

## Effects of Progesterone and Testosterone on the Hyaluronidase Activities and Sperm Characteristics in Sheep

Sadettin TANYILDIZI

Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Firat University, 23119, Elazığ - TURKEY

Tanzer BOZKURT

Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Firat University, 23119, Elazığ - TURKEY

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**Abstract:** The aim of this study was to determine the effects of progesterone and testosterone hormones on the hyaluronidase activities of serum and semen and on sperm characteristics in sheep. Thirty healthy Akkaraman sheep were used. The progesterone and testosterone were injected intramuscularly at the rate of 0.5 mg/kg and 5 mg/kg, respectively. Then the blood and semen samples were obtained from the sheep at 1, 2, 4, 24, 48, 72 and 168 h. The serum hyaluronidase activities of ewes and rams in progesterone and testosterone groups increased significantly ( $p < 0.01$ ,  $p < 0.001$ ) when compared with the control groups at all times. Additionally, there were significant ( $p < 0.001$ ) increases in the levels of semen hyaluronidase activity in the progesterone group except at 4 h. Furthermore, semen hyaluronidase activities in the testosterone group were detected to decrease significantly ( $p < 0.01$ ) in comparison with the control rams except at 4 h. The use of progesterone and testosterone caused a significant ( $p < 0.05$ ,  $p < 0.01$ ) increase in spermatozoa motility, respectively. However, sperm concentrations were determined to decrease significantly ( $p < 0.01$ ,  $p < 0.001$ ) in both groups. Although the values for semen volume decreased significantly ( $p < 0.05$ ,  $p < 0.01$ ) in the progesterone group, the same values were observed to increase significantly ( $p < 0.01$ ) in the testosterone group except at 48 h when compared with the control groups. The results of this study showed that there were no significant correlations between semen hyaluronidase activities and sperm characteristics. In conclusion, the use of both hormones did not have any deleterious effects on hyaluronidase activities but for the semen samples in the testosterone groups. However, progesterone and testosterone hormones cause harmful effects on the fertilization ability of rams due to decreased sperm concentration values.

**Key Words:** Hyaluronidase, Spermatozoa, Motility, Sheep

### Progesteron ve Testosteronun, Koyunlardaki Hyaluronidaz Aktiviteleri ve Sperm Özellikleri Üzerine Olan Etkileri

**Özet:** Progesteron ve testosteron hormonlarının koyunlardaki kan serumu ve semen sıvısı hyaluronidaz aktiviteleri ile sperm özellikleri üzerine olan etkilerinin belirlenmesi, bu çalışmanın amacını oluşturmaktadır. Araştırmada, otuz adet sağlıklı, Akkaraman ırkı koyun kullanıldı. Bu hayvanlara, 0.5 mg/kg dozunda progesterone ve 5 mg/kg dozunda testosteron kas içi yolla verildikten sonra 1, 2, 4, 24, 48, 72 ve 168. saatlerdeki kan ve sperm örnekleri alındı. Hem progesterone hem de testosteron gruplarındaki dişi ve erkek koyunların kan serumu hyaluronidaz düzeylerinin kontrol gruplarına göre sırasıyla, önemli ( $p < 0.01$ ,  $p < 0.001$ ) düzeylerde arttığı belirlendi. Bunun yanında, progesteron gruplarına ait semen hyaluronidaz düzeylerinde 4. saat hariç olmak üzere, kontrol gruplarına göre önemli ( $p < 0.001$ ) artış mevcuttur. Bundan başka, testosteron gruplarındaki semen hyaluronidaz aktivitelerinin 4. saat dışındaki zamanlarda önemli ( $p < 0.01$ ) düzeyde azaldığı belirlenmiştir. Progesteron ve testosteron kullanımı, sperm motilitesinde önemli ( $p < 0.05$ ,  $p < 0.01$ ) artışa neden olmuştur. Bununla birlikte, her iki guruptaki sperm konsantrasyonlarına ait değerlerin ise önemli ( $p < 0.01$ ,  $p < 0.001$ ) düzeylerde azaldığı tespit edilmiştir. Progesteron gruplarındaki semen hacmi değerlerinin önemli ( $p < 0.05$ ,  $p < 0.01$ ) düzeyde azalmasına rağmen, testosteron gruplarına ait aynı değerlerin 48. saat hariç olmak üzere, kontrol gruplarına göre önemli ( $p < 0.01$ ) derecede arttığı gözlenmiştir. Bu çalışmadan elde edilen sonuçlar, semen hyaluronidaz aktiviteleri ile sperm parametreleri arasında önemli korelasyonların olmadığını göstermektedir. Sonuçta her iki hormonun ilaç olarak kullanımı, testosteron gruplarındaki semen numuneleri hariç hyaluronidaz aktiviteleri üzerine herhangi bir zararlı etki oluşturmamaktadır. Ancak progesteron ve testosteron hormonları, sperm konsantrasyon değerlerinin azalttığından dolayı, koçların döl verimi yeteneği üzerine zararlı etkilere neden olacaktır.

**Anahtar Sözcükler:** Hyaluronidaz, sperma, motilite, koyun

## Introduction

Hyaluronan, a glycosaminoglycan of the extracellular matrix, is important for cellular proliferation and differentiation and has a structural role in connective tissue (1,2). Hyaluronidase, a lysosomal endoglycosidase mediating hyaluronan turnover, is thought to be important in the defence against certain pathogens and some malignancies and in wound healing (3,4). In addition, this enzyme facilitates the penetration of sperm through the oocyte's vestments (5,6).

Progesterone is used for menstruation expression, birth delay and against abortion in animals (7, 8). Testosterone is used against mammary tumours, sexual reluctance and decreased sperm counts (9-11).

Hirayama et al. (12) reported that low hyaluronidase activity in the acrosome might cause a decrease in the fertilizing capability of sperm. The aim of this study was to determine the effects of progesterone and testosterone on hyaluronidase activities of serum and semen and on sperm characteristics in sheep. In addition, it was investigated whether different semen hyaluronidase activities cause harmful effects on sperm motility and concentration.

## Materials and Methods

### Materials

Progesterone (Eifelfango, Bad Neuenahr, Germany), testosterone decanoate (Sigma Aldrich Co.), N-acetylglucosamine (Sigma-Aldrich Co.), hyaluronic acid (Merck Co.), potassium tetraborate (Sigma Co.), dimethylaminobenzaldehyde (Sigma-Aldrich Co.) were used. The other chemicals were purchased from Sigma.

### Animals

In this study, 30 Akkaraman sheep (15 ewes and 15 rams), weighing  $60 \pm 3.2$  kg and approximately 2 years old were used. The sheep were fed on grass supplemented with alfalfa hay. Drinking water was provided *ad libitum*. The sheep were allowed 20-30 days for acclimatization before use. They were divided into six groups as follows:

**Control groups:** The first group contained 5 ewes and the second group 5 rams.

### Experiment groups:

**A) Progesterone groups:** The third group included 5 ewes and the fourth group 5 rams.

**B) Testosterone groups:** The fifth group contained 5 ewes and the last group 5 rams.

### Administration of progesterone and testosterone and collection of serum and semen samples

Blood and semen samples were obtained from all animals to analyse the hyaluronidase activities of the control and experiment groups before drug application. The ram ejaculates had a sperm concentration of  $2.40 \pm 0.03 \times 10^9$  spermatozoa (mean  $\pm$  SEM) and a volume of  $0.75 \pm 0.04$  ml. The mean ( $\pm$  SEM) of sperm motility was  $75 \pm 1.02\%$ . The control animals received 1 ml olive oil intramuscularly. Progesterone was injected intramuscularly at 0.5 mg/kg (in 1 ml olive oil) to each animal in the progesterone groups. Testosterone decanoate was administered intramuscularly at 5 mg/kg (in 1 ml olive oil) to each animal in the testosterone groups. Blood and semen samples were obtained at 1, 2, 4, 24, 48, 72 and 168 h. The blood samples were collected from the jugular vein and semen samples were taken using an artificial vagina. The sperm volume, motility, concentrations and abnormal spermatozoa rate of rams were determined according to the method described by Hafez (13).

### Hyaluronidase Assay

Hyaluronidase activity was measured using the methods described by Wilkinson et al. (14) and Joyce et al. (15). The N-acetylglucosamine (NAG) solutions (50, 100 and 200 mg/l in water) were used for a calibration curve. Semen samples were centrifuged at 600 g for 5 min to separate sperm cells and supernatant was used. The blood serum and semen samples of the control and experiment groups were diluted 1 in 5 with 0.15 mol/l sodium chloride before assay. One millilitre of diluted samples was added to 0.1 ml acetate buffer (0.3 mol/l containing 0.45 mol/l sodium chloride) and 0.1 ml hyaluronic acid substrate was added to these mixtures and then incubated for 24 h at 37 °C in an incubator. After the reaction mixtures were taken, 60  $\mu$ l potassium tetraborate (0.8 mol/l in water, pH 10) was added. The reaction was terminated by heating block for 5 min. Then the mixtures were cooled in an ice-water bath before adding 2 ml of dimethylaminobenzaldehyde (Stock DMAB reagent-10% w/v in 12.5% v/v concentrated hydrochloric acid in glacial acetic acid; Stock reagent diluted 1 in 10 with glacial acetic acid before use) and then incubated for 20 min at 37 °C in a water bath. The

reaction mixtures were centrifuged immediately at 1500 g for 10 min and the absorbance of the supernatant was read at 582 nm within 30 min using a spectrophotometer. Hyaluronidase activity was expressed as the mean  $\mu\text{mol NAG}/\text{min}/\text{l}$ .

### Statistical analyses

Data are presented as the mean  $\pm$  SEM. Chi-square analysis was used to determine differences in the sperm motilities between the control and treatment groups. The Mann-Whitney U test was used for comparisons between the control and experiment groups of the other parameters. The Spearman rank correlation test was used to establish the relationship between hyaluronidase activities and sperm characteristics. All analyses were carried out by the SPSS statistical program (Win 10.0).

### Results

The serum hyaluronidase activities of ewes and rams in both the progesterone and testosterone groups were determined to increase significantly ( $p < 0.01$ ,  $p < 0.001$ ) when compared with the control group, respectively (Tables 1 and 2).

It was shown that the semen hyaluronidase activity values of rams in the progesterone group increased significantly ( $p < 0.001$ ,  $p < 0.01$ ) when compared with the control group at the 1, 24, 48 and 168 h. However, the same values were observed to decrease rather significantly ( $p < 0.01$ ) at 4 h (Fig. 1).

There were significant ( $p < 0.01$ ) decreases in the semen hyaluronidase activities of the testosterone group when compared with control group at 1, 2, 48, 72 and 168 h. Additionally, the same levels were determined to increase most significantly ( $p < 0.05$ ) in comparison with the control group at 4 h (Fig. 1). There was no significant correlation between the hyaluronidase activity of serum and semen in either the progesterone or testosterone groups.

After the administration of progesterone and testosterone to rams, significant increases ( $p < 0.05$ ,  $p < 0.01$ ) were determined for sperm motility when compared with the control group, respectively (Table 3). In addition, the values of sperm concentrations and semen volumes in the progesterone groups decreased significantly ( $p < 0.01$ ,  $p < 0.001$ ) in comparison with the control group (Tables 4 and 5). There were no

Time (hour)	Control (n=5)	Progesterone groups (n=5)	Testosterone groups (n=5)
1	30.17 $\pm$ 0.47	57.68 $\pm$ 1.33**	80.27 $\pm$ 2.53***
2	29.24 $\pm$ 1.28	53.65 $\pm$ 0.98**	63.21 $\pm$ 0.71***
4	35.56 $\pm$ 1.04	55.37 $\pm$ 2.36**	70.97 $\pm$ 2.65**
24	34.19 $\pm$ 0.42	55.91 $\pm$ 1.08**	42.28 $\pm$ 0.75**
48	33.25 $\pm$ 1.54	70.23 $\pm$ 3.03**	84.29 $\pm$ 4.29**
72	30.12 $\pm$ 0.69	51.05 $\pm$ 0.56**	132.43 $\pm$ 1.09***
168	31.73 $\pm$ 0.35	65.91 $\pm$ 2.16**	66.32 $\pm$ 1.12***

Table 1. The means ( $\pm$  SEM) of serum hyaluronidase activity in ewes from the progesterone, testosterone and control groups ( $\mu\text{mol NAG}/\text{min}/\text{l}$ ).

Significantly different from control groups, \*\* $p < 0.01$ , \*\*\* $p < 0.001$

Time (hour)	Control (n=5)	Progesterone groups (n=5)	Testosterone groups (n=5)
1	71.70 $\pm$ 0.26	87.08 $\pm$ 1.03**	107.43 $\pm$ 2.80**
2	70.53 $\pm$ 1.06	96.43 $\pm$ 1.05**	94.70 $\pm$ 1.59**
4	70.41 $\pm$ 0.89	94.04 $\pm$ 0.36**	115.90 $\pm$ 1.88***
24	71.03 $\pm$ 1.58	101.75 $\pm$ 2.70**	121.78 $\pm$ 1.69***
48	72.47 $\pm$ 1.71	119.0 $\pm$ 1.29***	108.16 $\pm$ 1.99**
72	72.33 $\pm$ 1.03	88.13 $\pm$ 0.84**	129.43 $\pm$ 0.77***
168	70.68 $\pm$ 1.23	86.34 $\pm$ 1.32**	88.03 $\pm$ 0.84**

Table 2. The means ( $\pm$  SEM) of serum hyaluronidase activity in rams from the progesterone, testosterone and control groups ( $\mu\text{mol NAG}/\text{min}/\text{l}$ ).

Significantly different from control groups, \*\* $p < 0.01$ , \*\*\* $p < 0.001$

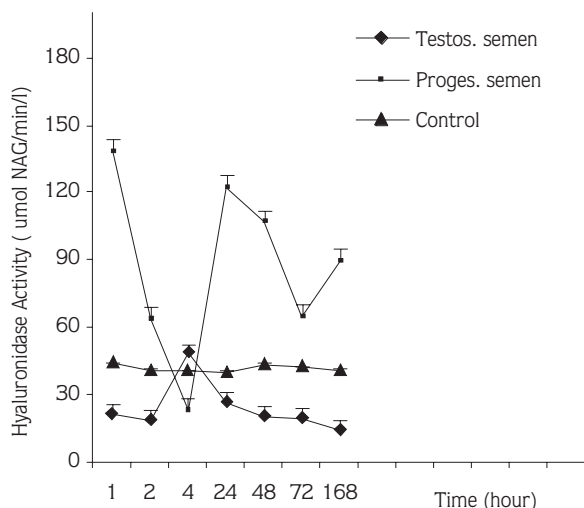


Fig. 1. Semen hyaluronidase activities (mean ± SEM) after administration of progesterone and testosterone (n = 15). The significant differences (p < 0.001, p < 0.01) were detected between the control and experiment groups.

correlations between semen hyaluronidase activities and sperm characteristics.

Time (hour) groups	Sperm motility of control groups (%)	Sperm motility of progesterone groups (%)	Sperm motility of testosterone groups (%)
1	75 ± 2.04	80 ± 3.25*	90 ± 1.45**
2	75 ± 1.25	80 ± 3.11*	85 ± 2.21**
4	80 ± 0.87	85 ± 1.16*	90 ± 1.46**
24	75 ± 1.06	80 ± 1.56*	85 ± 1.01**
48	75 ± 1.54	80 ± 1.03*	85 ± 1.24**
72	75 ± 1.64	85 ± 3.51*	85 ± 0.81**
168	75 ± 1.58	80 ± 2.13*	90 ± 1.08**

Significantly different from control groups, \*p < 0.05, \*\*p < 0.01

Time hour	Sperm concentration of control groups (x10 <sup>9</sup> )	Sperm concentration of progesterone groups (x10 <sup>9</sup> )	Sperm concentration of testosterone groups (x10 <sup>9</sup> )
1	2.45 ± 0.04	1.95 ± 0.03**	2.26 ± 0.05**
2	2.35 ± 0.05	1.87 ± 0.04**	2.10 ± 0.03**
4	2.06 ± 0.07	1.55 ± 0.05**	1.55 ± 0.06**
24	2.15 ± 0.06	0.80 ± 0.02***	1.65 ± 0.08**
48	1.85 ± 0.04	0.85 ± 0.03***	1.55 ± 0.06**
72	2.05 ± 0.04	1.14 ± 0.02***	1.60 ± 0.02**
168	1.85 ± 0.08	0.70 ± 0.06***	1.40 ± 0.08**

Significantly different from control groups, \*\*p < 0.01, \*\*\*p < 0.001

Sperm concentrations were determined to decrease significantly (p < 0.01, p < 0.001) in the progesterone and testosterone groups (Table 4). Although semen volume values decreased significantly (p < 0.05, p < 0.01) in the progesterone group, the same values were observed to increase significantly (p < 0.01) in the testosterone group except at 48 h compared with the control groups (Table 5). The rates of morphological abnormality did not show significant differences when compared with the control group. The percentages of abnormal spermatozoa were between 4.12% and 5.56% in the progesterone group and between 3.45% and 5.02% in the testosterone group.

### Discussion

Progesterone and testosterone are used as drugs in animals. Testosterone is especially used for the prevention of sexual reluctance (10,11). Semen hyaluronidase activity is an index of fertilization ability and there has been no investigation into the effects of

Table 3. The means (± SEM) of sperm motility in the progesterone, testosterone and control groups. (n=15)

Table 4. The means (± SEM) of sperm concentration in the progesterone, testosterone and control groups. (n=15)

Time hour	Semen volume of control groups (ml)	Semen volume of progesterone groups (ml)	Semen volume of testosterone groups (ml)
1	0.75 ± 0.02	0.60 ± 0.02*	0.90 ± 0.03**
2	0.75 ± 0.06	0.55 ± 0.05*	0.86 ± 0.01**
4	0.68 ± 0.11	0.50 ± 0.02*	0.96 ± 0.04**
24	0.70 ± 0.05	0.40 ± 0.04**	0.96 ± 0.06**
48	0.65 ± 0.05	0.50 ± 0.05*	0.65 ± 0.02
72	0.70 ± 0.03	0.40 ± 0.02**	0.92 ± 0.04**
168	0.72 ± 0.02	0.40 ± 0.03**	0.90 ± 0.03**

Table 5. The means ( $\pm$  SEM) of semen volume in the progesterone, testosterone and control groups. (n=15)

Significantly different from control groups, \*p < 0.05, \*\*p < 0.01

progesterone and testosterone on sperm characteristics and hyaluronidase activities. For these reasons, in this study the effects of both hormones on the fertilization ability of rams were investigated. To achieve this, the rams were divided into three groups. Progesterone and testosterone were injected into the rams and semen samples were obtained at different times. The results of each group were discussed.

The hyaluronidase enzyme is one of the acrosomal enzymes released from the sperm head during the fertilization process (15). In this study, it was clearly demonstrated that in the testosterone group, though the semen hyaluronidase activities decreased at 1, 2, 24, 48, 72 and 168 h, sperm motility and semen volume values were observed to increase rather significantly ( $p < 0.01$ ) when compared with the control groups over the same times. Additionally, the semen hyaluronidase activities of the progesterone group decreased at 4 h; however, the percentages of sperm motility and the values of semen volume were determined to increase at the same time. Furthermore, no significant correlation was found between hyaluronidase activities and sperm characteristics. These results showed that decrease in semen hyaluronidase activity did not cause deleterious effects in spermatozoa motility or semen volume.

Large doses of progesterone and testosterone inhibit the release of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and gonadotrophin-releasing hormone (GnRH) whereas low concentrations permit their release (11). LH stimulates the release of testosterone from Leydig cells. The testosterone hormone is necessary for certain steps in spermatocytogenesis as it acts through cells in the

seminiferous tubules to stimulate spermatogenesis. The findings of this study revealed that treatments with progesterone and testosterone of rams cause significant ( $p < 0.01$ ,  $p < 0.001$ ) decreases in sperm concentrations. These decreases may indicate that both hormones decrease sperm production depending on the decreased testosterone production of Leydig cells.

Hyaluronan is a large component of the extracellular matrix (1). The turnover of hyaluronan requires endoglycosidic breakdown by lysosomal hyaluronidase and a deficiency of hyaluronidase has been thought to be incompatible with life (3). The findings of this study indicated that progesterone and testosterone significantly increased ( $p < 0.001$ ) the serum hyaluronidase activities of sheep. The elevation of serum hyaluronidase activities in the progesterone and testosterone groups may explain why these hormones decrease the stability of the lysosomal membranes and cause the release of hyaluronidase into serum. It was reported that serum hyaluronidase activity showed a rise at the beginning of gestation and then fell to a minimum by the last week of pregnancy in rats. Additionally, the activity of the enzyme increased after birth and was close to normal 21 days post-partum (16). It is known that the levels of progesterone increase during the gestation period in mammalian serum. The findings of this study indicate that the use of progesterone causes an increase in hyaluronidase activity in serum. Therefore, it can be suggested that the rise of hyaluronidase activity in pregnant animals is due to the increase in progesterone in blood serum.

A variety of methods based on measuring changes in the viscosity (17) and turbidity (18) of solutions of

hyaluronic acid have been described for the determination of hyaluronidase activity in serum and semen. The colorimetric method described by Wilkinson et al. (14) was used in this study. This method is based on the release of saccharides with N-actylglucosamine as end groups from hyaluronic acid. The method was preferred in this investigation because it is simpler, cheaper and not time consuming compared with the above methods.

The results of this study showed that although there were significant ( $p < 0.01$ ) increases in the values of semen volume in the testosterone group, the same values diminished in the progesterone group. For this reason, it can be suggested that increased semen volume depends on the effect of testosterone. These findings were supported by McDonald and Capen (19), who stated that the testosterone hormone promotes the growth, development and secretory activity of the accessory sex organs of the male. Additionally, in this study, there were highly significant ( $p < 0.001$ ) decreases in the sperm concentration levels of rams in the testosterone group when compared with the control group. Decreases in

sperm concentration values depend on the elevation of semen volumes of the same samples.

These findings indicate that female and male sex hormones have significant effects on the hyaluronidase activities of serum and semen and on sperm characteristics in sheep. There was no relationship between semen hyaluronidase activities and sperm characteristics. The use of these sex hormones as a drug increased the values of sperm motility in both progesterone and testosterone groups. In addition, progesterone decreased the sperm volume and concentration values. Furthermore, while testosterone increased semen volume, it caused a decrease in sperm concentration. In conclusion, progesterone and testosterone hormones did not have any deleterious effects on hyaluronidase activities but for the semen hyaluronidase activities of the testosterone group and these hormones have harmful effects on sperm concentration values. Further detailed studies are required on how the decrease in semen hyaluronidase activity interacts with fertilization ability.

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