TESTICULAR STEREOLOGY OF LAMBS SUPPLEMENTED WITH ORGANIC AND INORGANIC ZINC

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Summary

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The present study was aimed to investigate the effects of organic and inorganic zinc supplements on histomorphometric features of the lambs' testis using stereological methods. Twelve Sanjabi ram lambs weighting 20–25 kg at an average age of 3–3.5 months were divided into three groups. The first group received basal diet while the diets of the second and third groups were supplemented with 40 mg/kg DM zinc methionine and zinc sulphate, respectively, for 70 days. The data revealed that body weight gain was significantly ($P \leq 0.05$) lower in inorganic zinc group compared to the control and organic zinc groups. Significant increase ($P \leq 0.05$) in the testis weight, testis volume, tubule volume, tubule length, tubule diameter and increased number of Leydig cells were observed in organic zinc supplemented lambs compared to both the control and inorganic zinc groups. Both organic and inorganic zinc groups exhibited significantly increased height of germinal epithelium compared to the control group, but there was no significant ($P > 0.05$) difference between them. Interstitial tissue volume did not show significant changes. It can be concluded that the organic form of dietary zinc (Zn methionine) showed a better response in improving testis structural parameters as compared to the inorganic form of Zn (zinc sulfate).

Key words: inorganic zinc, morphometry, nutrition, organic zinc, testis

INTRODUCTION

Reproductive performance of livestock in the tropics is determined by four factors: genetic merit, physical environment, nutrition and management. Evidence from the literature and practical experience suggests that nutritional factors are perhaps the most crucial, in terms of their direct effects on the reproduction, and the potential to moderate the effects of other factors. Thus, adequate nutrition could encourage mediocre biological types to reach their genetic potential, alleviate the

negative effects of a harsh physical environment, and minimise the effects of poor management techniques (Smith & Akinbamijo, 2000).

Zinc is indispensable for microorganisms, plants, animals and humans for many physiological functions such as growth and reproduction (Bedwal *et al.,* 1991; Bedwal & Bahuguna, 1994; Prasad, 2008).

Zinc might play a noteworthy role in the physiology of spermatozoa. Zinc in seminal plasma stabilises the cell membrane as well as nuclear chromatin structure of spermatozoa. Further, it may also have an antibacterial function (Lin *et al.,* 2000). The zinc content of the prostate gland, the seminal fluid and ejaculated sperm are very high and testicular zinc is essential for spermatogenesis (Vallee & Falchuk, 1993). Kvist *et al.* (1988) found a constructive relationship between zinc in sperm nuclei and the resistance of the chromatin to de-condense after exposure to a detergent. They also observed that the infertile men had lesser degree of sperm chromatin stability and lesser sperm zinc content than the fertile donors and suggested that a low content of nuclear zinc would damage the structural stability of the chromatin and thus increases the susceptibility of the male genome.

Several studies showed that nutrition and zinc deficiency can result in the reduction of testicular growth and retardation of tubule development in ram lambs throught different mechanisms (Martin & White, 1992; Martin *et al.,* 1994; Hotzel *et al.,* 1997; 1998).

Natural sources of zinc for farm animals are primarily bran, grains and fodder yeasts. Zinc occurs most frequently in the forms of inorganic salts such as zinc chloride, zinc sulfate and zinc oxide, and may also occur in the form of organic salts (Henry *et al.,* 1992).

The traditional inorganic forms of trace minerals rapidly dissociate in the rumen and are free to interact with antagonists, resulting in the loss of the trace minerals prior to absorption by the animal (Henry *et al.,* 1992; Ward *et al.,*1996). The effectiveness of organic forms resides in their higher activity and biological availability (Kinal *et al.,* 2004). Digestibility of Zn from bonds with proteinates, yeast cells, lactates and etc., is several times higher compared to inorganic compounds. Considerable interest in the use of chelate or organic trace elements in the diet of ruminants was supported by contributions for improved growth, reproductions and health in ruminants fed by an organic form of trace elements (Spears, 1996).

In the present study, the effects of organic and inorganic zinc sources on the testicular structures of lambs including testis weight and volume, seminiferous tubule volume, interstitial tissue volume, tubule length and diameter, germinal epithelium height and total number of Leydig cells were studied using modern stereological methods. Details of design-based and unbiased stereological methods are presented.

MATERIALS AND METHODS

Animals

Twelve Sanjabi ram lambs weighing 20–25 kg at an average age of 3–3.5 months from the same pedigree were used. The animals were housed in individual pens under natural lighting at Razi University, Kermanshah city, Iran where winter photoperiod was 10 h light: 14 h dark.

Experimental design

Before starting the experiment, the lambs were treated with common anti-parasitic drugs albendazole, triclabandazole, niclosamide and ivermectin) and vaccinated against enterotoxaemia. At the start of the experiment, after 16 h of fasting, the animals were weighed and divided into three groups each including four lambs. The control group received basic diet containing 33 mg/kg DM zinc and the second and third groups were given the basic diet plus 40 mg/kg DM of organic Zn methionine and inorganic zinc sulfate, respectively.

Diet

The basic diet for all groups was a total mixed ration consisting of hay, straw, shredded wheat and barley, soybean meal, wheat bran, salt, sodium bicarbonate, dicalcium phosphate and mineral supplements. Diet for experimental groups was calculated using NRC (2007) tables and considering the maintenance requirements for animals and 200 g/day weight gain. The diet had 10.46 MJ/kg metabolisable energy and 14.5 g/kg DM crude protein. Food was prepared daily and provided three times a day at 9:00 AM, 2:00 PM and 6:00 PM. Fresh water was available *ad libitum*. The duration of the experiment was 70 days plus an acclimatisation period of 2 weeks for adaptation to the experimental diet.

Stereological study

At the last day of the experiment, the animals were weighed and then slaughtered.The left testes were removed and immediately fixed in 10% buffered formaldehyde. After 2 weeks, the epididymis and tunica vaginalis parietalis were removed, the testis weighed and the primary volume was measured using the immersion method (Silva & Merzel, 2001).

A known fraction of the tissue was sampled systematically at random from each testis in a careful stepwise sampling procedure: 1) each testis was cut into 10-mm thick slabs, providing 8–10 slabs (Fig. 1A); 2) every 4th slab was sampled systematically randomly and cut into 5-mm thick bars providing 5–7 bars (Fig. 1B); 3) every 2nd bar was sampled and cut into cubes (Fig. 1C); 4) every 4th to 6th of these cubes approximately (8–10 cubes) were sampled.
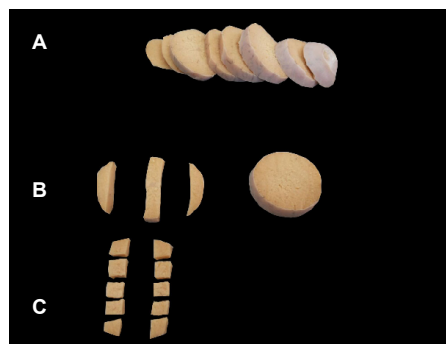

Fig. 1. Fractionator method. **A**: the testis was cut into 10 slabs; **B**: every 4th slab was sampled systematically randomly and cut into 5-μm bars providing 5–7 bars; **C**: every 2nd bar was sampled, cut into cubes and every 4th to 6th of these cubes were sampled.

The reference or final volume of the testis should be estimated in a stereological study to prevent reference trap (Braendgaard & Gundersen, 1986; Gundersen *et al.,* 1988). In this study, the final testis volume was estimated after tissue processing, staining and shrinkage estimation on the some sections without need to consecutive sections. Estimation of shrinkage requires isotropic uniform random sections. These were obtained from the sampled cubes by the orientator method (Fig. 2).

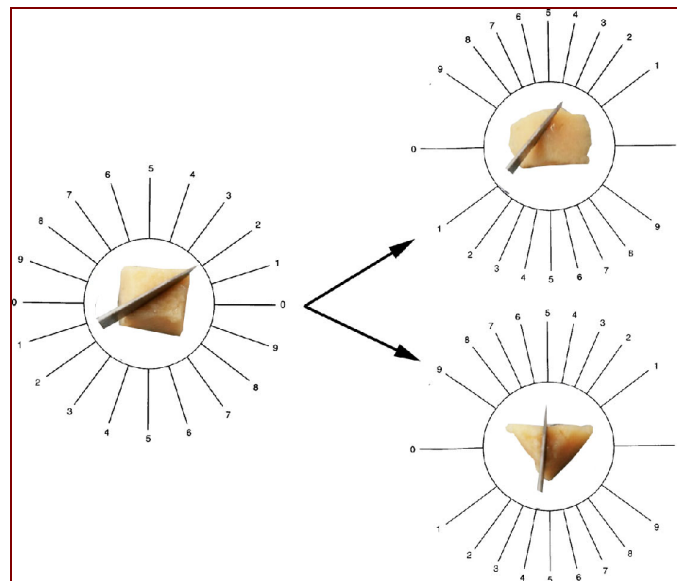Totally, 7–10 slabs were obtained from each testis through this method. A

Fig. 2. Orientator method to obtain the isotropic uniform random sections.
Left: the testis is placed on a circle divided into 10 equal distances. A random number between
0 and 10 is selected and the testis is sectioned into two halves, with a blade at that direction here.
Right: cut surface of the each half of the testis is then placed on the 0–0 direction of the second
circle with 10 unequal cosine-weighted divisions and the second cuts done (here, 3 and 5).

circular piece was sampled from a testis slab and the area of this piece was calculated. The slabs and circular piece were processed, sectioned at 5 μm and stained with haematoxilyn and eosin (Fig. 3). After staining, the area of the circular piece was measured again and tissue shrinkage (Nyengaard, 1999) was calculated as:

$$Shrinkage = 1 - (\frac{AA}{AB})^{1.5}$$

where AA and AB are the area of the circular piece after and before tissue processing. The total volume of the organ (the reference space) was then estimated as:

$$V = Vprimary \times (1 - Shrinkage)$$

All obtained sections were analysed using a videomicroscopy system equipped with a microscope (CX2; Olympus, Tokyo, Japan) connected to a video camera Dino-Lite (Dinocapture ver. 5; 30.5 mm;

AnMo Electronics Corp., New Taipei City, Taiwan), a Dell Pentium 4 PC (Dell Inc., Round Rock, TX) and a flat monitor to determine the parameters. For estimating

Fig. 3. From each testis, 7 to 10 slabs were obtained. A circular piece was sampled from a testis slab and its area was then calculated.

each parameter, 10–14 microscopic fields were examined in each testis.

Estimation of volume of the tubules and interstitial tissue

The fractional volume of seminiferous tubules and interstitial tissue were estimated using a point probe (with an area of 100 cm^2 and containing 25 points) and following formula:

$$Vv = \frac{\sum Pstructure}{\sum Preference}$$

where $\sum Pstructure$=sum of points hitting to the structures of interest; $\sum reference$= sum of points hitting to the reference space.

The final (absolute) volume was obtained by multiplying the volume density by testis volume (Gundersen *et al.,*1988; Nyengaard, 1999).

Estimation of the length and diameters of the tubules

The length density of the seminiferous tubules was estimated by superimposing an unbiased counting frame with an area of 100 cm^2 on the monitor live images (Gundersen *et al.,* 1988; Nyengaard, 1999). The length density was estimated as:

$$Lv = \frac{\sum Q}{a(frame) \times \sum frame}$$

where $\sum Q$=sum of counted tubules, a(frame) is the probe area, 547,600 μm^2, $\sum frame$ = total number of the counted frames. Finally, the total length of tubules, (L), was calculated by multiplying the length density (*Lv*) by the total volume of the testis. The diameter of tubules was measured perpendicular to the long axis where the tubule was the widest (Fig. 4).
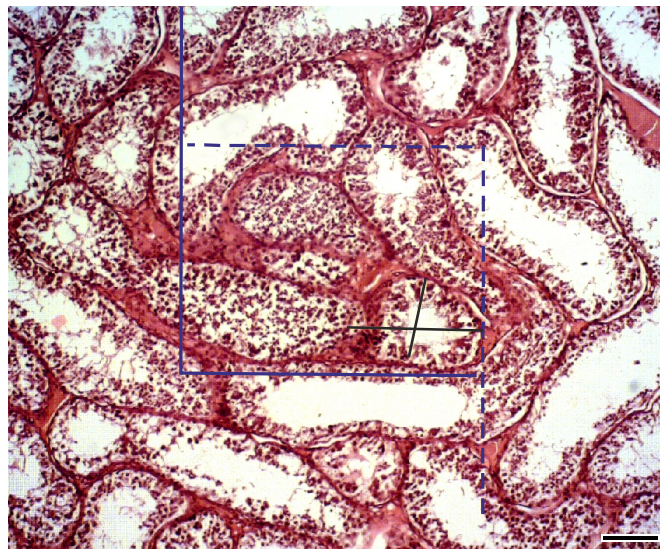

Fig. 4. Estimation of length density of the tubules using counting probe. The tubule structures completely or partly inside the counting frame but only touching the top and right lines are considered (here 2). The tubule profiles touching the bottom and left lines and its extensions are ignored. The diameter of the tubules is measured on the tubules sampled by the counting frame. The diameter is measured perpendicularly to the long axis where the tubule is widest; scale bar=50 μm.
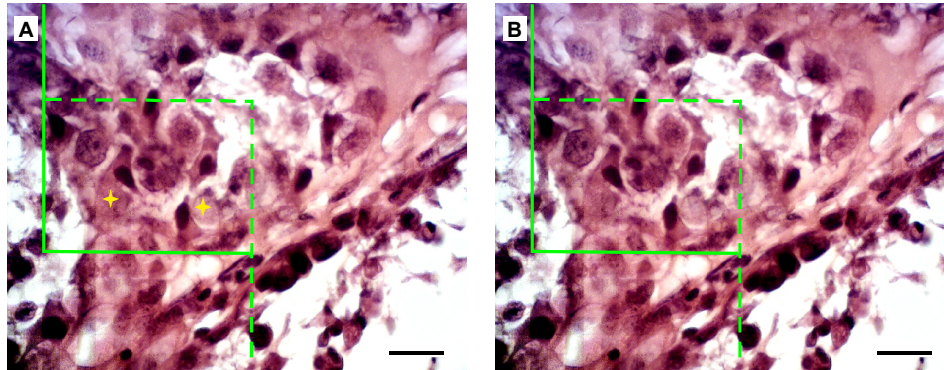
Fig. 5. Physical disector method for estimating the numerical density of Leydig cells. The images of a pair section (first and second sections) were used as the reference plane (A) and look-up plane (B), respectively. An unbiased counting frame is superimposed on the sample sections. Cellular profiles are counted if they are contained completely or partly in the frame and do not touch the exclusion (unbroken lines) as well as if they are disappeared in the look-up plane (here 2 cells); scale bar=5 µm.

Estimation of Leydig cells number

Total number of Leydig cells per testis was estimated using physical disector/fractionator combination method (Sterio, 1984). Approximately 10 pairs sections – every 400 µm or every 80th section and its pair were sampled from each testis. Two disector probes 740×740 µm with exclusion lines the left and lower borders and inclusion lines the right and upper borders were superimposed on the images of the first section as reference plane (Fig. 5A) and the 7th section as look-up plane (Fig. 5B) at a total magnification 400×. The counting rules of physical disector were applied. Thus, a cell was counted if it was presented in reference plane but not in lookup plane as well as didn't touch the exclusion lines. At least 200 cells per testis were counted. The numerical density of glomeruli was estimated using:

$$Nv = \frac{\sum Q}{\sum P \times a(frame) \times h}$$

where: $\sum Q$ = sum of the counted cells, *a* (frame) = probe area, $\sum P$ = total number

of the examined fields and *h* = disector height.

The total number of the Leydig cells was estimated by multiplying the number counted of cells by the inverse of the sampled fractions. The Leydig cells were recognised in the interstitium as relatively large ovoid shaped cell with an eccentric nucleus containing a prominent nucleolus and peripherally localised chromatin.

Estimation of the height of germinal epithelium of the tubules

The height of the germinal epithelium of seminiferous tubules was estimated as described by Nyengaard (1999).

$$H = \frac{Vv}{Sv}$$

where Vv and Sv were the volume density and surface density of the germinal epithelium respectively. The volume density of the germinal epithelium was obtained by the point counting method. The surface density of the germinal epithelium was estimated using a linear test probe (Fig. 6).
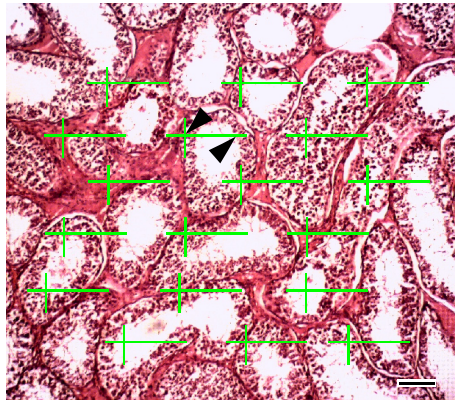
Fig. 6. The total number of points (upper arrowhead) superimposed on the germinal epithelium ($\sum p$), the length of each line (l/p), number of intersections (lower arrowhead) of linear test probe with the inner surface of the germinal epithelium ($\sum l$) are calculated. The surface density (Sv) is then estimated by the formula: $Sv = 2 \times \sum l / \sum p \times l / p$; scale bar=50 μm).

Statistical analysis

The data are expressed as mean and standard deviation. Kolmogorov-Smirnov test was used to test distribution normality. Statistical comparison between group means were done by one-way ANOVA followed by Tukey's *post host-hoc* test. P≤0.05 was considered as significant.

RESULTS

Body weight, volume and testis's weight

Final body weight did not show any significant difference between the control and organic zinc group. However, the body weight of lambs of inorganic zinc group was significantly (P≤0.05) lower than those of control and organic zinc groups. Testis weight and volume increased significantly (P≤0.05) in organic zinc group in comparison with control and inorganic zinc groups. The increase of testis weight and volume in lambs supplemented with inorganic Zn was also significant compared to the control group (Table 1).

Tubule and interstitial tissue parameters

The data showed that tubule volume in animals fed organic and inorganic zinc supplements has increased substantially (P≤0.05) in comparison with controls. Furthermore, the difference between organic and inorganic groups was significant (P≤0.05). Similar results were obtained for tubular diameter and tubule length of the experimental groups. Height of germinal epithelium increased significantly (P≤0.05) in organic and inorganic Zn groups compared to the control group, but there was no obvious (P>0.05) difference

Table 1. Body weight (kg), testis weight (g) and testis volume (cm³) in control lambs and lambs supplemented with either organic or inorganic zinc (mean ± SD; n=4)

Groups	Parameter			
	Initial body weight, kg	Final body weight, kg	Testis weight , g	Testis volume, cm³
Control group	22.5± 2.9	40 ±1.7	135± 5.5	112 ±14.6
Organic Zn group	22.6± 2.2	38±2	162±8.3[a,b]	149.2±8.4[a,b]
Inorganic Zn group	22.6±1.8	36.5±1.7[b]	141.9±10.3[a]	131.4±5.3[a]

[a] P≤0.05 organic or inorganic zinc *vs* control; [b] P≤0.05 organic zinc *vs* inorganic zinc.

Table 2. Absolute volume of the seminiferous tubules, (TV, cm^3), interstitial volume (ITV, cm^3), tubular length (TL, m), tubular diameter (TD, μm), height of the germinal epithelium (EH, μm) and Leydig cell number (×10^8) in control lambs and lambs supplemented with either organic or inorganic zinc (mean ± SD; n=4)

Groups	Parameter					
	TV	ITV	TL	EH	TD	LCN
Control group	90.8±10.4	22.5±4.2	1701±142	62.4±8.8	148.3± 22	55±9
Organic Zn group	137.0±7.8[a,b]	25.4±6.5	2478±323[a,b]	87.2±4.5[a]	185.6±17.1[a,b]	72±12[a,b]
Inorganic Zn group	113.8±12[a]	20.1±6.9	2010±218[a]	78.0±6.9[a]	166.0±10.8[a]	62±8

[a] P≤0.05 organic or inorganic zinc *vs* control; [b] P≤0.05 organic zinc *vs* inorganic zinc.

between them. Interstitial tissue volume did not change significantly (Table 2).

Leydig cell number

The number of Leydig cells of organic group increased significantly (P≤0.05) in comparison with control and inorganic Zn groups. However, the increase in Leydig cells number in inorganic Zn group was not statistically significant compared to the controls (Table 2).

DISCUSSION

The present study shows the comparative effects of organic and inorganic dietary zinc supplements on the testicular tissue of lambs using modern stereological methods. Based on our hypothesis, as expected, the organic zinc supplement had better effects on the structural parameters of lamb's testis. Initial body weight of groups did not differ significantly, but at the end of the experiment the inorganic group exhibited significantly lower final body weight as compared to the organic and control groups, while the lambs which had received organic zinc showed final body weight similar to the controls. It is

well acknowledged that organic zinc supplements are more palatable than inorganic ones (Malcolm-Callis *et al.,* 2000). Therefore, this could have decreased the feed intake and consequently, the final body weight in the inorganic Zn group (based on non presented data, mean dry matter intake in the inorganic group was by 285 g/day lower than that of the control group). Several studies on different animal species showed that zinc deficiency has been associated with many development disorders including poor appetite, failure to thrive and growth retardation (in terms of body weight gain) (Merrells *et al.,* 2009). Growth retardation in zinc deficiency cannot be attributed to a single factor and seems to be probably multifactorial. Accordingly, organic zinc supplements had no effects on growth performance as shown by Wright & Spears (2004) in calves and Fadayifar *et al.* (2012) in lambs. In contrast, Mallaki *et al.* (2015) reported that 20 mg organic zinc in lambs' diet could significantly increase dry matter intake and feed conversion ratio (FCR) compared to control and zinc sulfate group.

In the present study, the volume of seminiferous tubules has increased significantly following organic zinc supplement consumption, whereas interstitial tissue volume did not change significantly. Tubules are the main constituents of testes and their hypertrophy leads to increased testicular volume and weight. According to the results, testis volume and weight were increased significantly in organic Zn group. It should be noted that, although the increase of testis weight and volume in inorganic zinc group was significant compared to the controls, the effect of organic supplement was more pronounced. It was revealed that zinc deficiency could completely block testicular development in young rams going through puberty (Martin *et al.,* 1994). Further, more studies show that much of this effect was not a specific response to zinc deficiency, but was caused by the reduction of appetite leading to deficit in energy and protein needed for growth and pubertal development (Martin & White, 1992).

Length and diameter of the tubules are the other estimated parameters. When an increase in tubule volume or hypertrophic changes is seen in the tubules, this might be also due to increased length and diameter. Using applied stereological methods it was possible to evaluate the different aspects of hypertrophic changes and provide quantitative parameters. These parameters were increased in zinc supplemented lambs, but the increase was more pronounced in lambs fed organic zinc.

Germinal epithelium height, in the present work, was considered as a quantitative parameter. Since it is a flexible and irregular structure, its simple direct measurement would not lead to an unbiased estimation. Some factors such as orientation of tissue and cutting angle would influence the height. Thus, to obtain an un-

biased height, the volume density of the epithelium was divided by its surface density. The results showed that organic and inorganic zinc supplements can improve this parameter similarly without significant difference between respective groups. It has been reported that zinc-deficient rams had poorly developed tubules with little or no lumen and a large proportion of interstitial tissue, but the epithelium height was not affected. In the case of diets with insufficient Zn, retarded development of testicles and impaired spermatogenesis has been observed as well as poor sperm quality (Martin *et al.,* 1994).

Zn supplementation of diet affected the number of Leydig cells counted per testis and the difference between the organic and inorganic supplements was significant. Hotzel *et al.,*(1998) showed that nutrition at sub- and supra-maintenance levels had no marked effect on Leydig cells number.

Based on the present data, organic and inorganic zinc supplements had similar effects on germinal epithelium height. On the other hand, the impact of organic zinc supplement on Leydig cells number and their proliferation was better than the inorganic one. Therefore, the mechanism contributing to this event must be clarified. Perhaps, Leydig cells culture in medium containing organic zinc and other *in vitro* experiments could elucidate this issue.

The physical disector method was used to estimate the Leydig cells number. It is important to note the advantages of the physical disector/fractionator combination as a method for counting cells. With this method, cells are counted using the disector principle in a known and predetermined fraction of the testis. No assumptions of cellular shape, size, uniformity of size, or orientation are required.

Furthermore, the estimates are not influenced by shrinkage/swelling artifacts associated with tissue processing.

It has been suggested that organic minerals have an increased bioavailability, resulting in an increased absorption in the gastrointestinal tract (Spears, 1996). Higher absorption was observed for zinc sulphate than for zinc oxide (Kinal, 2000). However, higher absorption and utilisation of zinc in its organic forms is confirmed by Kinal (2005) in lambs. Upon use of the organic form, statistically significant differences of the Zn content in the serum of calves (Kinal *et al.,* 2004) and piglets (Novotný *et al.,* 2005) were observed.

Since the effects of dietary organic and inorganic zinc supplements on histomorphometric characteristics of testes have not been studied in any species with which the present data can be compared, further studies investigating these effects in different ruminant species are needed to confirm these findings.

CONCLUSION

It can be concluded that the organic form of zinc (Zn methionine) showed a better response in improving testis volume, tubular volume, tubule length, tubule diameter and Leydig cell number compared to the inorganic Zn sulfate. This might be due to the better bioavailability of Zn methionine comapred to Zn sulfate, and as a result, better absorption, distribution and uptake of Zn in the Zn methionine supplemented group accounted for its better effect over Zn sulfate.These findings provide a set of reliable quantitative date regarding to the effect of organic zinc supplementation on testes and will contribute to a better understanding of the nutrition-reproduction relationship in small ruminants.

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