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The Utilization of Organic Copper and Zinc in the Feeding of Sheep During the Pre and Post-Partum Period

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Abstract: In this study, the effects organic zinc and copper mineral supplementation in Kivircik sheep during the pre and post-partum period was investigated by decreasing the normally given inorganic mineral level by 25%. The control group (n = 8) received copper sulfate and zinc sulfate and the treatment group (n = 8) received copper chelate and zinc chelate. The difference between the mean live weight and total weight gain between the groups was not significant. The mean serum copper and zinc values, skin copper and zinc values and wool zinc value were numerically higher in the treatment group. Additionally, the mean wool copper and the skin dry matter values were higher ($p<0.05$) in the treatment group. At the end of the study, the mean feces copper ($p<0.05$) and zinc levels ($p<0.01$) were significantly lower in the treatment group. During examination of histological structure of the skin, researchers observed that the Str. papillare was thin ($p<0.001$) in the treatment group. No other differences between the skin features of the treatment and control group were observed. In addition, the decrease in the thickness of the Str. papillare may be a result of tightening due to the positive effect of organically ingested copper and zinc on connective tissue fibers.

Key words: Organic mineral, copper, zinc, feeding, skin features, treatment

INTRODUCTION

It is well known trace mineral deficiency and diseases affiliated with mineral deficiencies are prevalent worldwide. Inorganic salts (oxides, sulfates) are generally added to the diet to prevent deficiencies (Spears, 1996; Ryan *et al*., 2002). However because trace minerals usually antagonize other elements within the diet, they are not generally added to the diet in high levels. High-level utilization poses a risk of toxicity and causes pollution by increasing discharge in the feces (Ryan *et al*., 2002; Leeson, 2003; Javed, 2005; Tayefi-Nasrabadi *et al*., 2008).

Because organic minerals are absorbed without alterations except, for large molecule proteinates and are stored in their same organic forms because no antagonism is formed it has been stated the absorption and bioavailability of organic minerals are high (Nockels *et al*., 1993; Boland, 2003; Spears, 2003). It is also that known organic minerals are not discharged by feces as much as inorganic minerals because they are found in smaller levels in the diet (Nocek *et al*., 2006; Bao *et al*., 2007; Nollet *et al*., 2008; Wagner *et al*., 2008).

In this study, the effects of using organic Copper (Cu) and Zinc (Zn) (chelate) on the serum, wool, skin and feces mineral levels as well as skin dry matter and skin histological structure was compared by decreasing the recommended inorganic mineral level by 25%. Due to the intensity of metabolic activity, the experiment was performed during pre and post-partum periods.

MATERIALS AND METHODS

A total of 16, 3-5 years old Kivircik sheep within the last 20 days of their pregnancy were used for this study. The sheep were divided into two groups; control (n = 8) and treatment (n = 8). The control group was given 7 mg kg^{-1} Dry Matter (DM) copper-sulfate and 20 mg kg^{-1} DM zinc-sulfate (NRC, 1985). The treatment group was given 5.25 mg kg^{-1} DM copper-chelate (Cu-2-hydroxy-4-methylthiobutyrate) and 15.0 mg kg^{-1} DM zinc-chelate (Zn-2-hydroxy-4-methylthiobutyrate) in an organic form which is 25% less than the inorganic mineral level. The ration was organized to provide the nutrient requirements of the sheep when considering pregnancy and lactation

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Table 1: The ration composition and Cu and Zn values within the wheat hay and intensive forage that were given to pregnant sheep

Ration composition (%)	Treatment group (organic mineral)	Control group (inorganic mineral)
Wheat hay	55.75	55.75
Maize	30.41	30.41
Soybean meal (44% HP)	10.79	10.79
Vitamin-mineral mix*	3.05	3.05
ME (kcal kg^{-1} DM)	1932.20	1932.20
CP (g kg^{-1} DM)	109.60	109.60
Wheat hay Cu (kg DM, ppm)	7.58	7.58
Wheat hay Zn (kg DM, ppm)	8.54	8.54
Intensive forage Cu (kg DM, ppm)	6.23	6.23
Intensive forage Zn (kg DM, ppm)	17.40	17.40

*In 1.0 kg of the vitamin-mineral mix, there is 16,000,000 IU vitamin A, 3,200,000 IU vitamin D3, 32000 mg vitamin E, 80 g salt, 320 g DCP, 640 mg manganese, 1120 mg iron, 16 mg iodine, 3.20 mg cobalt, 6.40 mg selenium, 16 mg molybdenum and 256 mg magnesium. Also, there is 640 mg zinc and 224 mg copper in the inorganic mix and 480 mg zinc and 168 mg copper in the organic mix

Table 2: The ration composition and Cu and Zn values within the wheat hay and intensive forage that were given to lactating sheep

Ration composition (%)	Treatment group (organic mineral)	Control group (inorganic mineral)
Wheat hay	43.53	43.53
Maize	30.85	30.85
Soybean meal (44% HP)	23.22	23.22
Vitamin-mineral mix*	2.40	2.40
ME (kcal kg^{-1} DM)	2112.78	2112.78
CP (g kg^{-1} DM)	162.30	162.30
Wheat hay Cu (kg DM, ppm)	7.58	7.58
Wheat hay Zn (kg DM, ppm)	8.54	8.54
Intensive forage Cu (kg DM, ppm)	7.39	7.39
Intensive forage Zn (kg DM, ppm)	17.13	17.13

*In 1.0 kg of the vitamin-mineral mix, there is 24,000,000 IU vitamin A, 4,800,000 IU vitamin D3, 48000 mg vitamin E, 120 g salt, 320 g DCP, 80 g $CaCO_3$, 960 mg manganese, 1920 mg iron, 24 mg iodine, 4.80 mg cobalt, 9.60 mg selenium, 24 mg molybdenum and 384 mg magnesium. Also, there is 960 mg zinc and 336 mg copper in the inorganic mix and 720 mg zinc and 252 mg copper in the organic mix

(NRC, 1985). The rations given to the sheep are shown in Table 1 and 2. The treatment was initiated on the 20th day before birth and was completed 45 days after birth. The sheep were fed individually.

The intensive forage and vitamin-mineral compound were weighed daily for each sheep and given as a single meal. Wheat hay was given as two meals after the intensive forage was finished. A total of 770 g of intensive forage and 1000 g of hay were given daily to pre-partum sheep and 1500 g of intensive forage and 1160 g of wheat hay and *ad libitum* water was given during lactation.

The sheep were individually weighed at the beginning and end of the study. Serum was separated by taking blood from the vena jugularis and the Cu and Zn levels were determined spectrophotometrically (Shimadzu Corp. UV-1601, Australia) using a kit (Randox, Cu:Cu2340 and Zn:Zn2341, Ardmore, United Kingdom).

Intensive forage and wheat hay samples were taken at the beginning of the study and at lactation and wool samples (close to the skin) were taken at the beginning and end of the study from the sheep's shoulder, rib and hind. The skin samples were taken from the shoulder, rib and hip regions and the feces sample was taken from the rectum with the aid of a finger at the end of the study. The Cu and Zn levels of the samples were determined by using ICP (Inductively Coupled Plasma Spectro-Optima 2100 DV ICP/OES, PERKIN ELMER). The dry matter levels of the skin samples were also determined.

Skin biopsy samples (1 cm^2 section) for histological examination were taken from the hip and rib regions. The tissues taken from the hip region for the general examination and histometric measurements were fixed in Maximow's solution. The tissue samples taken from the rib region for the fat determination on the surface of the skin were fixed with formol-calcium solution.

The tissues taken from the hip region were blocked in paraffin after routine tissue follow-up. The tissues were divided into two sections; one section was placed vertically and the other was placed parallel to the surface. The paraffin tissue blocks were serially cut into 7 μm thick sections. Crossman's triple staining method was used for general histological examination and histometry, Gordon and Sweet's silver impregnation method was used for reticular fiber demonstration and Verhoeff's Hematoxylen staining method was applied to identify elastic fibers (Culling *et al*., 1985). In addition, the 10 μm frozen sections from the rib regions were stained with oil red O to determine the fat content (Culling *et al*., 1985).

After the general histological examinations, histometric data were obtained from the sections with the aid of an interactive image analyzing system (Lecia DC200) connected to a research microscope (Lecia DMLB). Six sections were used for each animal. Measurements were taken from three random areas on the section.

The thickness of the total skin, epidermis, the layers of the dermis, stratum papillare and stratum reticulare were measured in the sections that were vertical to the skin's surface. The primary and secondary hair follicle numbers in a unit area (mm^2) were determined in the sections that were taken parallel to the surface. Three primary follicle and five secondary follicle diameters that were round or nearly round were measured in every section. The statistical analyses were performed using the SPSS© 15.0 package program. The differences of the group means for the examined parameters in the group were determined using Student's t-test.

RESULTS AND DISCUSSION

The difference between the groups' mean live weight values and mean total live weight gain values at the beginning and end of the study was not significant

Table 3: The mean live weight and mean total live weight gain values at the beginning and end of the study (kg)

Weight (kg)	Treatment group (n = 8)	Control group (n = 8)	t-value
Initial live weight	40.90±1.05	40.18±1.22	0.444
Final live weight	40.75±0.86	40.03 ±1.24	0.472
Total live weight gain	0.156±0.39	0.150± 0.35	0.012

$\bar{X}\pm S_{\bar{x}}$ shows the ±values

Table 4: Serum Cu and Zn values at the beginning and end of the study (μg dL^{-1})

Serum level	Treatment group (n = 8)	Control group (n = 8)	t-value
Initial Cu	104.82±5.670	126.42±8.890	2.046
Final Cu	371.62±18.12	335.13±19.91	1.355
Initial Zn	98.01±2.910	109.83±3.290	2.689*
Final Zn	148.80±7.160	129.53±12.11	1.369

*p<0.05; $\bar{X}\pm S_{\bar{x}}$ shows the ±values

(Table 3). The studies that some researchers (Rojas *et al*., 1995; Yost *et al*., 2002; Wagner *et al*., 2008) performed on different species also demonstrated no statistically significant difference between the groups' mean live weights. At the beginning and end of the study, the difference between the mean serum Cu values was not statistically significant. However while the mean serum Cu value was numerically higher in the control group at the beginning of the study, the treatment group's mean serum Cu value was higher at the end of the study (Table 4). Similarly, although the mean serum Zn value was higher ($p<0.05$) in the control group at the beginning of the study it was numerically higher in the treatment group at the end of the study (Table 4).

Rojas *et al*. (1995) showed that the serum Zn value significantly ($p<0.05$) increases in lambs given organic minerals and Ryan *et al*. (2002) demonstrated that the plasma Cu value ($p<0.001$) and plasma Zn value ($p<0.05$) in sheep given lower levels of organic minerals were significantly high. Similar findings were reported by Eckert *et al*. (1999) for copper proteinate in sheep and by Mondal *et al*. (2008) for the organic forms of copper, zinc and manganese that were given at a 50% lower level. Additionally, Rojas *et al*. (1996) in cattle, Salama *et al*. (2003) in goats and Spears and Kegley (2002) in calves showed that the organic and inorganic minerals' serum levels are similar.

At the end of the study, the wool Cu value in the treatment group was significantly ($p<0.05$) higher than in the control group. However, there was no difference in the mean wool Zn value between the control and treatment groups (Table 5). Additionally, the wool Zn level in both the treatment and control groups decreased at the end of the study in comparison to the levels at the beginning of the study (Table 5).

The reason that the mean wool Zn values in both the treatment and control groups was high at the beginning of the study could be a result of the Zn levels found in the region's soil and plants. Because the sheep were not

Table 5: Wool Cu and Zn values at the beginning and end of the study (ppm)

Wool level	Treatment group (n = 8)	Control group (n = 8)	t-value
Initial Cu	6.49±0.270	6.38±0.580	0.177
Final Cu	10.25±0.570	7.32±0.990	2.544*
Initial Zn	173.17±15.78	181.11±14.90	0.366
Final Zn	81.15±04.49	80.15±2.660	0.192

*p<0.05; $\bar{X}\pm S_{\bar{x}}$ shows the ±values

Table 6: Skin Dry Matter (DM, %) and skin Cu and Zn values (ppm)

Skin level	Treatment group (n = 8)	Control group (n = 8)	t-value
Final DM	31.103±0.003	30.2700±0.004	2.185*
Final Cu	4.970±0.530	3.7100±0.440	1.796
Final Zn	37.510±7.470	32.1800±5.810	0.563

*p<0.05; $\bar{X}\pm S_{\bar{x}}$ shows the ±values

Table 7: The mean Cu and Zn values in the sheep feces (ppm)

Feces level	Treatment group (n = 8)	Control group (n = 8)	t-value
Final Cu	26.95±1.26	37.75±4.93	2.12*
Final Zn	44.84±6.44	76.40±7.65	3.14**

*p<0.05; **p<0.01; $\bar{X}\pm S_{\bar{x}}$ shows the ±values

taken out to graze during the study, the mean wool Zn level in both groups was lower at the end compared to the beginning. Ryan *et al*. (2002) showed that adding Zn to the diet of Texel sheep significantly ($p<0.05$) increases the wool Zn level; however, giving the Zn in forms of a sulfate or an amino acid chelate did not give a significant difference. Wright and Spears (2001) determined that the wool of the calves given zinc proteinate accumulate a higher level of Zn compared to calves given zinc sulfate but that the difference was not statistically significant. It has previously been determined that adding Zn that has been chelated with amino acids to the diet enables hair to grow longer in dogs and that the Zn level is higher in comparison to zinc oxide (Lowe *et al*., 1994).

The difference between the mean skin dry matter was statistically significant ($p<0.05$) in the treatment group. The difference in Cu and Zn values in the groups' skin was not statistically significant but the mean Cu and Zn values found in the skin were numerically higher in the treatment group (Table 6).

Rojas *et al*. (1995) also observed no significant difference ($p>0.05$) between the mean Zn values of the organic and inorganic groups in the skin of sheep. Interestingly, research has shown that organic minerals concentrate more in the organs and tissues and are held more within the body (Henry *et al*., 1992; Rojas *et al*., 1995; Hatfield *et al*., 2001; Ryan *et al*., 2002; Salama *et al*., 2003).

In this study, the mean Cu and mean Zn values found in the feces of the treatment group were significantly lower compared to the control group (Table 7) ($p<0.05$

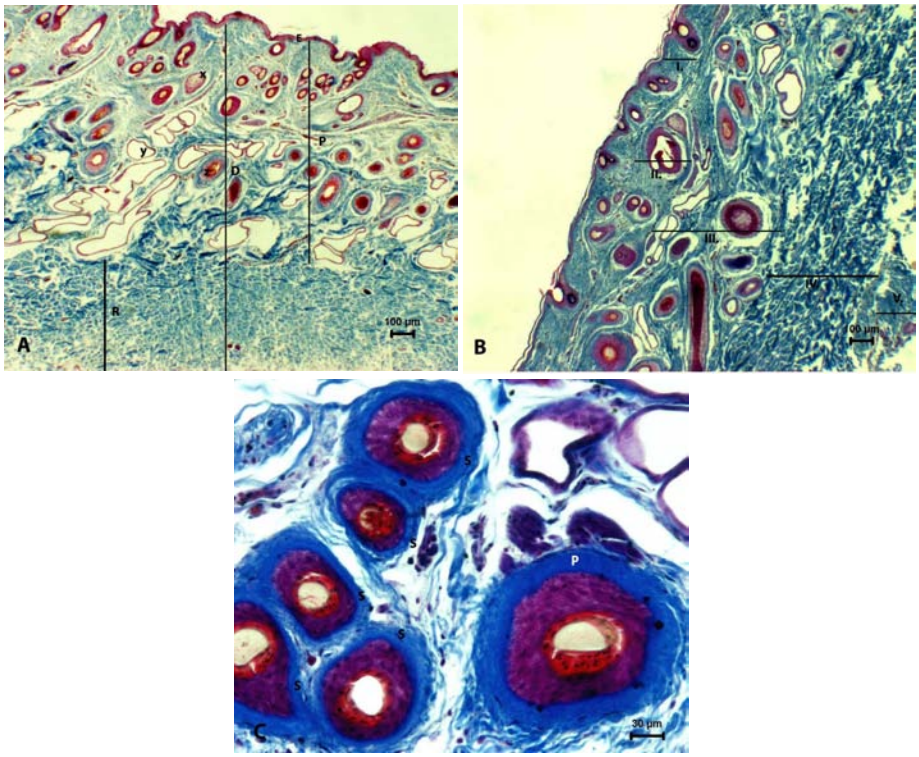

Fig. 1: The histological appearance of the skin in Kivircik sheep using the triple staining method; A) layers of the skin: Epidermis (E), Dermis (D), Str. Papillare (P), Str. Reticulare (R); B) the sections of Str. Papillare (section I-III) and Str. reticulare (section IV-V): section I is where the neck of hair follicles are found, section II is where the sebaceous glands and body of hair follicles are found, section III is where the bulbi of the hair follicles and body of sweat glands are found, section IV begins from the end of where the sweat glands and collagen fiber bunches are found, section V is where the collagen fiber bunches are thin and close to the hypodermis; C) appearance of the hair follicles. Primary follicles (P), Secondary follicles (S)

and $p<0.01$, respectively). Similar results have been demonstrated in other studies. Research by Nocek *et al.* (2006) on cows by Wagner *et al.* (2008) on calves and by Mondal *et al.* (2008) on male calves suggests that the absorption and bioavailability of organic trace minerals is higher.

Thus, when added to the animals' rations in lower levels, there is less discharge via the feces and thereby less pollution. Similar results have been seen in pigs (Coffey *et al.*, 1994; Lee *et al.*, 2001; Case and Carlson, 2002; Armstrong *et al.*, 2004; Carlson *et al.*, 2004) and steer (Ward *et al.*, 1993).

Histological appearence of the skin: During the histological examinations, researchers observed both in samples of the control and treatment groups that the main layers of the skin carried histological features in accordance with classical information (Fig. 1A). When the locations of the textures found in the dermis were considered, five separate sections were defined; three sections in the Stratum (Str.) papillare and two sections in the Str. reticulare (Fig. 1B). Although, there are studies on Kivircik sheep skin histology and its comparison with other types (Ozfiliz *et al.*, 1997), there are no other reports that have studied the skin's histological structure after feeding. The data on the thickness of the epidermis, Str. papillare and Str. reticulare layers and the total thickness of the skin are shown in Table 8. Researchers observed that the Str. papillare was thinner ($p<0.001$) in the treatment group. The primary and

Table 8: Mean total skin, epidermis, Str. papillare and Str. reticulare thicknesses in the control and treatment groups (µm)

Characteristics of skin	Treatment group (n = 8)	Control group (n = 8)	p-value
Epidermis	22.44±0.700	21.78±0.430	NS
Stratum papillare	884.62±27.44	1079.43±43.56	***
Stratum reticulare	857.83±45.71	753.57±28.31	NS
Total skin thickness	1764.43±51.53	1838.33±53.74	NS

***$p<0.001$; NS: Not Significant; $\bar{X}\pm S_{\bar{x}}$ shows the ±values

Table 9: Primary and secondary follicle diameters (μm) and numbers (number mm^{-2})

Characteristics	Treatment group (n = 8)	Control group (n = 8)	p-value
Primary follicle diameter	131.36±2.73	135.37±3.35	NS
Secondary follicle diameter	75.60±1.23	78.48±1.83	NS
Primary follicle number	2.89±0.13	2.76±0.13	NS
Secondary follicle number	13.48±0.52	13.85±0.81	NS

NS: Not Significant; $\bar{X}\pm S_{\bar{x}}$ shows the ±values

secondary follicle (Fig. 1C) numbers and follicle diameters are shown in Table 9. No significant difference was observed between the treatment and control groups. The epidermal thickness of the Kivircik sheep has been shown to be 36.67 and 13.45 μm. Additionally, the thickness of the epidermis in Karnobat sheep which are identical to Kivircik sheep is 18.8 μm (Stankov *et al*., 2004). In this study, the epidermal thickness in the control and treatment groups were 21.78 and 22.44 μm, respectively and no difference was observed between groups.

The stratum papillare in the dermis was 1079.43 μm in the control group and 884.62 μm in the treatment group. The value recorded in the control group was lower than the findings of other studies. This difference may be because the material was collected during February and the animals had been given milk. In the research conducted on Karacabey Merino sheep, Zik stated that the thickness of the epidermis and dermis and furthermore, the lipid layer in the epidermis is thinner during the winter.

In addition, this study found that the Str. papillare thickness was significantly less in the treatment group ($p<0.001$) compared to the control group. This difference may be a result of the effect of Cu and Zn on the collagen and elastin in the skin. Lysyl oxidase, an enzyme that is dependent on Cu, plays a role in the extra-cellular processing of collagen and elastin. Cu is essential for the functional activity of this enzyme (Kosonen *et al*., 1997).

It has been previously stated that lysyl oxidase plays a role in elastin and collagen cross-linking (Percival, 1997; Werman *et al*., 1997; Rucker *et al*., 1998; Maki, 2002). The cross-linking provides the stability of the collagen fibers and the elasticity of the elastin fibers (Maki, 2002).

It is also known that Zn plays a central role in the metabolism and repair of the skin and ligaments (Chien *et al*., 2006). In this study, the difference in the dry matter percentage of the skin samples was significant ($p<0.05$) between the control and treatment groups (Table 6). Taken together, researchers suggest that the decrease in the thickness of the Str. papillare may be a result of possible tightening due to a positive effect of Cu and Zn taken organically on connective tissue fibers. The primary and secondary hair follicle amounts determined in a 1 mm^2 area in the control group was 2.76 and 13.85, respectively.

The amount of the primary and secondary follicles as determined by was 8.07 and 33.21, respectively and that determined by Ozfiliz was 1.77 and 22.12, respectively. The primary and secondary follicle diameters were 135.37 and 78.48 μm, respectively.

Daglioglu determined the primary and secondary follicle diameters to be 105.28 and 65.74 μm, respectively; however, Ozfiliz showed that these values were 124.44 and 81.67 μm, respectively. The differences in the sheep's skin between the present research and other studies are thought to result from the body part that the sample was taken from and the season during which the sample was taken. There was no difference in primary and secondary hair follicle numbers or diameters between the control and the treatment groups in this study.

Connective tissue fibers: The collagen fibers were in small bundles in section I of the Str. papillare and were thicker and more scarce in section II. The thickness of the collagen fibers were noticeably increased in section III (Fig. 1B and 2A). No difference was observed between the treatment and control groups.

The elastic fibers were most dense in the Str. papillare and around the hair follicles and sebaceous glands in section II (Fig. 2B). The elastic fibers were less dense between the sweat glands and hair follicles in section III (Fig. 2C). A small amount of elastic fibers were also found in the Str. reticulare. The treatment group's elastic fibers were more distinctive.

The reticular fibers were high in number. They were found in the basal membrane and around the hair follicles (Fig. 3A), sebaceous glands, muscle cells and sweat glands (Fig. 3B). No difference was observed between the control and treatment groups.

While small amounts of lipid layers were observed in some locations in the epidermis, no difference was observed between the control and treatment groups.

The findings of this study regarding the connective tissue fibers are in accordance with the findings of Ozfiliz. The difference between Ozfiliz and data from the present study is that a small lipid layer was observed in the skin samples stained with oil red O. However, no differences were observed between the control and the treatment groups.

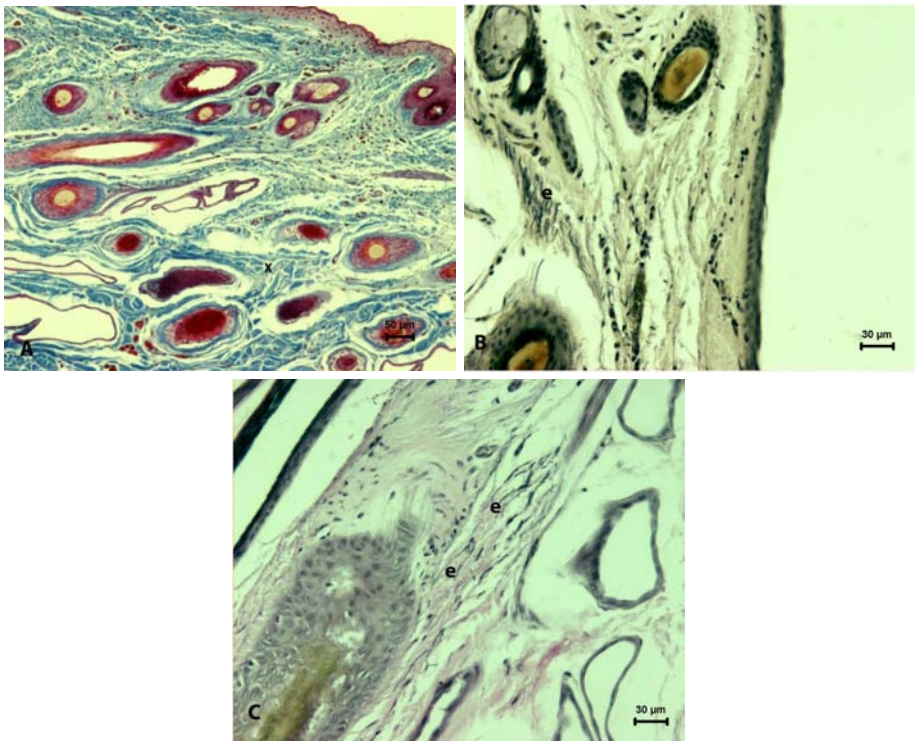

Fig. 2: A) appearance of the collagen fibers in section III (x) using the Triple Staining method; B) and C) appearance of the elastic fibers in sections II (e) and III (e) using Verhoeff's Hematoxylin Staining method

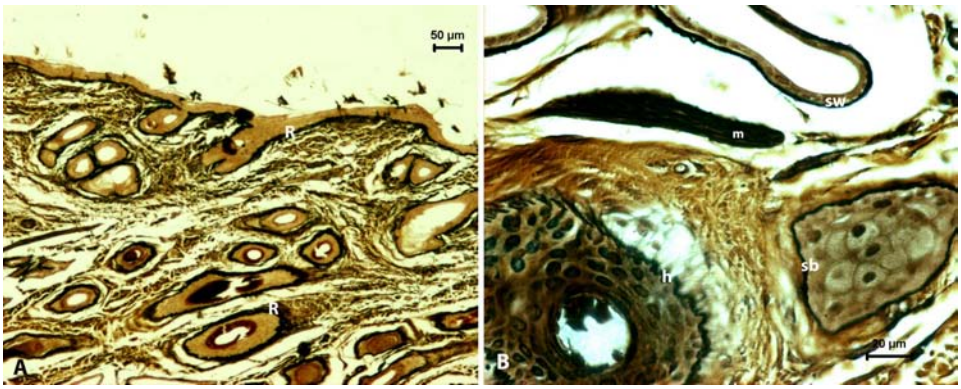

Fig. 3: Appearance of the reticular fibers in the skin of Kivircik sheep using Gordon and Sweet's silver impregnation method; A) reticular fibers in the basal membrane and around the hair follicles (R) and B) reticular fibers around the sebaceous glands (sb), muscle cells (m), hair follicles (h) and sweat glands

CONCLUSION

According to the examined parameters although, 25% less organic Cu and organic Zn was added to the sheep's diet, similar and even better results were observed for some parameters compared to inorganic Cu and Zn and organic minerals were found at lower levels in the feces. In addition, researchers suggest that the decrease in the thickness of the Str. papillare may be a result of possible tightening due to a positive effect of Cu and Zn taken organically on connective tissue fibers.

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