnification 150 ×

Twelve adult mongrel dogs of both sexes, free of heart worm disease, weighing 18 to 25 kg were used in the study. They were divided into two groups:

1. Thirty minutes spinal cord ischemia, 72 hours survival (n = 6).

2. Thirty minutes spinal cord ischemia, six days survival (n = 60).

Ischemia was carried out by a high thoracic aorta occlusion with a tourniquet applied on the vessel through a left-sided thoracotomy (Fig. 1). After releasing the tourniquet, the lungs were inflated by the help of the anaesthesiologic apparatus and the thoracotomic wound was closed by suturing tissues in anatomical layers. Manipulations with experimental animals were performed in general anaesthesia induced intravenously and maintained endotracheally, continual physiological monitoring was carried out throughout the experimental procedures, as described in detail elsewhere (17).

The operations were carried out under sterile conditions and basic surgical principles were strictly observed. After completing the experimental procedures, consisting of thirty minute spinal

cord ischemia, the dogs were placed in separated, disinfected compartments, covered with warm blankets and followed up to the point of complete awakening from the anaesthesia. Then they were offered drinking water ad libitum and a normal diet.

After 72 hours (six animals of the first studied group) and six days (six animals of the second group) survival, the dogs were killed by a deep, intravenous general anaesthesia (50 mg. kg^{-1} pentobarbital) by perfusion (3,000 ml saline) and fixation (the same volume of 10 % neutral formaldehyde). The principal parts of the central nervous system (i. e. *spinal cord, medulla oblongata* and brain stem) of each experimental animal were removed *in toto*, 24 hours postfixed with formaldehyde, then specimens comprising L3—S1 spinal cord segments were cut in 30 μm thick sections and processed according to the Nauta staining method (12). As controls served the sections from identical parts of central nervous system of three non-ischemic animals used in the previous study (17).

RESULTS

The short-time (thirty minute) ischemia followed by reperfusion caused an irreversible lesion of spinal cord gray matter cells of each dog used in the experiment. Complete paraplegia which, developed in animals of both studied groups, was characterised by hind-limbs and tail palsy, loss of urinary bladder and rectal sphincters control, as well as sexual incompetence (Fig. 2). Surgical wounds healed *per primam intentionem,* no intrathoracic complication was noticed at the time of transcardial perfusion-fixation procedures.

Light-microscopic observations of 30 μm thick sections from L3–S1 spinal cord segments from the first studied group (thirty minute ischemia, 72 hour reperfusion), impregnated according to the Nauta staining method, showed a massive occurrence of large, round or elliptical axonal terminals of variable density. The enlarged presynaptic knobs (boutons) were encircled by clear halos and attached to thin terminal fibres (Fig. 3). Their diameter exceeded 4 μm. The majority of boutons were localised in the central part of spinal cord gray matter, i.e. Rexed's laminae V–VII (14). In contrast neurons of Rexed's laminae VIII and IX were affected significantly less frequently and somatic argyrophilia of large motoneurons of anterior spinal cord horns was not noticed. The only light-microscopic symptom of irreversible damage of these large cells was dissolution of nuclei and nucleoli, which became almost undiscernible from the surrounding cytoplasm (Fig. 4).

Neuropathological changes observed in 30 μm thick sections from L3–S1 spinal cord segments of six dogs after thirty minutes of high thoracic aorta occlusion followed by six days of survival were characterised by a terminal stage of apoptosis occurring in all gray matter layers, but a narrow rim of normally appearing tissue covering posterior spinal cord horns. All Rexed's laminae were packed with intensively argyrophilic, polymorphic fragments (Fig. 5). The highest density of these dark bodies was concentrated in lamina VII, slightly less expressed was the disintegration of spinal cord neural structures in laminae V and VI.

DISCUSSION

Neuronal death is the end result of many neurological disease processes, whether they are degenerative, traumatic, or ischemic in nature (1, 4, 10, 16, 20). Regardless of the etiologic factors, which started the cascade of events leading to disintegration of central nervous system structures, the vast majority of investigators comply with the theory of a multi-step process, including persistent depolarization of cell membranes, energy depletion from repolarization, deregulation of intracellular calcium, formation of reactive oxygen species, lipid peroxidation, loss of cellular ionic gradients, oedema formation, nuclear DNA fragmentation, and rupture of cell membranes (2, 9, 13, 15, 18).

Many interventions have been attempted to interrupt this cascade with unreliable efficacy in the treatment of trauma, stroke or neurodegeneration (5, 16, 18, 19). This is possibly because they are late interventions to an early element of the apoptotic process (17, 19, 20). We believe this assumption confirms the rapid development of profound spinal cord neuronal changes induced by thirty minutes of ischemia and reperfusion in previous (17) as well the present study. Perhaps the most reliable demonstration of the clinical and theoretical significance of problems connected with central nervous system ischemic injury has been a vast number of papers published on this topic in scientific journals around the world during the last ten years.

This interest reflects a reasonable apprehension of the public from not only the already high, but further continually growing incidence of atherosclerosis with its serious, often even fatal, complications (10). There are hundreds of such articles indexed in the *Current Contents* and *Medline.* However, only a few have payed attention to the histologic presentation of neuronal damage characteristic for ischemic lesion of the brain tissue (stroke) or spinal medulla (6, 11, 18—20).

The clinical manifestation of spinal cord transversal lesion is paraplegia. This term refers to a deficit of sensori or motor, as well as vegetative inervation of affected part of the body. It is characterized by a lack of voluntary control of pelvic and lower extremities muscles, rectal and urinary bladder sphincters, and sexual impotence (8). A very similar clinical picture develops in animals following spinal cord or *cauda equina damage (1, 4).* The main causes of this complication are fractures or luxations of vertebrae, intraspinal mass lesions (neoplasms, bleeding, abscesses), less frequently an interruption of spinal cord blood flow (1, 3—5, 11, 13, 15, 18).

The temporary cross-clamping is a conditio sine qua non in any aortic or principal vessels surgery (10, 13). However, the awakening from the anaesthesia after an

uneventful, technically straightforward procedure with a stroke or an irreversible paraplegia, means a catastrophe for the patient and a nightmare for the surgeon (4, 5, 9, 10, 17). The risk of this complication is about 10 % in elective thoracoabdominal aortic operations, but significantly increases in patients struck with a rupture of an atherosclerotic aneurysm of the vessel (8).

The reason is haemorrhagic shock causing rapid decrease of blood pressure in a person with preexisting, latent insufficience of spinal cord vascular network together with its supplying arteries, potentiated by necessary aortic occlusion during the surgical reconstruction of the vessel (7, 8). The experimental model utilised in our study was developed with the aim of imitating as much as possible the situation in humans (3, 7, 17).

Originally we considered the use of smaller animals, as they are preferred in experimental studies nowadays (5, 6, 11, 15, 18, 20). We had both, rabbits as well as rats at our disposal. However, interventions in the thoracic cavity of rabbits or rats need a special apparatus for maintaining artificial ventilation and an endotracheal intubation is so difficult, that endotracheal cannula often can be introduced through a tracheotomy only (15, 19, 20). Moreover, in rabbits spinal cord lesion leads to a complete urinary retention, which causes the early death of the animal.

On the other hand, every manipulation with a larger animal is much simpler, the surgical situation in a thoracic cavity is closer to the situation in humans, results are more reliable. That is why sheep, dogs, or cats have been used in many experimental studies on central nervous system structures quite frequently up to the present (1, 4, 16). Having suitable equipment for operations on dogs and sufficient experience with treatment of these animals following even complex experimental procedures, we decided to use them in the study with longer post-surgical survival-period again (17).

An important finding of the previous study was the ability of the Nauta staining method to visualize and hence to localize spinal cord neurons whose somata and dendrites were becoming argyrophilic due to a thirty minute ischemic and a short reperfusion injury (17). The results of the present work have confirmed the indisputable advantages of the method in mapping of further development of spinal cord of spinal cord neuronal damage induced by thirty minutes of high thoracic aorta occlusion.

Following 72 hours of survival (reperfusion period), an unusual type of synaptic degeneration, characterized by massive occurrence of enlarged boutons encircled by clear halos, was found. This phenomenon could be a light-microscopic manifestation of ionic shifts and membrane depolarization processes, which are considered an integral part of a cascade of events leading to neuronal death.

The density and laminar distribution of boutons clearly correlated with the distribution of small argyrophilic neurons localized in the deep dorsal horn layers and *zona intermedia*, detected after thirty minutes of reperfusion (17). The results of our experiments have shown the high vulnerability of these spinal cord gray matter cells. With regard to the fact, that many small neurons and interneurons form synapses with axons of descending supraspinal tracts, the damage induced by ischemia and reperfusion very probably plays a decisive role in eventual development of postischemic paraplegia from a flaccid paralysis to a spastic palsy (11, 13, 15).

The dark argyrophilic fragments filling Rexed's laminae of semithick (30 μm) sections cut from lumbosacral spinal cord segments of dogs exposed to thirty minutes of ischemia followed by six days of survival, processed by the Nauta staining method, represented the light-microscopic picture of disintegration of gray matter neurons.

CONCLUSIONS

Prognosis of paraplegia induced by ischemia and reperfusion is unfavourable. The reason is a rapid onset and progressive character of spinal cord neuronal changes leading without an immediate, appropriate treatment to the profound and irreversible damage of gray matter structures.

REFERENCES

1. Beneš, V., jr., Rokyta, R., 1988: Experimental spinal cord injury: lumbar vertebra resection to shorten the gap between spinal cord stumps. *Acta Neurochir.* (Wien), 90, 152—156.

2. Danko, J., Kalanin, P., 2002: Intracellular calcium accumulation and CNS injury. *Proceedings of the 5th Košice Morphological Day with International Participation,* 117—118.

3. Evans, H. E., 1993: *Miller's Anatomy of the Dog.* W. B. Saunders Co., Philadelphia, London, Toronto, Montreal, Sydney, Tokyo, 1113 pp.

4. Hitchon, P. W., Dyste, G. N., Osenbach, R. K., Jensen, A. E., 1990: Response of spinal cord blood flow and motor and sensory evoked potentials to aortic ligation. *Surg. Neurol.,* 34, 279—285.

5. Kakimoto, H., Kawaguchi, M., Sakamoto, T., Inone, S., Furuya, H., Nakamura, M., Konishi, N., 2003: Evaluation of rapid ischemic preconditioning in a rabbit model of spinal cord ischemia. *Anaesthesiology,* 99, 1112—1117.

6. Kluchová, D., Rybárová, S., Miklošová, M., Lovásová, K., Dorko, F., 2001: Comparative analysis of NADPH-diaphorase positive neurons in the rat, rabbit and pheasant thoracic spinal cord. A histochemical study. *Eur. J. Histochem.,* 45, 239—249.

7. Krauss, W. E., 1999: Vascular anatomy of the spinal cord. *Neurosurg. Clin. N. Am.,* 10, 9—15.

8. Lederle, F. A., Johnson, G. R., Wilson, S. E., Ballard, D. J., Jordan, W. D., jr., Blebea, J., Littoy, F.N., Freischlag, J. A., Bandyk, D., Rapp, J. H., Salam, A. A., 2002: Rupture rate of large abdominal aortic aneurysms in patients refusing or unfit for elective repair. *JAMA,* 287, 2968—2972.

9. Lin, R., Rosenborough, G., Dong, Y., Williams, G. M., Wei, C., 2003: DNA damage and repair system in spinal cord ischemia. *J. Vasc. Surg.,* 37, 847—858.

10. Loftus, C. M., 2000: Overview of shunt controversy. In **Loftus, C. M., Kresovik, T. F.** (eds.): *Carotid Artery Surgery.* Thieme Medical Publisher, New York, 409—420.

11. Maršala, M., Yaksh, T. L., 1994: Transient spinal ischemia in the rat: characterization of behavioral and histopathological consequences as a function of the duration of aortic occlusion. *J. Cereb. Blood Flow Metab.,* 14, 526—535.

12. Nauta, W. J. H., 1957: Silver impregnation of degenerating axons. In **Windle, W. P.** (ed.): *New Research Techniques of Neuroanatomy.* Charles C. Thomas Publisher, Springfield Il., 17—26.

13. Nedeltchev, K., Loher, T. J., Stepper, F., Arnold, M., Schroth, G., Mattle, H. P., Sturzenegger, M., 2004: Long-term outcome of acute spinal cord ischemia syndrome. *Stroke,* 35, 560—565.

14. Rexed, B., 1954: A cytoarchitectonic atlas of the spinal cord in the cat. *J. Comp. Neurol.,* 100, 297—379.

15. Sakamoto, T., Kawaguchi, M., Kurita, N., Horiuchi, T., Kakimoto, M., Inoue, S., Furuya, H., Nakamura, M., Konishi, N., 2003: Long-term assessment of hind limb motor function and neuronal injury following spinal cord ischemia in rats. *J. Neurosurg. Anesthesiol.,* 15, 104—109.

16. Sutherland, G. R., Perron, J. T., Kozlowski, P., Mc Carthy, D. J., 2000: AR – R 15 896 AR reduces cerebral infarction volumes after focal ischemia in cats. *Neurosurgery,* 46, 710—720.

17. Šulla, I., Maršala, J., Lukáč, I., 2003: A light-microscopic study of an early phase of paraplegia induced by ischemia and reperfusion in dogs. *Folia Veterinaria,* 47, 2002—2003.

18. Takahashi, G., Sakurai, M., Abe, K., Itoyama, Y., Tabayashi, K., 2003: MCI-186 prevents spinal cord damage and effects enzyme levels of nitric oxide synthase and Cu/Zn superoxide dismutase after transient ischemia in rabbits. *J. Thoracic Cardiovasc. Surg.,* 126, 1461—1466.

19. Vanický, I., Urdzíková, L., Saganová, K., Maršala, M., 2002: Intrathecal methylprednisolone does not improve outcome after severe spinal cord injury in the rat. *Neurosci. Res. Com.,* 31, 183—191.

20. Vosko, M. R., Busch, E., Burggraf, D., Bueltemeier, G., Hamann, G. F., 2003: Microvascular basal lamina damage in thromboembolic stroke in a rat model. *Neurosci. Letters,* 353, 217—220.

Received March 15, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2, 81—86, 2004

THE FUNDAMENTALS OF MODERN MARKETING AND THEIR INFLUENCE ON VETERINARY SERVICES PERFORMANCE

Buleca, J., jr., Urbančíková, N.*, Bugarský, A., Švický, E.

University of Veterinary Medicine, Komenského 73, 041 81 Košice
***Faculty of Economics, Technical University of Košice, Letná 9, 040 01 Košice**
The Slovak Republic

buleca@yahoo.com

ABSTRACT

The extension of the European Union brings the new challenges to all service providers. The new opportunities arise but they are accompanied by possible dangers as well. Service providers have to cope with many problems in order to increase their business performance. Veterinary medicine services providers are among them. This paper deals with the fundamentals of modern marketing and their utilisation in the area of veterinary services provision in order to improve the quality of veterinary services and to enhance their efficiency and effectiveness. The role of marketing in modern firm is explained and marketing mix and its tools are described with the special focus on the specific features of veterinary services in The Slovak Republic. The paper is result of research that has been carried out in Košice region in the year 2003 in which time frame the veterinary surgeries in this region have been surveyed.

Key words: marketing; marketing mix; marketing research; veterinary service

INTRODUCTION

In the most industrialised economics, expenditures on services are growing. There are several reasons for this. The most important of them are, for example, the creation of the more sophisticated products using the advanced technologies, the growth of per capita income causing a greater percentage of expenditure on luxuries that very often require intensive services and the strong trend towards out-sourcing. Up coming competitors increase the importance of the and marketing in services. The area of veterinary medicine services is one of them.

The current veterinary profession offers services mostly through private small business activities, for profit and independent business (14). The assumption is that there are more than 100,000 veterinary surgeons providing services in European Union countries and about half of million all over the world (2). Žaja (19) puts stress on the decreasing tendency of agriculture in the gross domestic product (GDP) in the period of transformation (5.3 % at present) and the reduction of the number of persons employed in the agriculture in overall employment. These facts affect also veterinary surgeons and other employees connected with veterinary activities in the area of animal production (13). A deep understanding of the importance of efficiency and effectiveness in the business performance and application of marketing mix tools represent one condition for successful adaptation to transformed market conditions (9, 16).

MARKETING IN THE MODERN SERVICE PROVIDER COMPANIES

Cowell (3) has stated that what is significant about services are the relative dominance of intangible attributes in the make-up of the "service product". Services are special kind of product. They may require special understanding and special marketing efforts. Pure services do not result in ownership although they may be linked to a physical good.

Table 1. Service characteristic components description

Component	The description	
1. Intangibility	– a deed, performance or effort	– difficulty in evaluation
	– use tangible cues	– benefits of non-ownership
2. Inseparability	– simultaneous production and consumption	– importance of service provider
	– selection, training and rewarding of staff	– avoid inter-customer conflict
3. Variability	– standardization difficult	– selection, training and rewarding of staff
	– evaluation systems	– use of reliable equipment
4. Perishability	– consumption cannot be stored	– match supply and demand
	– use of part-time staff	– multi-skilling
	– participation by consumer	– different pricing
	– stimulation of off-peak demand	– comfortable waiting area
	– reservation system	

Many offerings, however, contain an element of tangible and intangible. For example, a veterinary service provide the examination which is intangible, but also offers the veterinary medicine which are tangible. The distinction between physical and service offerings can, therefore, be best understood as a matter of degree rather than in absolute terms.

The service characteristic consists of four components: intangibility, inseparability, variability and perishability (see Table 1).

Marketing is a complex of activities and processes that serves to recognition or support of the customer needs and requirements, for the development of a corresponding product and for the communication and consequential distribution of the product to customer which results into mutual exchange leading to long term satisfaction of customer and from this point of view also to the satisfaction of the organisation and whole society. It can be defined also as a managerial process responsible for identification, anticipation and satisfaction of clients in the profit making process (9). Westwood (18) says that marketing includes also customer demands, retrieval and comparisons of organisational products with those demands in order to make a profit. It is also very often connected to the concept of deceptive, enticing or dubious acitivities which is in conflict with marketing philosophy – the creation of a certain value which is valuable for customers and which they appreciate (16).

Marketing strategy is the selection of a target market, the choice of a competitive position and the development of an effective marketing mix to reach and serve the chosen market. Formulation of an *institutional marketing strategy* includes decisions about:

1. The service provider's current programs and markets whether to maintain, build, or drop them.
2. Future new programs and market opportunities.
3. Analysis of competitors.
4. Positioning of the institution in relation to competitors.
5. Segmenting the market and selection of target markets.
6. Designing of the marketing mix.

The definition of the *core marketing strategy* consists of:

A) Segmenting

Segmenting is the ability to divide a market into distinct and meaningful groups of consumers which merit separate products/services and/or marketing mixes.

B) Target marketing

A target market is a fairly homogeneous group to whom a veterinary services providers wish to appeal. Target marketing is a marketing mix tailored to fit some specific target customers. Mass marketing aims at satisfying all groups of customers with the same marketing mix.

C) Positioning

Positioning is about a particular surgery, its programs and services occupying a strategic place in people's minds. A position describes how a person or group perceives the veterinary surgery in relation to other veterinary surgeries. It is the art of developing and communicating meaningful differences between one's own offer and those of competitors serving the same target market.

The veterinary service providers marketing strategy should flow naturally from the earlier stages of the strategic marketing planning process. Successful service providers will have a unique view of itself and its role in the marketplace. An effective marketing strategy will have the following characteristics:

– Customer centered – its main focus will be meeting the needs and wants of its audience.

– Visionary – it will articulate a future for the surgery that offers a clear sense of where the organisation is going, what it will look like and what it will achieve.

– Differentiated – it will be differentiated from its competitors and will offer target markets unique reasons to prefer its perceived offerings.

– Sustainability – it will be sustainable in the face of reaction from competitors.

– Easily communicated – core elements of the strategy will be simple and clearly communicated to both the target market and the internal staff.

– Motivating – it will have the enthusiastic commitment of all those who will carry it out.

– Flexibility and adaptability – it will be sufficiently broad to allow for diversity in implementation and allow for modification if necessary.

Alongside to the traditional marketing tools, which are product, price, place and promotion there are three other elements to take into account. These elements include physical evidence, process and people and are included to 7 Ps mix.

In the 80's the model of 4 Ps formed the basis of marketing and its importance was significant. The model was both used establishing and maintaining the customer relationships. But the extended marketing mix is the issue of today.

Services are a special kind of product. They may require special understanding and special marketing efforts (11).

A marketing mix presents optimal combination for utilization of the marketing tools in the process of product and service offers creation. It includes marketing tools, parameters of target market and also production, distribution, contracting and communication policy. The success of a business subject in the market is conditioned by a well advised chosen system of marketing elements that act as a unit. The company has to choose those elements and use them for customer satisfaction on the one hand and also for making a profit on the other hand. They are: product, price, promotion and place.

The choice of elements depends on line of work, area of activity, overall evaluation of macro- and micro-environment. The combination of the elements that creates a marketing mix is modified and is different for each organisation. The marketing mix reflects the organisation goals, capacity, capability and the influence of external environment. The organisation will be successful only when all its tools will work in harmony. If only one tool does not work the success of organisation is at risk (6, 15).

THE SPECIFICS OF VETERINARY SERVICES MARKETING

Act number 10/1992 *"About Private Veterinary Surgeons and The Slovak Chamber of Veterinarians"* has been primary determined and adjusted after the transition to the market economy in the present period of veterinary medicine services. The law considers the private practitioners those of them who are registered in the Registry of Veterinary Surgeons in The Slovak Republic. The Slovak Chamber of Veterinarians manages this registry in The Slovak Republic. The Chamber issues a certificate to veterinary surgeon that entitles him/her to practice the veterinary medicine and also can reduce this to a specific field or district. The Chamber is self-ruling body that associates veterinary surgeons for outside needs as an independent for profit profession (17).

Next from the number of Acts that adjusts veterinary services providing is Act 337/1998 *"About Veterinary Solicitude and about Modification and Replenishment of Some Other Acts"*. The Act determines the rules and conditions for veterinary care in order to preserve and improve animal health, environmental protection, human health protection, production and gaining of health safe and biologically valuable animal food. Besides this the power of state government bodies and regional self-governmental bodies in the area of veterinary solicitude and tasks, management of veterinary inspection and veterinary control is specified there. Based on this Act the state administration bodies for veterinary medicine are:

a) Ministry of Agriculture of The Slovak Republic

b) State Veterinary and Food Administration of The Slovak Republic

c) The Regional Veterinary and Food Administrations

d) The County Veterinary and Food Administrations

Based on the analysis completed by governmental, private and other subjects the significant accumulation of the expenses for veterinary services is expected until 2015 (2). The ageing of population is one of the possible reasons for a decreasing demand for veterinary services. This is more visible nowadays not only at the academic field but also between veterinary practitioners and least but not last at client's side. The older people are supposed to own domestic animals more seldom. On the other hand it is compensated by fact that older people are willing to spent more money on veterinary services. It has influence on the changes in the veterinary service client structure, the demand will growth up especially on the small animal service side. Consecutively the small descend of veterinary surgeons number is expected mostly in the area of big animals (5).

The surveys carried out outside The Slovak Republic showed that owners of the domestic animals stated factors important for the selection of the veterinary services providers as follows:

1. veterinary surgeon is gentle and friendly
2. veterinary surgeon informs us well
3. veterinary surgeon has a good reputation, is known as high quality service provider
4. the previous positive experience regarding veterinary surgeon
5. the range of offered services
6. the location of the service provider ambulances
7. suitable working hours
8. the recommendation of the friends and relatives
9. the price of services
10. other factors

THE CREATION OF THE OPTIMAL MARKETING MIX FOR THE VETERINARY SERVICE PROVIDERS

A *marketing* mix presents optimal combination for the utilisation of the marketing tools in the process of product and service offers creation. It includes marketing tools, parameters of target market and also production, distribution, contracting and communication policy. The combination of the elements that creates marketing mix is modified and is different for each organisation. The marketing mix reflects the organisation goals, capacity, capability and the influence of external environment.

In view of specifics of service marketing marketing research in the veterinary service market should be focused on individual basic components of service marketing mix, which means 8 Ps: *product, place, price, promotion, process, physical evidence, people, productivity* and *quality*. The project of market research must be designed in the way that increases the level of managerial decision. The aim of market research is to create the model of service provider behavior in the frame

Table 2. A questionnaire for research of present status of marketing in veterinary services (shortened form)

Questions
1. We research customer needs before developing new products and services.
2. Our VET surgery considers customer "buying points" when promoting products.
3. Our VET surgery takes action to make sure that every customer is a satisfied customer.
4. Our VET surgery takes care about image building.
5. Our VET surgery widens the range of the services.
6. Our VET surgery offers services also at the customer households.
7. Our VET surgery utilises Internet to gain information.
8. Our VET surgery ensures the patient transportation.
9. Our VET surgery draws up a pricing strategy for every new product marketed.
10. Our VET surgery tries to decrease the costs of every service offered.
11. Our VET surgery offers the price reductions.
12. Our VET surgery obtains customer information and uses it to influence decisions.
13. Our VET surgery finds reasons to keep in touch with customers.
14. Our VET surgery tries to turn one-off customers into regular ones.
15. Our VET surgery tries to find out why we have lost a customer.
16. Our VET surgery attempts to win back lost customers.
17. Our VET surgery listens what customers say.
18. Our VET surgery pays attention to the little details that make all the difference.
19. Our VET surgery sets objectives for publicity campaigns.
20. Our VET surgery measures the overall effectiveness of a publicity campaign.
21. Our VET surgery tries to shorten customer stay in the waiting room.
22. Our VET surgery places some costumers before others (urgent cases, employees…).
23. Our VET surgery puts symbols to the objects its uses (building, car, clothes, interior…).
24. Our VET surgery produces the information materials (leaflets) about its activities.
25. Our employees permanently educate themselves.
26. Our VET surgery tries to see if there is anything we can learn from a customer's complaints.
27. Our VET surgery sets standards to ensure effective customer care.
28. Our VET surgery measures performance against the standards of customer care.
29. Our VET surgery monitors the number of customer complaints that we receive.
30. Our VET surgery takes the complains of customers very seriously.
31. Our VET surgery monitors competitive services carefully.

of market. The systematic approach has to be applied during project preparation. Three methods can be used in order to collect qualitative and quantitative data – observation, experiment and public opinion survey (8).

The following recommendation for the marketing mix for veterinary services providers is based on the research that included questionnaires submitted to veterinary practitioners with surgeries located in Košice. 15 veterinary practitioners took part in the research, which represents almost 79 percent of all Košice veterinary ambulances. The questionnaire was divided into 8 parts representing an individual specific area of the service marketing mix. Each part contains the different number of questions regarding each marketing mix components.

Table 3. The evaluation of the positive answers at level 4 for marketing mix components

MARKETING MIX COMPONENT	%
PRODUCT	41.33
PLACE	13.33
PRICE	24.44
PROMOTION	39.33
PROCESS	33.33
PHYSICAL EVIDENCE	10.00
PEOPLE	46.67
PRODUCTIVITY AND QUALITY	30.00

RESULTS AND DISCUSSION

The results of the questionnaire survey (see Table 3 and Fig. 1) will be discussed based on the individual part of marketing mix that they represent:

1) Product

The veterinary practitioner as a manager must select the feature of the core product and supplementary services that surround them with respect to the customer wishes.

In the product area the veterinary surgeries should research the customer needs and his/her motivation for buying products. One of the crucial elements for purchase is also the permanent adjustment of the range to the customer needs and current trends (7, 8).

2) Place and time

The delivery of the product to the customer includes the decision about place and time of delivery, sometimes also the physical or electronic distribution channels depending on delivered service nature. The surgery should consider service offering at the customer house, or at the place selected by customer. It is worth to think about

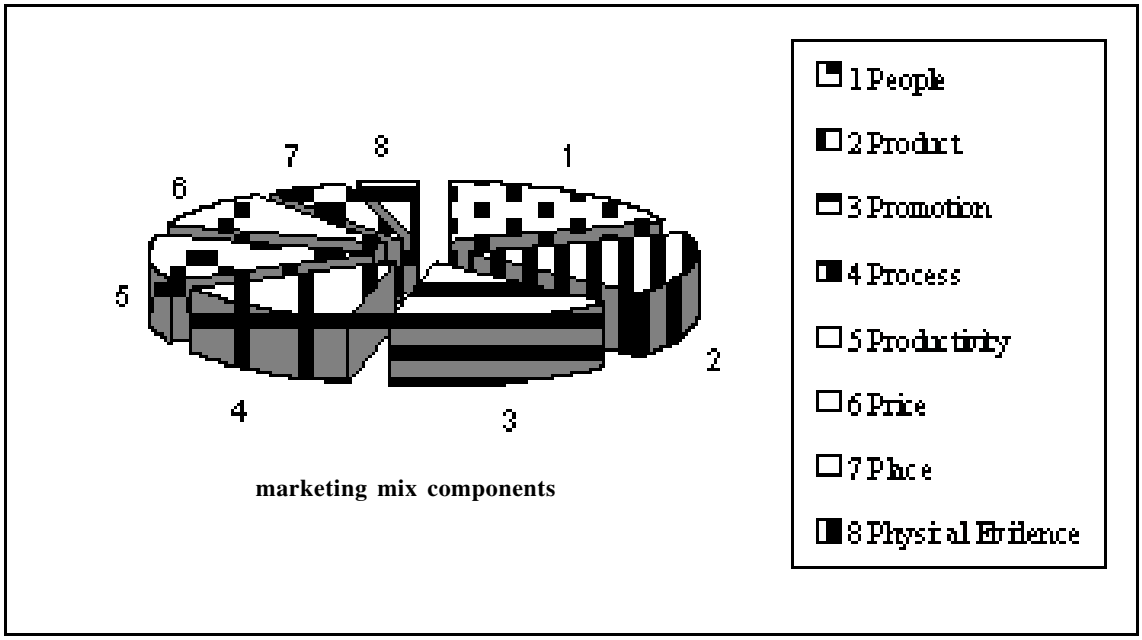

Fig. 1. The graphic representation of marketing mix components

arranging of the patient transportation to the veterinary surgery. The utilisation of the Internet also for the providing information and/or announcements for customers should be the commonplace in the information society age (9).

3) Price and other user costs

This component addresses the user service costs for customer during the obtaining of the benefit from service product. The veterinary surgeons as a service managers should recognise and, if possible, also try to assure the minimization of the costs and all other financial expenses, time, intellectual or physical effort and negative sensual experience of their clients (4).

It is necessary to gain the basic knowledge regarding pricing strategy through education in this area and apply them to practice. The monitoring of competitor prices must create the integral part of unit pricing. A sophisticated system of price reduction helps to assure the client loyalty. It should be kept in mind that to recruit new customers is five times more expensive than retain old ones.

4) Promotion

Any of the marketing programmes cannot be successful without an effective communication plan. It plays three important roles: opens up the necessary information and advises, helps to persuade the target customers about specific product value and encourages them to take action at a specific time (14, 17).

The key problem seems to be the task of promotion, method of promotion and measurement of promotion effectiveness. In spite of statements that should support an idea about sufficient space giving by veterinary surgery to customers and their needs, consecutively only 14 percent of surgeries with settled goals in the promotion area exist. It indicates that the product promotion and communication mix questions appear to be a point of weakness.

5) Process

The creation and delivery of the product to the customers require design and implementation of the effective processes. The process describes the methods and sequence by which the service system works. It is useful to describe individual processes in veterinary surgeries and to analyse them. The assessment of the weak points leads to a more effective way of surgery performance. A detailed description of the processes is an unnecessary condition for quality management introduction (7).

6) Physical evidence

The appearance of building, car, interior, personal, clothes, trademark, printed materials and other visible forms offers tangible proof of style and quality of offered services. Veterinary surgeries as service providers should be very careful regarding physical evidence management because this item can create profound impressions on the customer (5). The area of physical evidence is underestimate in Slovakia. It is partly caused by the financial situation of veterinary surgeries but, primarily because of a misconception of its importance for the delivered services (10).

7) People

Veterinary services, as with most of services, depend on the direct interaction between customer and personnel. The character of these interactions broadly influences

customer service quality perception. The quality of services tends to be considered *via* the quality of people providing services. In order to provide high quality services the development of a substantial effort in the area of choice, education and motivation of personnel is necessary. It involves also the endeavour to keep the high level of organisational culture especially in direct contact with customer level (but not only) (10).

The area of people is key service area. The behaviour of the staff to customers is one of the small things that make big difference. Based on the survey it seems that veterinary surgeries devote sufficient attention to this part of marketing mix. In order to support or disprove this assumption from veterinary surgeries side the realisation of deep interview and customer surveys are strongly recommended (12).

8) Productivity and quality

Veterinary surgeries cannot afford to isolate elements of productivity and quality. An increase in productivity is important for keeping control of costs, but veterinary practitioners must be aware that if improper reduction at the service level is carried out, services can be refused by customers (and also by own staff). A definition of service quality is important for product differentiation and customer loyalty building. The fact of the matter is that investment to quality requires understanding of the dependency between cost decline and revenue growth (1).

The measurement of the success can be possible only in the case that those criteria are settled unequivocally and are measurable. The establishment of criteria and standards to measure the effectiveness of patient care and also the criteria to check surgery staff efficiency is necessary (13). Surgeries should have a written obligatory procedure how to deal with customer complaints.

RECOMMENDATION AND CONCLUSION

The evaluation of the research based on the statistical approaches shows that veterinary surgeries in Košice reached only 31 percentage of the optimal situation. It is necessary to emphasie that profound understanding and application of marketing mix tools is only one part of the marketing strategy of veterinary services. The strategic marketing process includes the planning phase (marketing audit, the goals determination, the strategy formulation and marketing plan design), implemantation phase (plan implementation, the marketing organisation creation) and check-up phase (reached results measurement, comparison with plan, evaluation, correction and deviation utilisation) (2). The stated recommendations are only the first step to successful complex process implementation.

REFERENCES

1. Alsbury, A., Jay, R., 2002: *Marketing – the Best Practices* (In Czech). Computer Press, Prague, 275 pp.

2**. Bugarský, A., Lešník, F., Pilipčinec, E., Prosbová, M., 2002:** *Veterinary Medicine – the Perspectives* (In Slovak). UVM Košice, 59 pp.

3. Cowell, A., 1989: Marketing in travel and tourism – Middleton, VTC. *Journal of the Market Research Society,* 31, 141—142.

4. Drucker, P. F., 1999: *The Practice of Management.* Heineman, London, 125 pp.

5. European Commision, Directorate-General for Agriculture, 2002: Agricultural Situation in the Candidate Countries: *Country Report on the Slovak Republic* (online), Brussel, Available on Internet: <http://europa.eu.int/comm/agriculture/external/enlarge/publi/countryrep/slovak.pdf>

6. Foret, M., Prochádzka, P., Vaculík, J., Koprivová, K., Foret N., 2001: *Marketing – Basics and Practices* (In Czech). Computer Press, Prague, 159 pp.

7. Horáková, H., 2003: *Strategic Marketing* (In Czech). Grada Publishing, Prague, 167 pp.

8. Housden, M., 1997: *Succesful Market Research* (In Slovak). Open Windows, Bratislava, 98 pp.

9. Janečková, L., Vaštíková, M., 2000: *Service Marketing* (In Czech). Grada Publishing, Prague, 179 pp.

10. Kotler, Ph., 2001: *Marketing Management* (In Czech) (10th edn.). Grada Publishing, Prague, 718 pp.

11. Lovelock, Ch., Wright, L., 2002: *Service Marketing and Management* (2nd edn.). Prentice Hall, New Yersey, 436 pp.

12. Lyková, J., 2000: *Marketing Audit and Controll* (In Czech). Grada Publishing, Prague, 116 pp.

13. Oakley, R. G., 1989: The veterinary profession in the European Community: 1992 and beyond. *Vet. Rec.,* May 20, 527—529.

14. Ott, R. S., 1990: Veterinary services for animal use in the United States: A conflict of paradigms. *J. Am. Vet. Med. Assoc.,* 197, 1134—1139.

15. Smith, P., 2000: *Modern Marketing* (In Czech). Computer Press, Prague, 511 pp.

16. Urbančíková, N., 2002: *Marketing of Lifelong Education* (In Slovak). Center of Lifelong Education, TU Košice, 73 pp.

17. Vaňo, B., Jurčová, D., Mészáros, J., 2002: *Prognosis of Inhabitant Development in the Slovak Republic till 2050* (In Slovak). Infostat, Bratislava, 121 pp. Available on Internet: < http://www. infostat.sk/vdc/pdf/prognoza 2050vdc2.pdf>

18. Westwood, J., 1999: *How to Make an Marketing Plan* (In Czech). Grada Publishing, Prague, 117 pp.

19. Žaja, J., 1998: Requests on the SAU graduates in condition of labour market in the Slovak Republic (In Slovak). Miscellany: *Application of Graduates on Labour Market.* SAU Nitra, 3—19.

Received August 20, 2003

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 87—89, 2004

HONEY ADULTERATION

Fiľko, J., Takáčová, D., Bugarský, A.

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

bugarsky@uvm.sk

ABSTRACT

There is a lot of counterfeit foodstuff in the world. We refer to problems connected with the adulteration of honey and the legal regulation of this kind of problem in The Slovak Republic, as well. We mention the most frequent methods of adulterating honey and the methods for ascertaining the authenticity of honey.

Key words: adulteration; authentification; honey; laboratory methods

INTRODUCTION

Adulteration is as old as a mankind. Adulteration is difficult to check as a great margin of profit is involved. That is why the occurrence of adulteration in the food industry is not rare. Adulteration affects all food commodities especially expensive or a large scale products with the purpose of achieving the highest illegal profit. It is supposed that more than $90 billion of adulterated products are sold worldwide.

The first regulation against the adulteration of food stuffs can be found 2,000 years ago (9). The first law on the protection of consumers from adulterated food stuffs were directed towards health. In every country every producer makes an effort to sell their products. Due to gigantic competition, the products, which have a brand, which is not anonymous and which have characteristic features distinct from others, have better chances of selling. This distinctiveness has to be legally protected.

HONEY ADULTERATION

American legislation links the adulteration of food stuffs with worse quality. Adulterated food stuffs are those (12):

1)

a) which bear or contain hygienically defective material,

b) if the hygienically defective material was added to the food stuffs,

c) which contain dirty, rotten, smelly or decomposed material,

d) which are not prepared, packed and stored in hygienic conditions,

e) which contain parts of sick or decayed animals,

f) which are not in hygienic packing.

2)

a) those, in which, partly or fully, the valuable components are neglected or which are partly or fully replaced by another or by a less valuable component,

b) those, in which the lower quality of the product is not declared,

c) those, in which such a component is added in without its declaration, with the aim of obtaining higher weight, volume or to improve the appearance of the final products,

d) those, to which were added any component through which the quality of the product or the biological value was reduced,

3) those, in which additive materials are not approved.

Adulterated food stuffs in The Slovak Republic are those of which the appearance, taste, structure or other signs are changed in respect of the decrease of their value and which are offered to a consumer with a recognised brand name at the same value or by other fraudulent methods (11).

The change of appearance, taste, structure or other signs, due to the intentional addition of certain material, is aimed at the increase of the volume, the weight of a product, too, a partial or total restriction of some valuable ingredients and their replacement.

The branding of products has to be in harmony with the structure of food stuffs according to the protection of the consumer against adulteration (11).

The food stuffs in circulation fraudulently branded are those:

1) which persuade a consumer into the deception:

a) from the point of the quality of food stuff, including character, identity, quality, content, quantity, durability, origin or the place of origin, mode of production or processing,

b) by the attribution of qualities or effects of food stuffs which they do not have,

c) by indicating that the food stuff has special quality, when, all food stuffs have the same,

2) by assigning to the food stuffs preventive, curative or healing qualities,

3) apart from special curative waters, referring to the determined special nutritious purposes of food stuffs where they have no such as qualities.

These provisions are referred as well as to the demonstration of food stuffs, mainly with regard to their shape, appearance, packing, used packing material, mode of arrangement, display and advertising.

We often encounter the term authenticity in the identification of adulterated food stuffs. Authenticity means the ascertaining of the origin, genuineness or unity of food stuffs with the original.

Authentic food stuffs must have a defined origin, content and quality and they must issue from specific sources. The necessary conditions for an authentic product means that all its components must also be directly commensurate with the metabolism of the vegetable and animal organism from which they originate.

Honey adulteration is a very common and contemporary problem in The Slovak Republic.

The requirements for the obtaining, production and import of honey and its products and their bringing into circulation as well as their alteration have been created in the *Decree of the Ministry of Agriculture of The Slovak Republic* and the *Ministry of Health service from the 10th August 2000 No. 2313/I/2000-1000*, which is issued by the Chapter of the *Alimentary Code of The Slovak Republic amending honey and its products (10).*

Adulterated honey according to this Chapter is:

1) honey in which the organoleptic characteristics are not the same as declared (transparency, consistency, colour, odour, taste),

2) honey which is obtained or processed by a mode other than claimed.

We can divide honey according to its origin and basis on the microscopic determination of pollen grains and electrical conductivity, as:

a) flower, b) honeydew, c) mixed.

As to the mode of obtaining and processing we divide honey as drawn, pressed, honey comb, drip and pasta (7).

3) honey, treated by illegal means (e.g. the highest temperature for honey treatment is 50 °C),

4) when components such as honey dew and nectar were used for honey preparation. It is impossible to add or remove any basic components,

5) honey branded with false information and data.

The average parameters of honey composition (1) are covered by Table 1:

Component	Average concentration	Scale	S_{rel} %
Humidity	17.2	12.2–22.9	8.7
Fructose	38.4	30.9–44.3	4.6
Glucose	30.3	22.9–40.8	10.0
Saccharose	1.3	0.3–7.6	66.4
Maltose	8.6	3.3–18.2	24.1
Total acids	0.57	0.17–1.17	35.1
Ash	0.17	0.02–1.03	88.8
Proteins	0.17	0.058–0.79	42.1
Prolin	0.048	0.015–0.14	38.5
pH	3.91	3.42–6.10	–

S_{rel} — relative conclusive declination

Honey as a food stuff commodity attracts a lot of producers and businessmen to adulteration with the aim of obtaining an illegal enrichment. One of the most frequent methods of honey adulteration is the addition of sugar solutions or syrups, the preparation of artificial honey or the preparation of water extractions of flowers with a saccharose solution in it (e. g. dandelion – *Taraxacum officinale*).

We can identify the addition of saccharose by the sugar profile and by the content of saccharose over 5 % (as to the *Codex Alimentarius*), even though in some types of honey a saccharose content of more than 10 % is allowed.

We can also identify saccharose adulteration indirectly, based on the lower content of other minor components of honey like proteins, minerals, vitamins, amino acids, enzymes, based on the augmentation of hydroxymethylfurfural content, which is present in invert sugars as a result of a reaction of fructose with acids, which participate in the hydrolysis of a saccharosis.

The honey is suspect if the content of hydroxyfurfural exceeds 200 $\mu g.g^{-1}$ up to 500 $\mu g.g^{-1}$. In practice bees are very often fed with saccharose. When the accumulation is not sufficient, saccharose is used for the replenishment of winter stores. If we replenish the higher stock, bees are not able to use up it. We can find then higher content of saccharose in such honey (4). The content of the rest of sugars is similar to real honey. We can find it in the lower content of components originating from flower nectar and the lower activity of acid phosphatase, amino acids, minerals, as well.

When we suspect adulteration by glucose syrup, we determine the quantity of glucose in honey. When the content is higher than 40 %, honey is adulterated (5).

Fructose syrup is prepared by enzymic isomeration of glucose to fructose in corn syrup. This product is similar to the structure of saccharides in honey. The determination of enzymicaly prepared sugar is difficult, because the production of hydroxymethylfurfural does not happen here. We can

identify the addition of enzymically prepared invert sugar by the microscopic examination of product of enzymic activity, by the polarographic determination of invert sugar or by the study of a content of disaccharides (isomaltose) and trisaccharides in honey as well. The normal proportions of maltose and isomaltose range from 0.39 to 0.51 (3). When maltose and isomaltose range over 0.51 in honey it shows adulteration by fructose syrup.

We can determine the botanical origin of honey by the microscopic examination of the pollen grains in it. Methods of evaluation and characterisation of pollen in various sorts of honey are published by the *International Commission of Biologic Science* (6). A less specific method is the identification of amino acids (8). There are determined aromatic compounds or phenol substances. Nowadays determinations of aromas by modern methods of gas chromatography are preferred.

The differentiation of honeydew from the honey of blossom flowers is possible to do by microscopic examination. The honeydew contents spore from black mould, fungi and higher number of pollen grains. The conductivity of honeydew is higher because of higher content of minerals and organic acids.

The maximal electric conductivity of honey (by *Codex Alimentarius*) recommended for human consumption is:

– flower honey: 55.0 $mS.m^{-1}$
– honeydew honey: 90—130 $mS.m^{-1}$
– mixed honey: 55—100 $mS.m^{-1}$

The use of sugar cane and corn syrup is determined by the analysis of its carbon isotope range. The verification of thermal treatment of honey is done by the determination of the hydroxymethylfurfural amount (normal content is below 10 $mg.g^{-1}$) (2). A higher value means that a higher temperature was used for treatment during processing.

CONCLUSION

We have mentioned only few methods (6, 8, 9) for the determination of adulterated and authentic honey. To determine the authenticity of honey we need reliable, verified and sensitive methods. The regular control of honey samples and the development of new laboratory methods can improve the situation in this field.

REFERENCES

1. Ashurst, P. R., Dennis, M. J., 1996: *Food Authentification. Blackie Academic&Professional,* London, Weinheim, New York, Tokyo, Melbourne, Madras, 399 pp.

2. Baranová, M., 1999: Honey. In *Hygiene of Vegetable Food and Food Additive Substances* (In Slovak). UVM Košice, 69-77.

3. Cranova, E., 1983: From research on fructose to honey knowledge (In Czech). *Bee world, 3,* 22—30.

4. Dobrovoda, I., 1986: *Honey. Bee Products and Health* (In Slovak). Príroda Bratislava, 11—95.

5. FAO, 1986: *Food and Nutrition Paper 14/8 – Manuals of Food Quality Control, Food Analysis: Quality, Adulteration and Test of Identity.* FAO, Rome, 326 pp.

6. Loveaux, J., Mauriyio, A., Vorwohl, G., 1978: Methods of melissopalynology. *Bee World,* 59, 139—153.

7. Pekár, I., 2002: Honey and requirements for its obtaining, producing and introducing to a circulation (In Slovak). *Včelárstvo,* 76, 152—153.

8. Singhal, R. K., Kulkarni, P. R., Rege, D. V., 1997: *Handbook of Indices of Food Quality and Authenticity.* Woodhead Publishing Limited, Cambridge, 561 pp.

9. Suhaj, M., Kováč, M., 1999: *Methods of Identification, Falsification and Authentification of Food* (In Slovak). VÚP Bratislava (morfologická príručka v elektronickej forme).

10. Decree of the Ministry of Agriculture of the Slovak Republic and the Ministry of Health Service from the 10th August 2000 No. 2313/1/2000-1000 (In Slovak).

11. Food Act No. 152/1995 Body of Laws, in subsequenced regulations in force (In Slovak).

12. www.nfpa-food.org; **May, 2003.**

Received April 2, 2004

FOLIA VETERINARIA

www.uvm.sk

FOLIA VETERINARIA, 48, 2: 90—91, 2004

CHLAMYDOPHILA-INFECTIONS, A POTENTIAL CAUSE OF ABORTION IN OUTDOOR SOWS (A Short Communication)

Docic, A.[1], Bilkei, G.[2]

[1]Corresponding author
[1, 2]Bilkei Consulting, Raubbühlstrasse 4, 8600 Dübendorf Switzerland

ABSTRACT

The present study reports on *Chlamydophila*-caused late abortions in 54 outdoor gilts and low parity sows in a high health unit. The serological and microscopical evidence of *Chlamydophila* spp., the lack of other pathogens and the subsequent beneficial use of chlortetracycline, indicate that the abortions were caused by *Chlamydophila* spp. Outdoor, stressful production system may have played an important role in the cause of disease of the animals.

Key words: abort; *Chlamydophila* spp.; outdoor; sow

INTRODUCTION

Genus *Chlamydophila* (C.) are Gram-negative bacteria that are widespread in mammalian and avian species and can multiply only inside living cells (5). The species of *C.* found in the pig include *C. psittaci*, *C. trachomatis* (*C. suis*) and *C. pecorum* (2). They exist outside cells as elementary bodies which survive well only when dry (2). Entering the cells, they form small reticulate bodies, which are seen as inclusions within the cell. Source of infections are aerosols, ingestion of infected material, vulval discharges in muddy pastures or mucosal contamination at reproductive activity (1). *Chlamydophila* spp. can cause a variety of clinical signs in the respiratory, intestinal or genital tracts and polyarthritis in pigs (2). Since the renaissance of outdoor production, C. infections causing abortions in sows have increased (1). Herd immunity is a key factor in the expression of the disease. The organism can be isolated along with other bacteria and may be present in chronic cases. A serologic test alone is not considered to be a great help in diagnosing the disease, as the organism has often been recovered from herds in which disease was clinically not apparent (2).

Corresponding author: Tel. 0041 1 820 02 26

CASE DESCRIPTION

In a German high health 1,000 sow (F1 of Large White x Landrace) commercial unit (Donaueschingen, Stuttgart county), client of our consulting office, a sudden increase of late abortion (days 97—108 of pregnancy) from previously 0.1 % (year 2002) to 8.4 % (first half year of 2003) ($P<0.01$) in gilts, and from 0.3 % (year 2002) to 4.2 % (first half year of 2003) ($P<0.05$) in parity one sows were recorded. Only outdoor-kept animals aborted. Higher parity females did not abort. None of the gilts or sows died.

All aborted females showed *conjunctivitis, pyrexia* (39.9 ± 0.3 °C), and anorexia for 3—5 days prior to and 2—4 days after abortion. Routine pre-trial screening with an ELISA, for the detection of C. antibodies in incoming gilts (from a large nucleus herd) were negative. In the present herd, the gilts and sows received a unit specific *Escherichia coli* (Vet Invest, Zagreb, Croatia) *Clostridium perfringens* types A and C, porcine parvovirosis, Aujeszky disease, swine influenza virus and erysipelas vaccines.

Chlortetracycline treatment (23 mg.kg^{-1}/day body weight *per os*, for fourteen days in the whole unit, regardless of the age of the animals) and dislocation of the whole outdoor unit onto a new pasture were implemented. On the newl pasture and after chlortetracycline treatment the abortions (July to October 2003) stopped (out of 256 late pregnant females no abortion occurred).

All aborting animals (n = 54) were subjected to routine diagnostic examination (Vet Invest, Zagreb, Croatia) as suggested by Quinn et al. (3): *Haemophilus parasuis, Escherichia coli, Streptococcus suis, Actinobacillus pleuropneumoniae, Brucella* spp., classical and African Swine fever and *Coxiella burnetti* were excluded by serology and by routine laboratory examination of post-abortion-discharges and foetal tissues.

Pseudorabies were ruled out by fluorescin antibody (FA) test. Porcine stress syndrome was excluded as neither stress nor the typical clinical signs of porcine stress-syndrome were recorded (nevertheless, outdoor keeping of swine is accompanied by some environmental stress). Swine erysipelas was excluded by bacteriological culture as late aborted foetuses, heart, lung, liver, spleen, kidney, on tryptose media (Vet Invest, Zagreb, Croatia) were negative for *Erysipelothrix rhusiopathiae*.

The heart, lymph nodes, kidney and alimentary tract of foetuses were examined by gross pathology and histology for *porcine circovirus*-2 (PCV-2) (Vet Invest, Zagreb, Croatia). In paraffin-embedded, formalin-fixed lung, kidneys, liver, pancreas and in lymphoid tissues, small amount of PCV-2 antigen were found in 3 of the 61 examined foetuses out of 54 abortions. Serum samples were tested for antibodies against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) by immunoperoxidase monolayer assay (IPMA), with an Ag ELISA for the detection of C. antibodies, and with microscopic agglutination test (MAT) for the detection of agglutining antibodies against nineteen *Leptospira* serovars (Vet Invest, Zagreb, Croatia).

In addition to those serological investigations, smears from tissue of aborted foetuses were prepared by modified Ziehl-Neelsen (MZN) technique (3) for microscopic examination for intracellular inclusions containing elementary bodies. Post-abortion vulval discharges (n = 54) were examined by Koster's stain (by fixing smears with carbol fuchsin, decolourised with acetic acid and counterstained with aqueous Löffler's methylen blue).

RESULTS AND DISCUSSION

Twenty seven (50 %) of the examined serum samples had antibodies against PRRSV ranged up to 1:2560. ELISA identified antibodies to *C. trachomatis* (*C. suis,* 2) in **all** serum samples, having titres 400-1024. The MAT was positive in **all** serum samples. Using Koster's stain, C. appeared as clusters of intracellular red dots against a blue background in all cases. **All** smears (n = 61) from the liver of the aborted foetuses were positive for inclusion bodies. Three of 54 serum samples were *Leptospira Bratislava* positive.

The laboratory findings of PRRS, PCV-2, *Leptospira* spp. were the evidence that this agents were also present in this unit. The overwhelming presence of C. titres supported the diagnosis of a C. infection in this unit. This diagnosis was further confirmed with the observation of intracellular elementary bodies typical for C. infections. Similar inclusions can be observed in cases of *Brucella* spp. and *Coxiella burnetti* infections. In the present case, *Coxiella* needs further investigation for determining its involvement as a complicating agent. Serological tests for *Brucella* spp. showed negative results. Antibody titres to *Leptospira* spp. are indicative of this micro-organism's possible involvement in three cases of abortions in this unit.

The clinical signs of C. infections are not distinctive. Nevertheless, it must be considered – especially in outdoor production and in low parity females – as a possible cause of late abortion (1). Most C. infections in the pig are not apparent and whether they become clinical or not, depends on the stain and dose of the organism and the age and immunological status of the pig. The presence of other pathogens at the same time in this unit **might** have also been crucial in stressful outdoor raising and winter-spring season.

A critical remark: Serology for C. infections alone is questionable in diagnosing C. infections, due to the widespread occurrence of natural infections with large numbers of different strains of C. spp. (4). In field situations, the serological titres in response to C. infections are usually low (1). Therefore, in the present case clinical signs, serology, and microscopic examination and disclosure of other pathogens and (last but not least) the success of chlortetracycline treatment allowed us to make the diagnosis of a C. caused aborts.

Outdoor pig production bears the danger of C. infections. All three C. species are found in mammals and birds that have the potential to contact outdoor pigs. All C. species can survive for month as elementary bodies in the environment. The major route of transmission of C. species to pig are inhalation, oral and venereal route. In outdoor production all those routes of infection are possible. Birds, flies, dust, infected muddy pasture or slurry may represent major causes of C. infections in pigs.

REFERENCES

1. Bilkei, G. 1995: Herd health strategy for improving the reproductive performance of pigs. Proceedings of the 8th Symposium of International Society for Animal Hygiene. *Hung. Vet. J.,* 10, 776—777.

2. Done, S. H., 2001: Chlamydial infections in pigs. P*ig Journal* ,47, 189—202.

3. Quinn, P. J., Carter, M. E., Markery, B. (eds.), 1999: *Clinical Veterinary Microbiology.* Mosby, London, 74—315.

4. Szeredi, L., Schiller, I., Sydler, T., Guscetti, F., Heinen, E., Corboz, L., Eggenberger, E., Jones, G. E., Pospischil, A. 1996: Intestinal *Chlamydia* in finishing pigs. *Vet. Pathol.,* 33, 369—374.

5. Vasquez-Cisneros, C., Wilsmore, A. J., Bolle, E. 1994: Experimental infections of pregnant sows with ovine *Chlamydia* strain. *Vet. Microbiol.,* 42, 383—387.

Received April 3, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 92—94, 2004

THE OCCURRENCE OF THE GENERA *Hypoderma*, *Cephenemyia* AND *Pharyngomyia* IN DEER IN THE SLOVAK REPUBLIC (A Short Communication)

Čurlík, J., Letková, V., Ciberej, J., Lazar, P., Goldová, M., Kočišová, A. Košuthová, L., Trávniček, M., Bhide, M., Lazar. G., Pošivak, J., Konjevič, D.*

University of Veterinary Medicine, Komenského 73, 041 83 Košice
The Slovak Republic
***Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb**
Croatia

curlik@uvm.sk

ABSTRACT

From 1997 to 2001 2,387 red deer (*Cervus e. elaphus*) and 1,871 roe deer (*Capreolus capreolus*) were examined for the larvae of the warble fly *Hypoderma diana*. The prevalence of infestation was 47.22 % (1,122 specimens) in red deer and 57.98 % (1,141 specimens) in roe deer. The average number of larvae per head was 84.13 in red deer and 67.43 in roe deer. From 1997 to 2001, 71 red deer (females and fawns) and 202 roe deer were examined for the larvae of the botfly *Cephenemyia stimulator*, *Pharyngomyia picta*. The prevalence of infestation was 36.62 % (26 specimens) in red deer and 45.05 % (91 specimens) in roe deer. The average number of larvae per head was 13.13 in red deer and 15.24 in roe deer.

Key words: bot fly (*Cephenemyia stimulator*; *Pharyngomyia picta*); *Capreolus capreolus*; *Cervus elaphus*; prevalence; warble fly (*Hypoderma diana*)

INTRODUCTION

Generally in free living animals 60—75 % of diseases are due to parasitic infections (3). Botfly and warble fly infestations are major and widespread parasitic infestations. In Europe only five species of botfly and warble fly infest wild bi-footed animals (6).

Family Hypodermatidae includes *Hypoderma diana* in red deer, fallow deer, roe deer and mouflon, whereas, *Hypoderma actaeon* is present in red deer in Central Europe.

In the family Oestridae the genera *Cephenemyia* (*C. stimulator, C. auribarbis*) and *Pharyngomyia* (*P. picta*) represent the groups of ectoparasites.

MATERIALS AND METHODS

1. The Warble fly – *Hypoderma diana*-infestation

In the course of May to December, 1997–2001 we screened 2,387 samples of red deer and, 1,968 samples of roe deer. Material was collected during hunting in the East of Slovakia. Screening was carried out in the abattoir (Zverex Šaľa, Mraziarne Kežmarok). Larvae were collected and sorted according to species (7).

2. The Botfly – *Cephenemyia stimulator, Pharyngomyia picta*-infestation

During the years 1997-2001 during the hunting period (16th May—31st December) a total of 71 red deer and 202 roe deer were observed for nasal botfly infestation. The heads of females and young animals were marked and submitted in processing plants. The heads were screened (by flushing the nasal cavity and by cutting in median plane) for larvae and were sorted according to species (5).

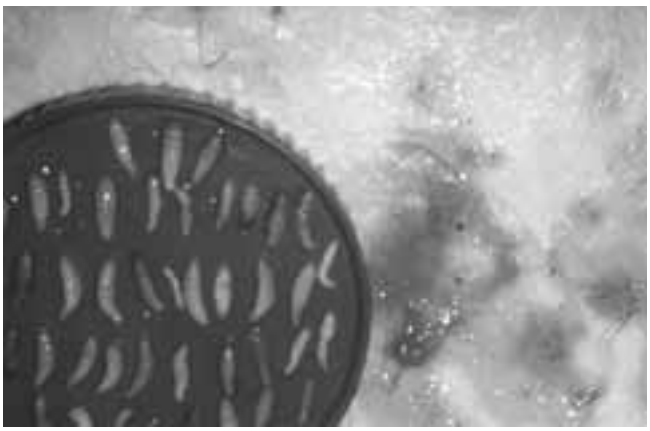

Fig. 1. *Hypoderma diana* larvae under skin of red deer

Fig. 2. *Cephenemyia stimulator* in the nasal cavities of roe deer

RESULTS

1. Warble fly – *Hypoderma diana*-infestation

Red deer

The prevalence of *H. diana* in red deer was 47.22 %. The average number of larvae (Fig. 1) was 84.13 (12—196). The high intensity of infestation in 194 red deer (above 100 larvae/head) was observed with 8.13 % prevalence (Table 1).

Table 1. Warble fly (*Hypoderma diana*)-infestation in red deer

Year	No. of samples screened	Number of positive samples	Prevalence %	Number of larvae (average)
1997	579	284	49.05	82.10
1998	1,756	793	45.16	93.05
1999	19	10	52.63	81.13
2000	21	10	47.62	79.21
2001	12	5	41.67	85.14
Total	**2, 387**	**1 122**	**47.22**	**84.13**

Roe deer

The prevalence of *H. diana* was 57.98 % (Table 2), with an intensity of infestation of 67.43 larvae (6—189).

Table 2. Warble fly (*Hypoderma diana*)-infestation in roe deer

Year	No. of samples screened	Number of positive samples	Prevalence %	Number of larvae (average)
1997	999	559	55.96	57.28
1998	872	520	59.63	64.32
1999	33	20	60.61	68.19
2000	35	20	57.14	71.56
2001	29	15	51.72	75.82
Total	**1,968**	**1,141**	**57.98**	**67.43**

2. Botfly infestation – *Cephenemyia stimulator, Pharyngomyia picta*

Red deer

During the year 1997—2001 we recorded a 36.62 % prevalence of *Pharyngomyia picta* (Table 3). Intensity of infestation was 13.13 larvae (9—47).

Table 3. Botfly (*Pharyngomyia picta*)-infestation in red deer

Year	No. of samples screened	Number of positive samples	Prevalence %	Number of larvae (average)
1997	16	3	18.75	17.14
1998	24	10	41.67	13.13
1999	20	8	40.00	12.21
2000	6	3	50.00	11.07
2001	5	2	40.00	12.08
Total	**71**	**26**	**36.62**	**13.13**

Roe deer

During the years 1997–2001 the prevalence of *Cephenemyia stimulator* larvae in roe deer was 45.05 % (Table 4) whereas the intensity of infestation was 15.24 larvae (4–21) (Fig. 2).

Table 4. Botfly (*Cephenemyia stimulator*)-infestation in roe deer

Year	No. of samples screened	Number of positive samples	Prevalence %	Number of larvae (average)
1997	77	35	45.45	17.35
1998	57	23	40.35	11.07
1999	52	24	46.15	13.21
2000	9	5	55.56	18.14
2001	7	4	57.14	16.42
Total	**202**	**91**	**45.05**	**15.24**

DISCUSSION

The prevalence of *Pharyngomyia picta* in red deer in Europe ranged from 13 % in Hungary (8), and in Poland up to 100 % (2). The prevalence of *Cephenemyia stimulator* in red deer in Spain was recorded at 58 % (4) whereas in Scotland it was 100 % (1). In Slovakia, during the five year (1997—2001) study we observed a total of 36 % *Pharyngomyia picta prevalence in red deer.*

The intensity of infestation of Hypoderma diana in roe deer in former Czechoslovakia was 27.5 larvae (5), which is much lower than our results (67 larvae). The maximum number of larvae from one red deer recorded was 220 (5). Whereas, in our check we recorded a maximum of 189 larvae from one animal.

CONCLUSION

The paper deals with the occurrence of botfly (*Cephenemyia stimulator, Pharyngomyia picta*) and warble fly (*Hypoderma diana*) in deer (*Cervus e. elaphus*, *Capreolus capreolus*) in the East Slovakia region. The prevalence obtained in our study alerts us to the importance of these infestations in deer. It is necessary to give prompt attention to the aspects of prevention and treatment of these infestations.

REFERENCES

1. Cameron, A. E., 1932: The nasal Bot Fly, *Cephenemyia auribarbis* of the red deer, *Cervus elaphus* L. *Parasitology,* 24, 185—195.

2. Droźdz, J., 1961: New data from biology of *Pharyngomyia picta* Meig. larvae (Diptera: Oestridae) *Cervus elaphus* parasite. *Wiadomośći Parasitologiczne,* 7, 373—379.

3. Farkaš J., Čepelák J., 1984: Present distribution of subcutaneous gadfly occurrence in red deer and roe deer in Slovakia. *Folia venatoria,* 14, 291—295.

4. Gill Collado, J., Valls, I. J., Fierro De Riva, Y., 1985: Estudio do las larvas de Oestridae, parásitas do Cervidae en Espaňa. *Boletim do la Sociadade Portuguesa do Entomologia* (Supplementum I), 467—475.

5. Lamka J., Suchý J., Štaud F., 1997: Efficacy of oral administered Ivermectin against larval stages of bot fly (*Cephenemyia stimulator*) in deer. *Acta Veterinaria Brno,* 66, 51—55.

6. Minář, J., 1982: A contribution to the knowkledge of distribution and ecology of botflies and warble flies in the cervids. *Folia Facultatis Sci. Universitatis Purkynianae Brunensis,* 23/7, 87—92.

7. Minář, J., 1980: Hypodermatidae, Oestridae, Gastrophilidae. In **Chvála, M.:** *Fauna ČSSR. Krev sající mouchy a střečci* (In Czech). Praha, Academia, Vol., 22, 391–446.

8. Sugár, L., 1974: The occurrence of nasal throat bot flies in wild ruminants in Hungary. *Parasit. Hung.,* 7, 181—189.

Received November 18, 2003

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 95—99, 2004

THE PREVALENCE AND CAUSE OF CAMEL MASTITIS (*Camelus dromedarius)* IN ERRER VALLEY, EASTERN ETHIOPIA

Mitiku, E., Asefa Asmare, A.

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

asefa_a@yahoo.com

ABSTRACT

For the study a total of 115 lactating camels were used. Grade 3 and above CM test reactions were considered to be mastitis. Sixteen (13.91 %) of the camels were found to be affected by clinical mastitis and eighteen (15.65 %) by subclinical mastitis. Of the sixteen identified blocked quarters, five quarters of three camels (2.61 %) were not associated with other mastitis positive quarters of the udder. All blocked quarters had a previous history of mastitis. Therefore, in this study a total of 32.17 % prevalence rate of mastitis in camels was detected. Out of 50 CM test positive quarters, 34 were graded as 3, twelve as 4 and four as 5. California mastitis test scores of 1, 2,3, 4, and 5 equated to average cell counts of 97,000, 333,000, 1150,000, 3100,000, and 13900,000, respectively. The bacteriological examination of CM test positive quarters revealed that *Staphylococcus aureus,* coagulase negative staphylococcus and *Pasteurella haemolytica* as important causative agents of camel mastitis. In grade 4 and 3 of CM test reactions, *Staphylococcus aureus* and *Pasteurella haemolytica* stimulated the highest mean cell count (1520,000 ± 200,000, 1325,000 ± 200,000, respectively). Therefore, although camels are reported to be relatively resistant to mastitis, this study revealed an apparently high prevalence of mastitis in camels managed traditionally by pastoralists. Moreover, most of the causative agents are of significance for human health. Thus the mastitis problem in camel needs more attention.

Key words: bacteriological examination; California mastitis test; camel; mastitis; quarter; somatic cell count

INTRODUCTION

The world one-humped camel population is about 16.5 million (17). Over 80 % of these camels are found in Africa and over 63 % of the African camels are found in the lowlands of East Africa. Ethiopia is blessed with 1.5 million head of camels. The camels mostly inhabit the arid and semiarid lands of the country.

In thevast areas of arid and semiarid lands drought accompanied by famine is a frequent phenomenon. It threatens the lives of a large number of people and robs them of their cattle and livestock. Nevertheless, the camel is considered more drought resistant than any other domestic animals (16). During the recent recurrent droughts in Africa, the camel population was only marginally affected compared with other species of domestic stock (22).

The camel is the most efficient domestic animal at transforming scarce arid and semiarid resources into valuable animal protein. It consistently produces food such as milk and meat in harsh climatic conditions where other livestock species would have difficulty even in surviving and where the risks and uncertainty of other forms of agricultural production have been demonstrated (2). The camel's major contribution to the socio-economics life of pastoralists is its milk production potential. In Ethiopia, Knoess (12) has recorded that camels produce more milk than all other local livestock under similar conditions.

Kebebew and Baars (10) have reported a mean daily milk production of 7.5 kg with total yield of 2104 kg per lactation in the Errer valley. In the same area Moges (15)

declared 5.6 kg of milk per day in the dry season and 7.5 kg per day in the wet season. The duration of lactation period is estimated to vary from nine to eighteen months, the average being around fourteen months (5).

Camel milk is highly nutritious. The daily protein requirements of an adult man are contained in 1.8 kg of camel's milk while 2.5 kg covers caloric requirements (6). Different authors give the fat content of dromedary milk as 2.9 % to 5.5 %, protein as 2.5 % to 4.5 %, lactose as 2.9 % to 5.8 %, ash as 0.35 % to 0.95 %, vitamin C as 2.3 % to 2.5 % (13). The high vitamin C content is very essential in areas where vegetables are rare or completely absent.

In any dairy animal, the quantity and quality of milk depends on genetic and non-genetic factors such as udder health. There is an extensive literature on bovine mastitis, but fewer research reports on ovine and caprine mastitis. Very little is known about mastitis in the camel, because the disease was thought to be rare in this species (4). Since camel milk is consumed fresh or acidified without heat treatment, it can be a public hazard to the consumer unless handled hygienically and the health status of the udder is determined regularly. Therefore, the purpose of this study is to investigate the prevalence of mastitis in the camel and to identify the bacterial causative agents of mastitis.

MATERIAL AND METHODS

The study area

The study was conducted in the Errer valley found in Eastern Ethiopia. The Valley is located 529 kilometres from Addis Ababa, the capital city. The altitude ranges from 1,300 metres to 1,600 metres above sea level. The area receives a bimodal type of rain. The precipitation ranges from 400 mm to 800 mm with two peaks, one from March to April and the other from July to September. The climate in thisregion is semi-arid with a long dry season extending from October through February. The Errer valley has high camel population and camel milk is used extensively as a staple food for agropastoralists and pastoralists.

Experimental animals

For the study a total of 115 lactating camels belonging to 36 herds were used. They were from five years to twelve years old. The camels were managed traditionally by the pastoralists. They were milked twice a day: in the morning from 6:00 am to 8:00 am and in the evening from 6:30 pm to 7:30 pm. Camels in the first week and the last stage of lactation were not included in the study.

Sampling and laboratory examination

Milk samples were collected from individual quarters during morning milking time. All quarters were first carefully examined by visual observation and palpation. The teat ends were cleaned thoroughly and properly dried. Then were disinfected with swabs dipped in 70 % alcohol, extruding the external sphincter by pressure to ensure that dirt and wax are removed from the orifice. The first few streams of milk from each quarter were examined on a strip cup for the presence of discoloration, clots, flakes or pus. Approximately two to three millilitres of milk were drawn into the plastic paddle cups for the California mastitis test (CMT). The CM test was conducted according to the method of Klastrup and Schmidt (11) immediately after collecting the sample.

About five to ten millilitres of milk were collected from the experimental animals into separate clean, dry and sterile tubes with airtight seals for somatic cell count (SCC) and bacteriological examination. Each sample was accurately identified with respect to number of the animal, quarter and date of collection. Samples were kept in an icebox and transported to the laboratory.

A total of 446-quarter milk samples were available for SCC and CMT. Out of 50 CM test positive quarters, 34 were randomly selected and their milk samples were used for bacteriological analysis. The SCC was carried out according to the method of Saloniemi (21) whereas the bacteriological examinations were undertaken following the methods of Sears *et al.* (23) and Quinn *et al.* (19).

Data analysis

Data were analysed using the general Linear Model of MINITAB software version 12 (14). For mean separation, the least significant difference method was employed (7).

RESULTS

The mastitis prevalence rate and number of quarters affected are given in Table 1. The incidence of clinical mastitis in the camel was determined based on a physical examination of the udder and milk, and bacteriological culturing of milk samples whereas incidence of sub-clinical mastitis was determined in addition to the microbiological examination by SCC and CM test results.

Accordingly, from 115 examined camels, sixteen (13.91 %) were found to be affected by clinical mastitis and eighteen (15.65 %) by subclinical mastitis. Nine camels had one or two quarters with a complete cessation of milk flow. Of the sixteen blocked quarters, eleven quarters of six camels were associated with other mastitis positive quarters while five blocked quarters of three camels (2.61 %) were not associated with other mastitis positive quarters of the udder. All blocked quarters had a previous history of mastitis. Therefore, in this study a total of 32.17 % prevalence rate of mastitis in camels was detected. It was also found that 59.46 % of the affected camels had only one mastitis positive quarter, 32.43 % two quarters and 8.11 % three quarters.

Some of the clinically mastitis quarters were either swollen or indurate, hot and painful to palpation. Furthermore the milk on the strip cup showed wateriness or bloodstaining as well as clots. No systemic reaction was observed.

Table 1. The mastitis prevalence rate among 115 examined camels and its distribution among quarters

Stage of mastitis	Affected camels	Prevalence rate %	Number of animals with mastitis positive quarters: One quarter	Two quarters	Three quarters
Subclinical mastitis	18	15.65	14	2	2
Clinical mastitis	16	13.91	7	8	1
Chronic mastitis	3	2.61	1	2	–
Total	37	32.17	22	12	3
%	–	–	59.46	32.43	8.11

Table 2. The results of the California mastitis test and respective somatic cell count

CMT grade	Number of quarters	SCC (Mean ± SE x 1000)
1	320	97 ± 36.7
2	76	333 ± 27
3	34	1,150 ± 160
4	12	3,100 ± 1,500
5	4	13,900 ± 4,200

California mastitis test and somatic cell count

The results of SCC and CMT of milk samples are given in Table 2. After mixing an equal amount of milk sample and the reagent, sodium lauryl sulphate, the reaction was graded immediately as 1, 2, 3, 4 and 5 depending on the amount of gel formation in the samples (11). Grade 3 and above reactions indicated the presence of mastitis. Therefore, 34 quarters were graded as 3, twelve as 4 and four as 5. California mastitis test scores of 1, 2, 3, 4, and 5 equated to average cell counts of 97,000, 333,000, 1,150,000, 3,100,000 and 13,900,000, respectively.

Table 3. Pathogenic bacteria detected in CM test positive milk samples

Microorganism	Clinical mastitis		Subclinical mastitis		Total	
	Number**	%	Number	%	Number	%
Staphylococcus aureus	2	16.77	7	31.82	9	26.5
CNS*	3	25	5	22.73	8	23.5
Pasteurella haemolytica	3	25	6	27.27	9	26.5
Pasteurella multocida	1	8.33	1	4.55	2	5.9
Bacillus species	0	0	1	4.55	1	2.9
Enterobacter	2	16.67	1	4.55	3	8.8
Escherichia coli	*1*	*8.33*	*1*	*4.55*	*2*	*5.9*

** — Coagulase negative staphylococcus; ** — Number of quarters*

Table 4. The somatic cell count-stimulating ability of bacteria identified in the CMT positive quarter milk samples

Identified bacteria	CMT grade 3 SCC × 1000	N	4 SCC × 1000	N	5 SCC × 1000	N
Staphylococcus aureus	1140 ± 122*	7	5200 ± 200*	2	–	0
CNS[1]	800 ± 39	5	1800 ± 290	3	–	0
Pasteurella haemolytica	1325 ± 200*	6	2600	1	15000 ± 300	3
Pasteurella multocida	600	1	2400	1	–	0
Bacillus species	720	1	–	0	–	0
Enterobacter	500	1	4100 ± 205*	2	–	0
Escherichia coli	950	1	–	0	12000	1

* — Significantly different ($p < 0.01$); [1] — Coagulase negative staphylococcus ; N — Number of quarters

Bacteriological examination

The results of the bacteriological examination of milk samples and somatic cell count stimulating ability of the bacteria are indicated in Tables 3 and 4, respectively. The bacteriological examination of CM test positive quarters revealed the dominant position of *Staphylococcus aureus,* coagulase negative staphylococcus and *Pasteurella haemolytica* as important causative agents of camel mastitis. In grade 4 and 3 of CM test reaction *Staphylococcus aureus* and *Pasteurella haemolytica stimulated the highest mean cell count (5,200000 ± 200,000, 1,325,000 ± 200,000, respectively; see Table 4).*

DISCUSSION

In the present study a considerably high incidence of mastitis (32.17 %) was detected. This value is less than the findings of Al-Ani and Al-Shareefi (1) who found a 38 % and Barbour *et al.* (3) 55.7 % incidence of mastitis in Iraq and Saudi-Arabia, respectively. As such mastitis is not rare in this species as reported by Bolbol (4).

By bacteriological examination of CM test positive milk samples, a high prevalence rate of *Staphylococcus areus* and *Pasteurella haemolytica* was found (31.82 %, 27.27 %, respectively). Ramadan *et al.* (20) and Quandil and Ouadar (18) have also noted that *Staphylococcus aureus* was prominent as a cause of mastitis in camels. Further *Staphylococcus aureus was found to be a significantly ($p < 0.01$) potent stimulator of an enhanced cellular population (5,200,000 ± 200,000) compared with other pathogenic agents.*

Further study is needed to know the effects of different species of bacteria on SCC especially in camels. In this study, a single infection was encountered. This seems likely because prior infection with one type of bacterium increases resistance to other mastitis producing pathogens by provoking an increase in the white blood cell content of the milk.

Pastoralists do not usually examine the foremilk before they begin milking. Thus milk from infected quarters, which does not show marked physical change may pass unnoticed and contaminate the normal milk and then enter the food chain. Under a high atmospheric temperature as in the Errer valley, the bacteria in the milk multiply very rapidly. Since camel milk is consumed in its raw state, the high bacterial content in it may pose a health hazard to humans. According to Graaf et al. (8), Heeschen (9), and Teshager and Bayleyegne (24) *Staphylococcus aureus* and *Escherichia coli* are of human health significance.

Except in the case of certain microorganisms like *Mycobacterium tuberculosis,* where the method of dissemination may be haematogenous, infection of the mammary gland usually occurs through the teat canal. Dust contaminated with camel faeces in the enclosure, which harbour pathogenic bacteria have probably played a major role in the epidemiology of camel mastitis. Gross contamination of the teats and thus the invasion and infection of the mammary gland tissues with pathogenic agents might have occurred primarily between milking during the night time when the camels are kept in an enclosure and are in contact with the faecal contaminated dust rather than during the day time when the camels are continuously at pasture. Unhygienic milking procedures, inappropriate milking order and injuries around the teat or udder caused by ticks, thorny bushes and very vigorous suckling by grown-up camel calves can be predispositional factors of camel mastitis.

From our results we can conclude that there is apparently high incidence of mastitis in camel managed traditionally by pastoralists and most of the causative agents are of human health significance, thus the mastitis problem in camel should be given more attention.

ACKNOWLEDGEMENT

The authors would like to thank the Alemaya University and Dire Dawa Regional Veterinary Laboratory for providing us facilities to carry out the study. Assistance of the National Camel Research Centre by providing fund is also highly appreciated. We are indebted to Professor B. P. Hedge for reviewing the manuscript.

REFERENCES

1. Al-Ani, F. K., Al-Shareefi, M. R., 1997: Studies on mastitis in lactating one-humped camel (*Camelus dromedarius*) in Iraq. *Journal of Camel Practice and Research,* 4, 47—49.

2. Asefa Asmare, A., 2000: Review Article: The Camel the prime source of food for human consumption in harsh arid and semi-arid areas (*Camelus dromedarius*). *Folia Veterinaria,* 44, 215—22.

3. Barbour, E. K., Nabbut, N. H., Frerichs,W. M., Al-Nakhil, H. M., Al-Mukayel, H. M., 1985: Mastitis in *Camelus dromedarius* in Saudi-Arabia. *Trop. Anim. Health Prod.,* 17, 173—179.

4. Bolbol, A. E., 1982: Mastectomy in she-camel. *Assiut Vet. Med. J.,* 10, 215.

5. Chamberlain, A., 1989: *Milk Production in theTropics.* Longman Groups, UK Limited, England, pp. 202—210.

6. Dahl, G., Hjort, A., 1979: Dromedary pastoralism in Africa and Arabia. In **Cockrill, W. E.** (ed.): *The Camelid: An All-purpose Animal.* SIAS, Upsala, Sweden, 144pp.

7. Gomez, K. A., Gomez, A. G., 1984: *Statistical Procedures for Agricultural Research.* (2nd edn.). Wiley-Interscience Publication, pp. 198—207.

8. Graaf, T., Romero Zuniga, de J. L., Caballero, M., Duringer, R. H., 1997: Microbiological quality aspects of cow's milk at small holder cooperative in Turrialba, Costarica. *Rev. Elev. Med. Vet. Pays Trop.,* 50, 57—64.

9. Heeschen, W. H., 1997: Milk hygiene and milk safety in the European and international markets. *Keiler milchwirtschaftliche Forschungsberichte,* 50, 53—57.

10. Kebebew, T., Baars, R. M. T., 1998: Milk production performance of pastorally managed camels in Eastern Ethiopia. Ethiopian Society of Animal Production: *Proceedings of the 6th Annual Conference on Women and Animal Production,* May 14—15, 1998, Addis Ababa, Ethiopia, pp. 184—191.

11. Klastrup, O., Schmidt, M. P., 1974: Nordic recommendation concerning mastitis control of quarter samples. *Nord. Vet. Med.,* 26, 197—204.

12. Knoess, K. H., 1982: Milk production potential of the dromedary. *Pakistan Vet.,* 12, 91—98.

13. Knoess, K. H., Makhudum, A. J., Rafiq, M., Hafeez, M., 1986: Milk production potential of the dromedary, with special reference to the province of Punjab, Pakistan. *World Anim. Rev.,* 57, 11—21.

14. Minitab, 1991: *MINITAB,* Minitab statistical software company, UK.

15. Moges. D., 2001: T*he Dromedary Camel: Supplementation for Higher Yield and Behavioral and Feed Preference on Range.* MSc. Thesis. Swedish University of Agriculture. Uppsala, Sweden, pp. 6—8.

16. Mohammed Salih, M. A., 1988: Production in the arid lands of the Sudan: National and local perception of the potential. In **Hyort, A.** (ed.): *Camel in Development of Sustainable Products in African Dry Lands.* Motala Grafiska, Motala, Sweden, pp. 19—29.

17. Payne, W. J. A., Wilson, R. T., 1999: *An Introduction to Animal Husbandry in the Tropics* (5th edn.). Blackwell Science Ltd., Osney Mead, Oxford, pp. 485—520.

18. Quandil, S. S., Ouadar, J., 1984: Bacteriological study of some cases of mastitis in the dromedary (Camelus dromedarius) in the United Arab Emirates (Preliminary Report). *Rev. Med. Vet.,* 135, 705—707.

19. Quinn, P. J., Carter, M. E., Markey, B., Carter, G. R., 1994: *Clinical Veterinary Microbiology.* Wolfe Publishing, London, pp. 327—344.

20. Ramadan, R. O., Hassan, A. M., Abdin-Rey, R., Al-Gansnawi, Y. A., Ardalla, C., Fayed, A. A., 1986: Chronic obstructive mastitis in the camels: clinico-pathological study. *Cornell Veterinarian,* 77, 132—150.

21. Saloniemi, H., 1995: Use of somatic cell count in udder health work. In **Kaatinen, L. and Pyralas, H.** (eds): *The Bovine Udder and Mastitis.* Faculty of Veterinary Medicine, University of Helsinki, Finland, pp. 105—110.

22. Schwartz, H. I., Dioli, M., 1992: *The one-humped Camel (Camelus dromedarius) in Eastern Africa.* Verlag Joseph Margraf, Germany. 282 pp.

23. Sears, P. M., Gonzales, R. N., Wilson, D. J., Han, H. R., 1993: Procedures for mastitis diagnosis and control. *Veterinary Clinics of North America Food Animal Practice,* 9, 445—467.

24. Teshager, S., Bayleyegn, M., 2001: Bacteriological quality of raw milk of camel (*Camelus dromedarius*) in Afar Region (Ethiopia). *Journal of Camel Practice and Research,* 8, 51—54.

Received April 2, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2 : 100—103, 2004

THE DETERMINATION OF MALONDIALDEHYDE IN EGG YOLK BY SOLID PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Marcinčák, S., Bystrický, P., Popelka, P., Nagy, J., Sokol, J.

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

marcincak@lycos.com

ABSTRACT

An assay for the determination of malondialdehyde (MDA) levels in egg yolk by HPLC is described. The method involves extraction of tissues with ice-cold 10 % trichloroacetic acid (TCA) and reaction of the TCA extract with 2,4-dinitrophenylhydrazine (DNPH). After separation of MDA-DNPH complex using solid-phase extraction C_{18} column samples were eluted with 750 µl of acetonitrile and aliquots of 20 µl acetonitrile were detected by HPLC on reversed-phase C_{18} column (3 µm, Nucleosil, 125 × 3 mm). The flow rate was maintained at 1.0 ml.min^{-1}. The products were eluted isocratically with the mobile phase containing acetonitrile : water : acetic acid (39 : 61 : 0.2; v/v/v) and the UV absorption for all samples with maximum response was observed at 307 nm. The retention time for MDA-DNPH was 6.5 min.

Key words: egg; HPLC; lipid oxidation; malondialdehyde

INTRODUCTION

The organoleptic properties of food are determined by various factors including quality and the chemical modifications to fat. Lipid oxidation is a major cause of quality deterioration during the storage of lipid-rich foods (10). Although fresh shell egg lipids are not easily oxidised, even during storage, oxidation is facilitated when eggs are processed, especially when high temperature treatments are involved. Nowadays, higher susceptibility in lipid oxidation can by related to the tendency to enrich eggs with ω-3 polyunsaturated fatty acids (PUFA) as a preventative of cardiovascular diseases (1, 11). An increased level of PUFA, however, poses a potential risk to auto-oxidation.

The oxidative changes that occur in food lipid systems are generally quantified by measurement of secondary degradation products (7, 12, 17). In practice, the most often used method is thiobarbituric acid (TBA) test. The TBA test determines the amount of malondialdehyde (MDA), a major secondary by-product of lipid oxidation, in a sample. Detection of MDA is based on spectrophotometric quantisation of the colour complex (pink) formed after reaction of MDA with two molecules of TBA (13, 17). However, the TBA method is not absolutely specific for MDA detection, and the other TBA reactive substances (TBARS) as a proteins, DNA, deoxyribose absorb also significantly at 532 nm.

The reaction between MDA and compounds other than aldehydes may lead to an overestimation of the amount of MDA present in biological materials by the TBA reaction and decreases the specificity of TBA analysis (5, 6, 9, 13, 14). To overcome this problem a number of methods were developed to estimate MDA-TBA adducts using HPLC (6, 7). Although these results are more reliable compared to the spectrophotometric methods of TBA detection, this HPLC technique has not gained popularity because of the length and the complexity of the methods (4).

The MDA and DNPH reaction produces DNPH derivatives with an intensive yellow colour and it makes HPLC detection easier. The other advantage is excellent elution and separation

of decomposed aldehydes on HPLC column (8). These factors increase the specificity of MDA detection and they improve the complex view in the process of oxidation in a sample (5).

The objective of the present work was to modify the HPLC detection of MDA after derivatisation with DNPH (16) and its application on the MDA analysis in egg yolk.

MATERIAL AND METHODS

Acetonitrile (ACN) and hexane were obtained from Merck (Darmstadt, Germany), 1,1,3,3-tetramethoxypropane (TMP) and butylated hydroxytoluene (BHT) from Sigma (Steinheim, Germany), DNPH was purchased from Fluka (Buchs, Switzerland), hydrochloric acid (HCl), trichloroacetic acid (TCA) and EDTA were purchased from LACHEMA CZ (Brno, Czech Republic). All other chemicals reagents were of analytical or HPLC grade.

BHT was prepared as 0.8 % in hexane. The DNPH reagent was prepared by dissolving 31 mg DNPH in 10 ml of 2 mol. l^{-1} HCl. EDTA solution was prepared diluting 0.3 % EDTA in deionised water. The standard of MDA was prepared by acid hydrolysis of TMP (11). The resulting MDA stock solution of 4.37 µg.ml^{-1} was further diluted with water to yield final concentrations of 4.37, 8.74, 17.5, 87.4, 437 and 1000 ng.ml^{-1} to get the standard curve. The fresh working standards were prepared from stock solutions daily.

Eggs were purchased from trhe local supermarket and divided into two groups. The first group was examined immediately after purchasing the fresh eggs. The second group was examined after thirty days of storage at 10 °C. The eggs were broken and the yolk was separated from albumen.

A yolk egg sample of 1.0 g was weighed in 50 ml centrifuge tube, and 1 ml of 0.3 % EDTA was added immediately. After gentle agitation, 5 ml of 0.8 % BHT was also added, and the tube was gently shaken again. Just before homogenisation, 8 ml of 10 % TCA (1 °C) was added to the tube and homogenisation was carried out for 15 seconds at maximum speed. After centrifugation (5 minutes at 3500 × *g*, 4 °C), the top hexane layer was discarded and the bottom layer was filtered through Whatman filter paper No. 4 (Whatman, England) into a 10 ml volumetric flask. 10 % TCA was used to make up the volume.

For derivation 100 µl of DNPH reagent was added to 2 ml of TCA extract or standard in 12 ml test tube. Samples were mixed and the reaction mixture with pH < 1.5 was incubated for 30 minutes at room temperature in the dark.

Three-millilitre Supelclean LC-18 columns (Supelco, Bellefonte, USA) were used for solid-phase extraction. The columns were activated by washing with 2 ml of acetonitrile and 2 ml of water. The samples were slowly passed trough the columns and washed with 2 ml of water. MDA-DNPH complex was eluted with 750 µl of acetonitrile and 20 µl of eluate was used for HPLC analysis.

Analytical HPLC separations were performed with a Hewlett-Packard Series 1050 liquid chromatograph equipped with a quaternary autosampler, variable wavelength detector operated at 307 nm, and an integrator HP 339 6II was used. The column Nucleosil (3 µm, 125 × 3 mm) C_{18} reverse-phase (Manchery-Nagel, Düren, Germany) was used. The flow rate was maintained at 1.0 ml.min^{-1}. The operation was isocratic with a mobile phase of acetonitrile : water : acetic acid (39 : 61 : 0.2; v/v/v). Both samples and standards were injected in the mobile phase. The volume injected was 20 µl. For determination of recoveries, MDA standards (58.3, 116.6 ng.g^{-1}) were added to the egg yolk samples.

RESULTS AND DISCUSSION

Fig. 1 represents the HPLC chromatograms of standard, reagent blank, and egg yolk sample where MDA was derived by DNPH. The complex MDA-DNPH was detected with an isocratic system at 6.5 minutes. The mobile phase (ACN : water : acetic acid, 39 : 61 : 0.2; v/v/v) was used for MDA-DNPH elution and the UV absorption for all the

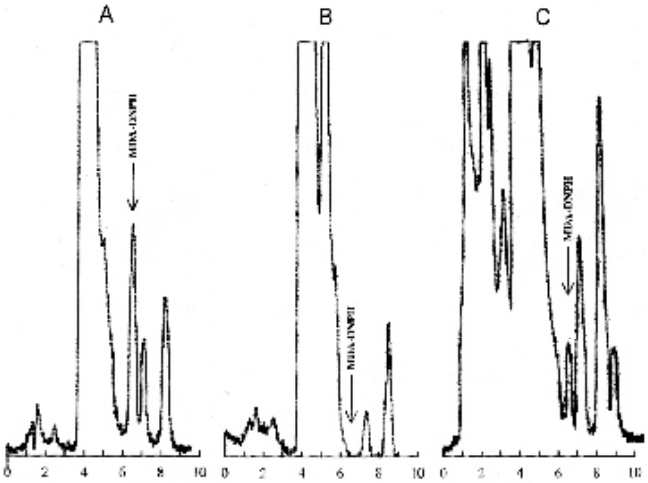

Fig. 1. Chromatograms of MDA: A) standard of MDA (MDA-DNPH), 87.4 ng.ml^{-1}; B) reagent blank; C) sample, 53.8 ng. g^{-1}. LC conditions: Nucleosil C_{18} reverse phase (3 αm, 125 × 3 mm), acetonitrile-water-acetic acid (39 : 61 : 0.2), flow rate 1 ml.min^{-1}, UV detection at 307 nm. Volume injected 20 µl

samples with maximum response was observed at 307 nm. At the optimised conditions, the peak MDA-DNPH is well separated from another peaks. The calibration curve showed a linear relationship between the MDA-DNPH area and the concentration of MDA in standard solutions in the range of 4.35—1000 ng.ml^{-1} (r = 0.9989). The detection limit of this method (20 µl injected) was 0.075 ng, which represents 4.25 µg.kg^{-1} for the egg yolk sample.

Cold extraction of MDA from the sample was done according to Grau *et al.* (12) with some modifications. Instead of 5 % TCA we used 10 % TCA ice cold at 1 °C. The time of homogenisation was shortened to fifteen seconds. Stock and working standard solutions of MDA were prepared according to Squires (19). Sample derivation was performed according to Cordis *et al. (5).*

Concentrations of MDA in fresh eggs (Table 1) were 19.15 ± 2.15 ng.g^{-1}. Eggs stored for thirty days at 10 °C produced 56.76 ± 6.08 ng.g^{-1} of MDA. The increase of MDA concentrations in stored eggs was three times higher than in fresh eggs, but the total amount of MDA

Table 1. The detection and recovery of MDA[1] from yolk eggs after 0 and 30 days of storage at 10 °C

Time of storage (days)	MDA added ($ng.g^{-1}$)	MDA found ($ng.g^{-1}$)	CV[2] (%)	Recovery (%)
0	0	19.15 ± 2.15	11.22	
	58.3	68.23 ± 5.63	8.26	84.20
	116.6	123.96 ± 9.47	7.64	90.35
30	0	56.76 ± 6.08	10.71	
	58.3	107.72 ± 6.27	5.82	87.42
	116.6	163.24 ± 7.36	4.51	91.32

[1] — Concentrations are presented as an average from six samples and standard deviations ($\bar{x} \pm SD$); [2] — CV-coefficient of variation

in both groups was significantly low. It means that lipid oxidation processes in eggs were also low and eggs previously described by some authors (3, 11) showed a high oxidative stability.

The recoveries were evaluated for yolk samples fortified at different known concentration levels of MDA standard and subsequently calculating the fraction of MDA recovered through the extraction procedure. Table 1 summarises the data on the recovery of MDA the spiked samples. Recoveries from yolk samples spiked with 58.3 $ng.g^{-1}$ were lower (84.2 % for fresh eggs and 87.4 % for eggs stored at 10 °C) than recoveries from yolk samples spiked with 116.6 $ng.g^{-1}$ (90.35 % for fresh eggs and 91.32 % for stored eggs).

The estimation of MDA from samples with a complex biological matrix is difficult. The method of MDA extraction from the sample has a serious influence on the result of MDA detection (12, 16, 17). In biological matrices MDA exists both free and bound form (-SH and $-NH_2$ groups of macromolecules such as proteins and nucleic acids). Bound MDA in biological samples can only be measured after acid or alkaline hydrolysis of the protein binding (4, 5, 16). Strong acid conditions, without higher temperatures, can lead to a release of bound MDA from its protein binding (12, 16). Rosmi- ni et al. (18) have reported that 10 % TCA solution produces the best recovery. We used acid hydrolysis with 10 % TCA which was found to be optimal.

Good protection against auto-oxidation can be assured when antioxidants (BHT) are added to the samples before the homogenisation process (2, 12). Addition of BHT before blending in the presence of hexane had a more pronounced effect on the suppression of lipid oxidation (2). Immediate addition of BHT to the sample after weighing is the most important step in the effective breaking of oxidation in the sample. The EDTA effect is important mainly in thermal treated products when metal ions are released (12). The binding of metal ions with EDTA eliminates oxidation as well as additional MDA production.

The final concentration of MDA depended on the length and temperature of homogenisation. The hexane layer is important to protect the sample against oxygen insertion and following oxidation, which can cause additional MDA production. Ice cold TCA (4 °C) can be used as a protection against temperature increases during sample homogenisation.

Centrifugation and filtration are important steps to remove meat constituents which can react with DNPH to form complexes and peaks produced by them interfering with the MDA-DNPH peak. The high temperature used in TBA methods produces additional MDA, which overestimates results and decreases the specificity of MDA determination. The quantification of MDA-TBA in milk powder, measured by HPLC gives values about twice as high as those obtained from MDA-DNPH analysed by HPLC with UV detection. This variety may be explained by the artificial formation of MDA during the acid-heating step required for TBA derivation (70 °C), despite the presence of BHT (10).

Cleaning of the derived samples on the C_{18} SPE cartridges before HPLC analysis removes all matter which can interfere during UV detection and increase MDA content. A similar effect has also been recorded elsewhere (15, 17). Application of a solid phase extraction step in the sample preparation is effective on account of increasing selectivity and column protection.

CONCLUSION

The HPLC method with cold extraction and derivation of the samples with DNPH is suitable and recommended for practical use in MDA determination. Elimination of higher temperatures at the phase of extraction and derivation rejected the possibility of additional MDA production and increased the method's specificity. To conclude, the addition of BHT and EDTA before homogenisation effectively protects samples against oxidation during homogenisation. This HPLC method modification in combination with DNPH derivation is a simple, accurate and cost-effective way for MDA determination of the important products of PUFA decomposition.

ACKNOWLEDGMENT

This study was supported by a grant VEGA SR No. 1/8237/01.

REFERENCES

1. Baucells, M. D., Crespo, N., Baroeta, A. C., López-Ferrer, S., Grashorn, M. A., 2000: Incorporation of different polyunsaturated fatty acids into eggs. *Poultry Sci.,* 79, 51—59.

2. Botsoglou, N. O., Fletoris, D. J., Papageorgiou, G. E., Vassilopolous, V. N., Mantis, A. J., Trakatellis, A. G., 1994: Rapid sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. *J. Agric. Food Chem., 4*2, 1931—1937.

3. Cherian, G., Wolfe, F. W., Sim, J. S., 1996: Dietary oils with added tocopherols: Effects on egg or tissue tocopherols, fatty acids, and oxidative stability. *Poultry Sci.,* 75, 423—431.

4. Cordis, G. A., Maulik, N., Das, D. K., 1995: Detection of oxidative stress in heart by estimating the dinitrophenylhydrazine derivative of malondialdehyde. *J. Mol. Cell. Cardiol.,* 27, 1645—1653.

5. Cordis, G. A., Das, D. K., Riedel, W., 1998: High-performance liquid chromatographic peak identification of 2,4-dinitrophenylhydrazine derivatives of lipid peroxidation aldehydes by photodiode array detection. *J. Chromatogr.* A, 798, 117—123.

6. Csallany, A. S., Guan, M. D., Manwaring, J. D., Addis, P. B., 1984: Free malonaldehyde determination in tissues by high-performance liquid chromatography. *Anal. Biochem.,* 142, 277—283.

7. Draper, H. H., Squires, E. J., Mahmoodi, H., Wu, S., Agarwal, S., Hadley, M., 1993: A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. *Free Rad. Biol. Med.,* 15, 353—363.

8. Esterbauer, H., Zollner, H., 1989: Methods for determination of aldehydic lipid peroxidation products. *Free Rad. Biol. Med.,* 7, 197—203.

9. Esterbauer, H., Schaur, R. J., Zollner, H., 1991: Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Rad. Biol. Med.,* 11, 81—128.

10. Fenaille, F., Mottier, P., Turesky, R. J., Ali, S., Guy, P. A., 2001: Comparison of analytical techniques to quantify malondialdehyde in milk powders. *J. Chromatogr.* A, 921, 237—245.

11. Galobart, J., Barroeta, A. C., Baucells, M. D., Codony, R., Ternes, W., 2001: Effect of dietary supplementation with rosemary extract and α-tocopheryl acetate on lipid oxidation in eggs enriched with ω-3 fatty acids. *Poultry Sci.,* 80, 460—467.

12. Grau, A., Guardiola, F., Boatella, J., Barroeta, A., Codony, R., 2000: Measurment of 2-thiobarbituric acid values in dark chicken meat through derivate spectrophotometry: Influence of various parameters. *J. Agric. Food Chem.,* 48, 1155—1159.

13. Jo, C., Ahn, D. U., 1998: Fluorometric analysis of 2-thiobarbituric acid reactive substances in turkey. *Poultry Sci.,* 77, 475—480.

14. Korimová, L., Máté, D., Turek, P., 2000: Influence of natural antioxidants on heat-untreated meat products quality (In Slovak). *Czech Journal of Food Science,* 18, 124—128.

15. Piette, G., Raymond, Y., 1999: Rancidity in processed meats. *Fleischwirtschaft international,* 3, 36—39.

16. Pilz, J., Meinke, I., Gleiter, Ch. H., 2000: Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. *J. Chromatogr.* B, 742, 315—325.

17. Raharjo, S., Sofos, J. N., 1993: Methodology for measuring malonaldehyde as a product of lipid peroxidation in muscle tissues: A review. *Meat Sci.,* 35, 145—169.

18. Rosmini, M. R., Perlo, F., Perez-Alvarez, J. A., Papan-Moreno, M. J., Gago-Gago, A., Lopez-Santovena, F., 1995: TBA test by an extractive method applied to Pate. *Meat Sci.,* 42, 103—110.

Received March 27, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 104—107, 2004

THE BENEFICIAL EFFECTS OF IRON-ENRICHED YEAST *Saccharomyces cerevisiae* BIOMASS IN POULTRY BREEDING

Gligić, Lj.[1], Palić, T.[2], Nikolovski, J.[1]

[1]Galenika a.d., Institute for Research and Development, Batajnički drum bb, 11080 Belgrade
[2]Faculty of Veterinary Medicine, University of Belgrade, Belgrade
Serbia and Montenegro

ljgligic@eunet.yz

ABSTRACT

Brewers' yeast biomass containing approximately 42 mg iron.g^{-1} was obtained by aerobic submersed fermentation of *S. cerevisiae* in an iron salt-enriched medium. Iron binds to yeast cell organelles and macromolecules forming complexes of Mr over 12 kD. Yeast biomass produced in this way and heat inactivated (BYFe), has served as a source of iron-containing molecules. In order to examine the absorption efficiency of iron from BYFe, broilers of Arbor Acres provenance were fed a standard diet supplemented with the pre-mixture containing ferrosulphate (control) and a diet supplemented with a certain amount of BYFe. Blood was collected 1, 7, 35 and 42 days after the onset of experiments and the red blood cell count, haemoglobin content and haematocrite measured. Besides, body weight gain, the food conversion and mortality rate were determined after 42 days. Statistical significance was evaluated using Student's *t*-test. A significant increase of all iron transport indicators was observed even after seven days in the BYFe group comparing to the control. Such tendency continued until the 35th day, but the increase was less conspicuous than after seven days. A significant body mass gain and a decreased mortality rate were recorded. This suggests that BYFe production on an industrial scale could be of special benefit in the improvement of poultry breeding.

Key words: iron bioavailability; iron-enriched yeast; poultry breeding

INTRODUCTION

The correct nutrition of animals means an intake of a corresponding amount of nutrient matters in optimal ratio and in bioavailable form. Iron intake is of special significance for the numerous functions of an organism (9) and insufficient absorption of this microelement from food leads to severe disturbances, including hypochromic anemia.

The importance of iron for most prokaryotes and eukaryotes is underlined by its ability to participate in many chemical reactions that involve the shift of electrons between molecules (reduction-oxidation reactions), as emphasized by Crichton and Charloteaux-Wauters (2), as early as 1987. In living organisms, iron is indispensable for the functioning of numerous enzymes and other iron-containing proteins. The natural iron transport process through cell membrane and formation of its complexes within a cell have been previously employed to accumulate large amounts of iron in brewers' yeast cells through an aerobic fermentation process.

Because of their solubility and bioavailability of iron storage proteins contained in iron-enriched brewers' yeast (4), we hypothesized that heat inactivated yeast biomass could be used as a food supplement in animal breeding. Connected with this, the aim of the present study was to assess the bioavailability of iron present in the preparations of iron-enriched brewers' yeast in poultry breeding in contrast with that of inorganic iron.

MATERIAL AND METHODS

Iron-enriched yeast containing organically bound iron (BYFe) was prepared by the fermentation of brewers' yeast *S. cerevisiae* in a semi-synthetic medium containing corn-steep liquor as a source of protein and $FeSO_4$ as a source of iron. The inoculum was prepared in "BIP" Belgrade Beverage Industry (Belgrade, Serbia & Montenegro). For these investigations a fermentor (20 l volume; Chemap CF 3000, Austria) was also used.

Stationary-phase cells were harvested by centrifugation, the pellet was dried in a vacuum oven (Heraeus FVT 420K, Germany) and then the yeast biomass was heat inactivated.

Protein content in the product was determined *via* nitrogen content (by sulphuric acid digestion) according to the *European Pharmacopoeia* (2001). The total ash was measured by firing the samples and moisture was estimated by drying the samples in an IR oven at 120 °C a MM460 Mettler oven, Germany) to a constant weight.

The iron content in dry yeast cells was determined by atomic absorption spectrophotometry (a Varian Techtron, model 1200, equipped with a carbon rod atomizer, U.S.A.).

Experiments with chickens

In the present work, 200 one-day-old broilers of Arbor Acres provenance, were used. During the experiments, all the technological scale of norms for broilers of this provenance were employed. The animals had free access to food and water.

The chickens were divided into two groups. The controls (100 specimens) were fed a standard chicken diet enriched with 1 % (w/w) vitamin-mineral pre-mixture (trade name Vezevit B1, a product of the Bureau for Veterinary Medicine, Zemun, Serbia and Montenegro). This pre-mixture contains 6 mg iron.g^{-1} in the form of $FeSO_4$. The second group (100 specimens) was offered the same food in which the inorganic iron was replaced by an equivalent amount of iron contained in iron-enriched brewers' yeast.

The blood was collected by heart puncture after 1, 7, 35 and 42 days after the onset of experiments, each group consisting of ten chickens.

Red blood cell count was determined by an automatic blood counter (a Haematology Analyzer, Abacus type, Diatron, Austria). Blood serum iron concentration was estimated by an automatic apparatus (a Secomam BP106 95334 Domont Cedex type basic, France) using the reagents produced by Elitech diagnostics (France).

A group of 42-day-old chickens (each group consisting of 60 chickens) was used to determine body weight gain, food conversion and mortality rate.

RESULTS

Brewers' yeast *S. cerevisiae* cells were multiplied under aerobic conditions of fermentation by a batch procedure using an iron-enriched semi-synthetic growth medium. This enabled an intensive iron incorporation. After the fermentation process, the yeast biomass was separated, dried to a constant weight and characterized by the parameters listed in Table 1.

The efficiency of transport of inorganic and organically bound iron in yeast cells was judged on the basis of blood analyses (red blood cell count, haemoglobin concentration and haematocrite) and comparison of the values found in the group of broilers fed standard diet and those offered food containing iron-enriched yeast instead of inorganic iron salt. The results of these analyses are summarized in Table 2.

From the data presented in Table 2, the volume of red blood cells (MCV in fl) and their saturation with haemoglobin (MCH in fmol) were calculated and presented in Table 3.

After 42 days of experiment, body weight gain, food conversion and mortality rate were determined, as well. The results obtained are shown in Table 4.

Table 1. Some features of BYFe preparation obtained by fermentation of brewers' yeast *Saccharomyces cerevisiae* under optimal growth conditions in an iron-enriched medium

PARAMETER	CONTENT
Proteins	50.40 ± 0.94 %
Lipids	0.35 ± 0.05 %
Ashes	22.10 ± 0.66 %
Moisture	2.31 % ± 0.048 %
Iron	42.0 ± 2.13 mg.g^{-1}

Table 2. Effects of iron-enriched yeast on red blood cell count, haemoglobin and haematocrite in broilers of Arbor Acres provenance

Experimental point (days)	Red blood cell count $\times 10^{12}.l^{-1}$		Haemoglobin $g.l^{-1}$		Hematocrite $l.l^{-1}$	
	control	BYFe-fed group	control	BYFe-fed group	control	BYFe-fed group
1 (n = 10)	1.9 ± 0.22	1.9 ± 0.23	160 ± 9.81	160 ± 9.80	0.38 ± 0.02	0.38 ± 0.02
7 (n = 10)	2.4 ± 0.21	**2.9 ± 0.22	170 ± 9.80	**200 ± 9.81	0.37 ± 0.02	*0.38 ± 0.02
35 (n = 10)	2.7 ± 0.22	**3.0 ± 0.22	200 ± 9.80	**220 ± 9.80	0.36 ± 0.02	**0.40 ± 0.02
42 (n = 10)	2.8 ± 0.21	**3.1 ± 0.21	210 ± 9.81	**230 ± 9.80	0.37 ± 0.02	**0.40 ± 0.02

** — $p < 0.001$; * — $p - 0.001$

Table 3. Values of parameters derived from red blood cell count and haemoglobin content in the control group and the group of broilers fed a standard diet supplemented with BYFe

Experimental point (days)	MCV (fl) Control	MCV (fl) BYFe-fed group	MCH (fmol) Control	MCH (fmol) BYFe-fed group
1 (n = 10)	200.00	200.00	52.27	52.27
7 (n = 10)	154.17	131.03	43.96	42.81
35 (n = 10)	133.33	133.33	45.98	45.52
42 (n = 10)	132.14	129.03	46.55	46.05

Table 4. The body weight, nutrient conversion and mortality rate of Arbor Acres provenance broilers 42 days after the onset of experiment

Parameter	Control	BYFe-fed group
Body weight (kg)	2.01 ± 0.20	*2.14 ± 0.20
Food conversion	1.99 ± 0.23	*1.92 ± 0.23
Mortality rate (%)	5.9	5.4

* — $p < 0.001$

DISCUSSION

Iron-enriched yeast biomass was prepared by the fermentation process of *S. cerevisiae* under optimal conditions as has been described recently (4). For this purpose, growth of brewers' yeast and iron accumulation in the cells were examined in a medium enriched with iron salts during the batch fermentation procedure. In this way, a biomass with a high content of organically bound iron (about 42 mg.g^{-1}) and a high nutritive value (over 50 % of protein, B complex vitamins, high content of other minerals and valuable molecules) was obtained. Babcock *et al.* (1) and Li *et al.* (5) have shown that *S. cerevisiae* can store iron in the vacuoles and mitochondria.

Feeding the chicken with the diet containing different amounts of iron, Šerman et al. (8) have concluded that 82.23 μg iron per g food represents an optimal iron dose not only for the growth and development of poultry, but also for their resistance against different infections. The results obtained throughout the present study have clearly demonstrated the beneficial effects of iron-enriched yeast (BYFe) on haematological parameters in chickens fed a standard diet supplemented with BYFe. The effect of iron uptake was the most marked after seven days of treatment in both groups of chickens, but it was higher in the group taking the food supplemented with organically-bound iron in yeast cells than in the group offered inorganic iron in the form of ferrosulphate, as judged by red blood cell count and haemoglobin concentration.

Haematocrite values in the control group slightly declined until the 35th day of age and then an increase was recorded. However, in the group fed the diet supplemented with iron-enriched yeast, a certain elevation of haematocrite value was observed after 35 and 42 days of treatment.

Haemoglobin concentration arithmetically increased in the controls while a logarithmic increase was observed in blood samples of the chickens fed iron-enriched yeast. This increase of haemoglobin concentration in the latter group in relation to the control was more prominent than the elevation of red blood cell count pointing to a higher iron concentration in the red blood cells.

It is well documented that the formation of stable, electro-neutral chelates protects microelements against chemical reactions during the food digestion process. Such protection enables the maintenance of satisfactory solubility throughout the entire digestive tract up to the site of absorption.

Fly and Czarnecki-Maulden (3) have examined the bioavailability of organically bound iron using several animal species (rats, chicken, dogs and cats) based on the determination of iron content in both haemoglobin and haemin. These authors have observed species-specific differences in iron uptake in haemoglobin and haemin. The bioavailability of haeme iron has been found to be higher in chickens than in other animal species and approximately the same as that of ferrosulphate. It is believed that proteins contained in the diet contribute to a better iron uptake from haeme. Positive erythropoiesis results obtained throughout the present study using BYFe suggest that the constituents of this preparation could play a role as nutritional factors in normal erythropoiesis enabling the maturation of progenitor cells during erythrogenesis into normal mature red blood cells.

Brewers' yeast represents a rich source of these factors, i.e. proteins, amino acids, B vitamin complex, etc.

As mentioned above, iron occurs in yeast in the form of haeme, as well as bound to proteins. In connection with this, Pizarro *et al.* (6) have recently attempted to establish whether iron chelates are absorbed by the same mechanism as non-haeme iron. The data of these authors has pointed to improved transport through the cell membranes of the intestinal endothelium of iron bound to macromolecules in the yeast cells.

Our results presented here corroborate and extend the findings of Pizarro *et al.* (6), recommending BYFe as a beneficial supplement that could be successfully applied not only in poultry breeding, but also in the prevention and treatment of some anaemias in veterinary medicine.

ACKNOWLEDGEMENT

This work was partly supported by the Ministry for Science, Technology and Development of the Republic of Serbia, contract BTN#443.

REFERENCES

1. Babcock, M., De Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Monteermini, L. *et al.,* 1997: Regulation of mitochondrial iron accumulation by Yfh1, a putative homolog of frataxin. *Science,* 276, 1709—1712.

2. Crichton, R. R., Charloteaux-Wauters, M., 1987: Iron transport and storage. *Eur. J. Biochem.,* 164, 485—506. *European Pharmacopoeia, 2001:* Fourth Edition, 107, 91.

3. Fly, D. A., Czarnecki-Maulden, L.G., 2000: Iron bioavailability from hemoglobin and hemin in chick, rat, cat and dog: A comparative study. *Nutr. Res.,* 20, 237—248.

4. Gligić, Lj., Vujović, N., Stevović, B., Manić, J., 2003: Optimal conditions for accumulation of bioavailable iron in *Saccharomyces cerevisiae* cells. *Boll. Chim. Farm.,* 142, 345—347.

5. Li, L., Chen, O. S., McVey Ward, D., Kaplan, J., 2001: CCC1 is a transporter that mediates vacuolar iron storage in yeast. *J. Biol. Chem.,* 276, 29515—29519.

6. Pizarro, F., Olivares, M., Hertrampf, E., Mazariegos, D. I., Arredondo, M., Letelier, A., Gidi, V., 2002: Iron bisglycine chelate, competes for the nonheme-iron absorption pathway. *Am. J. Clin. Nutr.,* 76, 577—581.

7. Rusov, Ć., 2001: Haematology of Birds (In Serbian). In *Eritrocitna loza.* Naučni institut za veterinarstvo Srbije (NIVS), Srbija i Crna Gora, 71—83.

8. Šerman, V., Mazija, H., Findrik, M., 1980: Investigation on the influence of the acquired immunity against the Newcastle disease. II. The influence of some iron doses on the production of antibodies against Newcastle disease (In Croatian). *Vet. Arhiv,* 4, 171—175.

9. Wrrigglesworth, J. M., Baum, H., 1980: In *Iron in Biochemistry and Medicine,* Vol-II, edited: **Jacobs, A.** and **Worwood, M.** Academic Press, London, New York, 29—86.

Received April 2, 2004

FOLIA VETERINARIA
www.uvm.sk

FOLIA VETERINARIA, 48, 2: 108—112, 2004

THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SULPHADIMIDINE RESIDUES IN EGGS

Kožárová, I., Hussein, K., Máté, D., Jevinová, P.

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

kozarova@uvm.sk

ABSTRACT

Sulphadimidine is widely used for the treatment of coccidiosis in laying hens at a dose of 2 g.l^{-1} for six consecutive days *via* drinking water. As a result, there is concern that the residues of this drug may be retained in the eggs and present a potential risk to human health. The aim of our study was to determine the residue concentrations of sulphadimidine in the whole eggs of laying hens during and after its oral administration up to the 15th day of the withdrawal period. Sulphadimidine residues were determined by a gradient high-performance liquid chromatography system with a photo-diode array detector at 275 nm. Our results indicate that the administration of sulphadimidine to the laying hens produced a rapid and sustained increase in sulphadimidine residues in eggs. The highest residue concentrations of sulphadimidine were found on the sixth day of its oral administration. After withdrawal, the residues declined rapidly, but they were still detectable on the tenth day of the withdrawal period. On day 11, the residues were below the established maximum residue limit (MRL) (0.1 mg. kg^{-1}). The limit of quantification (LOQ) for sulphadimidine was 0.3 mg.kg^{-1}, the detection limit (LOD) was 0.09 mg.kg^{-1}, and the recovery range from 91 to 98 %.

Key words: determination; eggs; HPLC; sulphadimidine residues

INTRODUCTION

Sulphonamides are one of the oldest groups of pharmacologically active substances used in veterinary medicine to date. Their discovery, in 1935, signified the beginning of a new era in the treatment of a wide range of bacterial diseases and a number of protozoan infections. In laying hens, sulphonamides are used for the treatment of coccidiosis. As a result, there is a concern that the residues of these drugs may be retained in the eggs and present a potential risk to human health (11, 12, 19).

Direct toxic or allergic reactions after administration of therapeutic doses of sulphonamides to humans have been described (4, 17). Besides these negative effects of sulphonamides or their metabolites on the human body have been reported, particularly after the long-term consumption of animal products containing their trace amounts. The accumulation of these trace amounts in animal products has resulted in a build-up of resistance and the development of hypersensitivity to sulphonamides (1, 17). Sulphonamides are known for their negative effects on the thyroid gland in relation to the development of thyroid gland tumours (2, 18).

In order to decrease the potential health risk to the consumer's health and ensure the reduction of sulphonamide residues in edible tissues and eggs to an acceptable level, they must be administered only in recommended concentrations and their respective withdrawal times must be observed. With regard to the pharmacokinetic properties of sulphonamides and the persistence of their residues in edible tissues and eggs of food-producing animals, the original 7-day withdrawal period has, since 1980, been increased to 15 days (3).

Current legislation has established the MRL of 0.1 mg.kg^{-1} for sulphonamides (all compounds of the sulphonamide group) in foods of animal origin (5, 6).

Various methods for the determination of sulphonamide residues have been reported in the literature. These methods are variable in terms of degree of sophistication and procedure. In the present two-tier testing system microbiological and immunological methodologies as the screening methods and high-performance liquid chromatography (HPLC) methodologies as the confirmatory ones have been used predominantly for the monitoring of sulphonamide residues in various biological matrices (1, 9, 11, 12, 14).

Sulphadimidine has been observed to be effective against coccidiosis in laying hens when it is administered orally *via* the drinking water at a dose of 2 g.l^{-1}. This paper describes the HPLC method for the determination of sulfadimidine residues in the whole eggs of laying hens. The eggs were examined during and after its oral administration up to the fifteen day of the withdrawal period. Residue analysis was performed using the methodology describes by Furusawa (7), Furusawa and Mukai (8), and Malisch (13).

MATERIAL AND METHODS

Animal treatment and processing of egg samples

Twenty-one 40-week old laying hens (hybrid Hisex Brown) weighing between 1.6–1.8 kg were used in the experiment. The laying hens were placed in individual animal-care approved cages with the free access to feed and water, and they were randomly divided into two groups, one experimental and one control group. The laying hens were fed with a complete mixed feed for productive layers HYD –10 *ad libitum.*

Sixteen laying hens from the experimental group received sulphadimidine *via* the drinking water for six consecutive days at the dose of 2 g.l^{-1} of water. Medicated drinking water was prepared fresh each day. The remaining five hens served as sulphadimidine-free control.

All eggs laid by hens during administration and after the withdrawal of sulphadimidine were daily collected, weighed, broken, homogenized, and stored in the polyethylene bags as a deep-frozen until the analysis.

Solvents and reagents

All solvents and reagents were analytical or HPLC grade. Methanol, acetonitrile, n-hexane, ethyl acetate, and acetic acid were obtained from Merck (Darmstadt, Germany), anhydrous sodium sulphate, sodium chloride, and sodium acetate from Lachema (Brno, Czech Republic), and de-ionized and re-distilled water was prepared on Milli-Q Plus (Millipore, France).

Standard solutions

Sulphadimidine (Sulphamethazine sodium salt, S 5637), as a standard, was obtained from Sigma Chemical CO. (St. Louis, MO, USA). A stock solution (0.5 mg.ml^{-1}) was prepared by dissolving 25 mg sulphadimidine standard in 50 ml methanol. Working solutions of sulphadimidine were prepared by serial dilutions with methanol. The stock and working solutions were stored in the refrigerator at 4 °C.

High-performance liquid chromatography

Analyses of the standard and extracted sulphadimidine residues were conducting using an HP 1090 liquid chromatograph with a photo-diode array detector (Hewlett-Packard, USA). The separation was performed on a LiChroCART RP-18e column (125—4, 5 µm) (Merck, Germany) using acetonitrile-acetate buffer (pH 4.6) (25 : 75, v/v) as the mobile phase at a flow-rate of 1.0 ml.min^{-1} at a temperature of 40 °C. The injection volume was 25 µl, and the detection was conducted at 275 nm.

Sample extraction and sample clean-up

An accurately weighed 50 g amount of the homogenized eggs was extracted with 100 ml acetonitrile on a mechanical shaker for fifteen minutes. The extract was filtered through a filtrate paper by using a Buchner's funnel. The residue on the filtrate paper was washed with 25 ml acetonitrile and the procedure was repeated once more. The collected filtrates were poured into a separating funnel. After adding 7 g sodium chloride, the combined filtrate was left until separated completely into two layers. The lower aqueous layer was discharged and the upper acetonitrile layer was dried with anhydrous sodium sulphate followed by filtration. Separated aqueous layer was extracted with 25 ml ethyl acetate.

After extraction, both layers the ethyl acetate and acetonitrile were collected in a 100 ml round-bottom flask and evaporated to dryness in a rotary vacuum evaporator (Laborota 4000, Heidolph, Germany) at 40 °C. The residue was redisolved in 1 ml methanol and 1 ml of mobile phase, and transferred to a centrifuge tube. The flask was washed twice with 2 ml n-hexane, and 4 ml n-hexane was added to the centrifuge tube. The mixture was centrifuged twice at 3000 rpm for ten minutes (Jouan BR 4I, France). The lower layer was filtered through a disposable syringe filter unit and transferred to the vial. Twenty-five ěl volume of the solution was injected into the HPLC system.

RESULTS

The mean residue concentrations of sulphadimidine in the whole eggs of laying hens during and after its administration for six days at a dose of 2 g.l^{-1} through the drinking water, determined by the HPLC method with photo-diode array detection, are shown in Table 1. The eggs were analysed up to the 15th day of the withdrawal period.

Table 1 and Table 2 show that the oral administration of sulphadimidine via the drinking water to the laying hens on the level indicated by manufacturer (2 g.l^{-1}) produced a rapid and sustained increase in sulfadimidine residues in the whole eggs. Sulphadimidine quickly reached high levels observed already on the first day following the beginning of administration. On this day, the mean sulphadimidine concentration in the whole eggs was

Table 1. Sulphadimidine concentrations in the whole eggs of laying hens during its oral administration for six days at 2 g.l^{-1} in the drinking water

Days of treatmen	1	2	3	4	5	6
mg.kg^{-1} ± SD	4.8 ± 0.53	11.6 ± 2.4	25.7 ± 3.2	38.1 ± 4.1	42.6 ± 4.6	43.1 ± 4.8

Legend: SD — standard deviation

Table 2. Sulphadimidine residue concentrations in the whole eggs of laying hens after its oral administration throughout the fifteen days of the withdrawal period

Days of the withdrawal period	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
mg.kg^{-1} ± SD	13.1 ± 2.7	5.15 ± 0.71	2.35 ± 0.36	2.01 ± 0.29	1.57 ± 0.24	1.15 ± 0.18	0.85 ± 0.09	0.59 ± 0.021	0.23 ± 0.013	0.12 ± 0.008	ND	ND	ND	ND	ND

Legend: SD — standard deviation; ND — not detectable

4.8 mg.kg^{-1}. The maximum sulphadimidine concentration was achieved on the sixth day of its oral administration. On day 6, the mean sulphadimidine concentration in the whole eggs was 43.1 mg.kg^{-1}.

As soon as the treatment was discontinued, the sulphadimidine residues declined rapidly during the first three days of the withdrawal period. On the first day of the withdrawal period the mean residue concentration of sulphadimidine in the whole eggs of laying hens was 13.1 mg.kg^{-1}, on the second day of the withdrawal period the mean residue concentration of sulphadimidine was 5.15 mg.kg^{-1} and on the third day of the withdrawal period the mean residue concentration of sulphadimidine was 2.35 mg.kg^{-1}. The sulphadimidine residues in the whole eggs of laying hens were detectable up to the tenth day of the withdrawal period and the mean sulphadimidine residue concentration was 0.12 mg.kg^{-1}. On the 11th day of the withdrawal period, the sulphadimidine residues were below the established MRL (0.1 mg.kg^{-1}). No residues of sulphadimidine were found in the whole eggs of laying hens produced by sulphadimidine-free control group.

Recoveries of sulphadimidine from spiked tissue samples at the concentrations of 0.1, 0.5, and 1 mg.kg^{-1} ranged between 91 % and 98 %. The LOQ for sulphadimidine was 0.3 mg.kg^{-1}, and the LOD was 0.09 mg.kg^{-1}.

The absorption spectrum of the sulphadimidine was measured by a photo-diode array detector, and the wavelength of 275 nm was chosen to monitor the maximum absorbance for sulphadimidine. Sulphadimidine was successfully detected within nineteen minutes, when the flow-rate was 1.0 ml.min^{-1}.

Figure 1 shows the chromatogram of the sulphadimidine standard (10 mg.kg^{-1}) obtained with the photo-diode array detector at 275 nm. The retention time was 18.895 min.

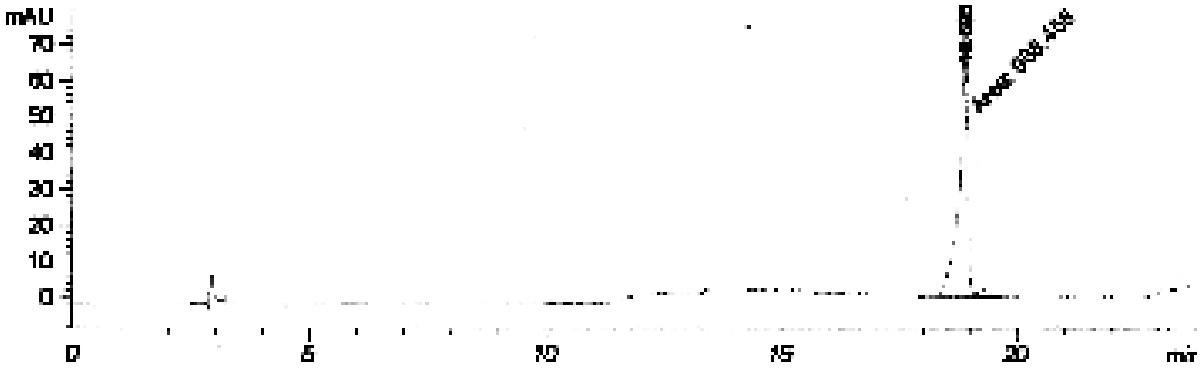

Fig. 1. The chromatogram of the sulphadimidine standard obtained with the photo-diode array detector at 275 nm

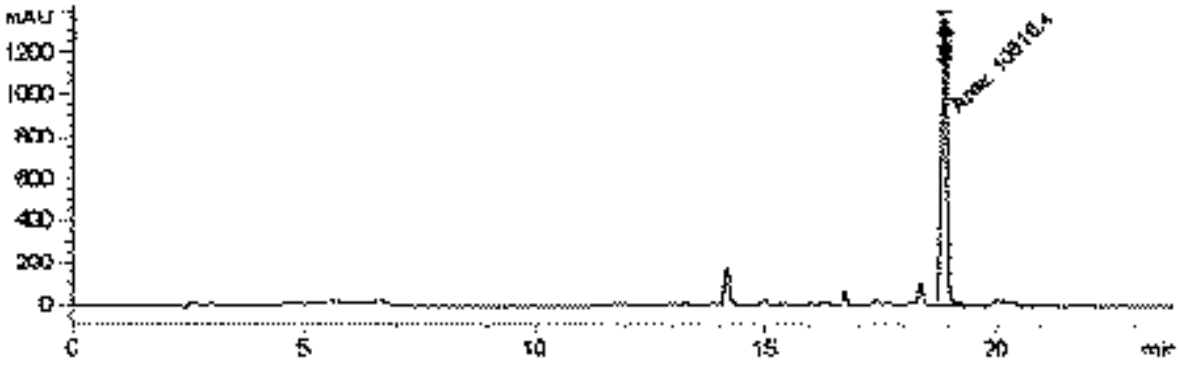

Fig. 2. The chromatogram of sulphadimidine in whole eggs examined on the third day of the withdrawal period obtained with photo-diode array detector at 275 nm

The chromatogram of sulphadimidine in the whole eggs examined on the third day of the withdrawal period, obtained with the photo-diode array detector at 275 nm, is shown in Figure 2. The retention time was 18.911 min. On this day, the residue concentration of sulphadimidine was 2.35 mg.kg^{-1}.

DISCUSSION

High-performance liquid chromatography has become the most widely used confirmatory technique for the determination of sulphonamide residues in foods of animal origin. The principal approach of the HPLC analysis involves extraction, sample clean-up and HPLC analysis steps (1, 9).

Traditionally, the extraction of sulphonamide from biological matrices, such as meat, milk, and eggs, has been done with organic solvents (1, 9). The extraction of sulphadimidine from whole eggs laid during the experiment was performed with acetonitrile and ethyl acetate. Some organic solvents also denature the sample proteins, which result in cleaner extracts, and also help to extract the drug residues bound to proteins (13). For effective deproteinization, a combination of acetonitrile and acetate buffer as the mobile phase has been used. To minimize the fat content, n-hexane was applied. After the rather time-consuming sample processing, the analysis was finally achieved utilising HPLC with photo-diode array detection.

The HPLC system equipped with a photo-diode array detector proved to be able to detect a wide range of molecules and to ensure the identification of the target compound. The absorption spectrum and the retention time provide the strong evidence of its identity (10).

Using the photo-diode array detector, the absorption spectrum of sulphadimidine standard in the mobile phase was measured for the selection of the HPLC monitoring wavelength. The measurement was conducted at 275 nm, which gave an average maximum absorbance for sulphadimidine. The spectrum of sulphadimidine obtained from the samples was practically identical to that of the standard.

The retention time of sulphadimidine decreased with increasing the pH value of the acetate buffer and the concentration of acetonitrile in the mobile phase. The best separation of sulphadimidine was achieved using acetonitrile-acetate buffer (pH 4.6) (25:75, v/v) as the mobile phase. Sulphadimidine was successfully detected within nineteen minutes, when the flow-rate was 1.0 ml. min^{-1}. The minimum detectable amount (signal-to-noise ratio > 5) was 0.09 mg.kg^{-1}.

In spite of the fact that the LOD of this method was 0.09 mg.kg^{-1}, the sulphadimidine residues in the whole eggs of laying hens were detectable up to the tenth day of the withdrawal period. From the foregoing data it can be concluded that the 15-day withdrawal period recommended for sulphonamides since 1980 should be sufficient to ensure the reduction of sulphadimidine residues in the whole eggs of laying eggs to the acceptable level of 0.1 mg.kg^{-1}.

As sulphadimidine was continuously administered through the drinking water, a decrease in both water intake and egg production was observed. The decrease in water intake is known for sulphadimidine at the dose of 2 g.l^{-1} because of the bitterness of the water (15). Similar results were recorded by Roudaut and Garnier (16).

The monitoring of sulphadimidine residues in foods of animal origin has been the critical point in the protection of the food chain against the penetration of residues of this drug from the point of view of hygiene and public health. The development of individual analytical methods is related to the specific needs of the final consumer and analytical technologies available at the time. The choice of method used in the laboratory will often be dictated by the availability of suitable expertise, facilities and equipment. The development of individual analytical methods suitable for detecting sulphadimidine residues at or below the levels of the concern, constitute an impressive challenge. Foods are a basic, everyday commodity, therefore the food and health aspects of the administration of sulphadimidine are very important (11, 12).

Acknowledgement: *This study was supported by a grant VEGA SR No. 1/0617/03.*

REFERENCES

1. Agarwal, V. K, 1992: High-performance liquid chromatographic methods for the determination of sulphonamides in tissues, milk and eggs. *Journal of Chromatography*, 624, 411—423.

2. Ahmed Mir, S., Ahmed, N., 1989: Drug residues in poultry meat and eggs – public health importance. *Poultry Adviser*, 22, 12, 53—58.

3. Ausberg, J. K., 1989: Sulpha residues in pork: An update. *Journal of Animal Science*, 67, 2817—2821.

4. Booth, N. H., 1988: Toxicology of drug and chemical residues in edible tissues of animals. In *Veterinary Pharmacology and Therapeutics*. **H. R. Adams** (ed.). Iowa State University Press, Ames, Iowa, USA, 1149—1205.

5. Council Regulation (EEC) No. 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Official Journal*, No. L 224, 18. 8. 1990, 1—8.

6. First chapter of The Codex Alimentarius of The Slovak Republic (In Slovak). *A Bulletin of the Ministry of Agriculture of The Slovak Republic*, XXXV, 2003, part 8, 50.

7. Furusawa, N., 1999: High-performance liquid chromatographic determination/identification of oxytetracycline and sulphadimidine in meat and eggs. *Chromatographia*, 49, 369—373.

8. Furusawa, N., Mukai, T., 1994: Simultaneous high-performance liquid chromatographic determination of residual sulphamonomethoxine, sulphadimethoxine and their N^4-acetyl metabolites in foods of animal origin. *Journal of Chromatography A*, 677, 81—85.

9. Guggisberg, D., Mooser, A. E., Koch., H., 1992: Methods for the determination of sulphonamides in meat. *Journal of Chromatography*, 624, 425—437.

10. Kishida, K., Furusawa, N., 2001: Matrix solid-phase dispersion extraction and high-performance liquid chromatographic determination of residual sulphonamides in chickens. *Journal of Chromatography A*, 937, 49—55.

11. Kožárová, I., Máté, D., Cabadaj, R., 2001: Methods for the determination of sulphonamide residues in milk and other animal products. In *Proceedings of Lectures and Posters of the International Conference "Hygiena Alimentorum XXII"*, Štrbské Pleso, High Tatras, SR, 5—7 June, 2001, 159—163.

12. Kožárová, I., Máté, D., Cabadaj, R., Hussein, K., 2002: Methods for determining sulphonamide residues in meat. In *Proceedings of Lectures and Posters of the International Conference "Hygiena Alimentorum XXIII"*, Štrbské Pleso, High Tatras, SR, 5—7 June, 2002, 65—67.

13. Malisch, R., 1994: Nachweismethoden für Rückstände von Arzneimitteln: Vorläugife Methode zur Bestimmung und zum Nachweis von Sulfonamiden in Muskelfleisch – HPLC-Verfahren. *Bundesgesundheitsblatt*, 6, 279—282.

14. Pavelka, M., Golian, J., 2001: The changes of detection of sulphonamides and their derivates in meat (In Slovak). In *Proceedings of Lectures and Posters of the International Conference "Hygiena Alimentorum XXIII"*, Štrbské Pleso, High Tatras, SR, 5—7 June, 2001, 150—153.

15. Puyt, J. D., 1995: Antibiothérapie en élevage de volaille. *Bulletin des GTV*, 5, 17—110.

16. Roudaut, B., Garnier, M., 2002: Sulphonamide residues in eggs following drug administration via the drinking water. *Food Additives and Contaminants*, 19, 373—378.

17. Spoo, J. W., Riviere, J. E., 1995: Sulphonamides. In *Veterinary Pharmacology and Therapeutics*. **H. R. Adams** (ed.). Iowa State University Press, Ames, Iowa, USA, 753—773.

18. Swarm, R. L., Roberts, G. K. S., Levy, A. C., Hines, L. R., 1973: Observation in the thyroid gland in rats following the administration of sulphamethoxazole and trimethoprim. *Toxicology and Applied Pharmacology*, 24, 351—363.

19. Šutiak, V., Šutiaková, I., Korének, M., Krokavec, P., Kozák, M., Šály, J., Neuschl, J., 2000: Current problems with drug use and the need for their solution in poultry and some other animals. In *Proceedings of Lectures and Posters of the International Conference "Hygiena Alimentorum XXI"*, Štrbské Pleso, High Tatras, SR, 1—3 June, 2000, 113—115.

Received May 10, 2004