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Research Article

An Investigation of the Effects of Ivermectin on Blood Serum, Semen Hyaluronidase Activities and Spermatological Characteristics in Sheep*

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Abstract: This study was carried out to investigate the effects of ivermectin on the hyaluronidase activities of blood serum and semen and on the sperm characteristics in sheep. In this investigation 18 sheep, at the age of 2 years and weighing between 50-60kg were used. After the administration of ivermectin subcutanously at a dose of 0.2 mg/kg, semen and blood samples were taken at different times. The hyaluronidase activities of blood serum and semen samples in sheep were determined to increase significantly ($p<0.001$) when compared with control groups from the first hour to the 120^{th} hour. Furthermore, there was a significant difference ($p<0.05$) between the hyaluronidase activities of serum and semen in sheep. After the injection of ivermectin subcutanously at a dose of 0.2 mg/kg, the values of sperm concentration were demonstrated to decrease highly significantly ($p<0.001$) in comparison with the control group. Although the semen volume levels of rams increased significantly ($p<0.01$) at the first, on the 48^{th} and 72^{nd} hours, the same levels were observed to decline significantly ($p<0.01$) when compared with control groups at 24, 96, 120 and 168 hours. In addition, the rates of sperm motility were established to diminish significantly ($p<0.01$) in comparison with the control group at all times except the first hour. No differences were observed of abnormal spermatozoa rates when compared with the control group. Furthermore, there was no correlation between hyaluronidase activities of serum or semen with sperm characteristics. These findings indicate that ivermectin increases hyaluronidase activity of serum and semen in sheep, but it decreases sperm motility and concentrations. In conclusion, the use of ivermectin is not suitable during ramming season and in rams used for breeding due to the deleterious effects on fertility.

Key Words: Ivermectin, Hyaluronidase, Sheep, Sperm

Koyunların Kan Serumu ve Sperma Hyaluronidaz Aktiviteleri ile Sperm Parametreleri Üzerine İvermektinin Etkilerinin Araştırılması

Özet: Bu çalışma, ivermektinin koyunlardaki kan serumu ve sperma sıvısı hyaluronidaz aktiviteleri ile sperm paremetreleri üzerine olan etkilerini araştırmak amacıyla yapıldı. Çalışmada 2 yaşında ve ağırlıkları 50-60 kg arasında olan 18 koyun kullanıldı. İvermektinin 0.2 mg/kg dozunda deri altı yolla uygulanmasından sonra değişik zamanlarda alınan kan serumu ve sperma örneklerine ait hyaluronidaz aktivitelerinin kontrol gruplarına göre 1. saatten 120. saate kadar çok önemli ($p<0.001$) oranda yükseldiği belirlendi. Ayrıca serum ve sperma hyaluronidaz aktiviteleri arasında önemli ($p<0.05$) bir farklılık bulunduğu tespit edildi. 0.2 mg/kg dozunda ivermektinin deri altı yolla enjeksiyonundan sonra alınan sperma örneklerine ait sperma yoğunluğu değerlerinin kontrol gruplarına göre oldukça önemli ($p<0.001$) oranda azaldığı belirlenmiştir. Koçların sperma hacmi değerlerinin ise kontrol grupları ile karşılaştırıldığında 1, 48 ve 72. saatlerde önemli ($p<0.01$) oranda yükselmesine rağmen, aynı değerlerin 24, 96, 120 ve 168. saatlerde yine önemli ($p<0.01$) oranda azaldığı gözlenmiştir. Bunlara ek olarak, sperm motilitesi oranlarının 1. saat hariç, tüm zamanlarda kontrol gruplarına gore önemli ($p<0.01$) oranda azaldığı saptanmıştır. Anormal spermatozoit oranlarının ise kontrol grubuna göre değişmediği gözlendi. Bunların dışında, serum ve sperma hyaluronidaz aktiviteleri arasında herhangi bir korelasyon bulunmamaktadır. Bu bulgular, ivermektinin koyunlarda serum ve sperma hyaluronidaz aktivitelerini artırdığını, sperm motilitesi ve yoğunluklarını ise azalttığını göstermektedir. Sonuç olarak , ivermektinin koç katımı sezonunda erkek hayvanlarda ve damızlık koçlarda kullanımı, fertilite üzerine zararlı etkileri bulunduğundan dolayı uygun değildir.

Anahtar Sözcükler: İvermektin, hyaluronidaz, koyun, sperm

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Introduction

Ivermectin is a drug that has been used against nematodes and ectoparasites (1,2). Ivermectin can diffuse to all tissue compartments except the central nervous system after being taken orally or in other ways (3).

Hyaluronidase has been found in a wide variety of mammalian tissues (4) lysosomes, blood serum, synovial fluids and semen but the origin of the serum hyaluronidase is not known (5). The acrosomal enzymes existing at the head of sperm facilitate the penetration into ovum. It was reported that there was a significant correlation between semen hyaluronidase activity and sperm concentration in male infertility (6,7).

Hyaluronidase can cleave the follicle cell layer of oocytes and the cumulus oophorus of animals (8). The low acrosomal enzyme activity causes a decrease in the fertilizing capability of sperm. Furthermore, while the hyaluronidase activity increased, the sperm concentration was reported to increase (9). This study was carried out to establish the effects of ivermectin on hyaluronidase activities of serum and semen and on sperm motility, concentration, volume and morphological abnormality of spermatozoa in sheep.

Materials and Methods

Animals

During this investigation, 18 Akkaraman sheep (9 ewes and 9 rams), aged between 2 and 3 years and weighing 50-60kg were used. The sheep were fed on grass supplemented with Lucerne hay and drinking water was provided ad libitum. The sheep were allowed 20-30 days for acclimatization before use. The sheep were divided into 4 groups; two groups were used as control; the first group contained 3 rams and the second included 3 ewes. The other groups were called ivermectin groups; the third consisted of 6 rams and the fourth contained 6 ewes.

Administration of ivermectin and the collection of blood and semen samples

Ivermectin (Devormec, Sanofi, Hungary) at a dose of 0.2mg/kg was given subcutaneously to 6 ewes and 6 rams. Blood and semen samples were taken from all animals to analyse the hyaluronidase activities of the control groups and other group animals just before ivermectin administration. After ivermectin was given subcutanously to rams (n=6) and ewes (n=6), blood and semen samples were taken from control and ivermectin groups after 1, 2, 4, 24, 48, 72, 96, 120, 144, 168, 216 and 264 hours. The blood samples were collected by jugular veinpuncture into 10ml vacutainer tubes and semen samples were collected by artificial vagina. The blood samples were stored at +4 °C until separation of serum for 2 hours.

Reagents

Azo acetylglucosamine (NAG) (Sigma chem.) standards for calibration: NAG is weighted at the doses of 50, 100 and 200 mg and dissolved in 1 l water. This reagent was used to prepare the calibration curve.

Acetate buffer: 0.3 mol sodium acetate (Sigma chem.) was dissolved in 1 l of 0.45mol/L sodium chloride (in water).

Hyaluronic acid (Sigma Chem.) substrate: 4mg hyaluronic acid was dissolved in 1 l water.

Potassium tetraborate solution (Merck co.): 0.8 mol potassium tetraborate was dissolved in 1 L. water

Stock Dimthylaminobenzaldehyde (DMAB) (Sigma chem.) reagent: 10g DMAB was dissolved in 12.5% V/V concentrated hydrochloric acid in glacial acetic acid. Stock reagent was diluted 1: 10 with glacial acetic acid before use

The determination of hyaluronidase activity

Hyaluronidase activity was measured using the methods described by Wilkinson et al., (10) and Joyce et al. (11). The blood serum and semen samples were diluted 1 : 5 with 0.15 mol/L sodium chloride before assay. 1ml of serum and semen samples were added to 0.1ml acetate buffer and 0.1ml hyaluronic acid substrate was added to these mixtures and then incubated for 24h at 37ºC in a thermostatically controlled room. The tubes were centrifuged at 500g for 5 min to remove spermatozoa and the supernatant was used for the experiment. 60µl potassium tetraborate solution was added to these mixtures and the reaction was terminated by heating at 100ºC in a heating block for 5min. The reaction mixtures were cooled in an ice-water bath and 2ml DMAB was added and then incubated for 20min at 37ºC in a water bath. The reaction mixtures were centrifuged immediately at 1500g for 10min. Then the supernatants of blood serum and semen samples were taken and measured at 582nm within 30min spectrophotometrically (11). Hyaluronidase activity was expressed as the mean µmol NAG/min/l.

The sperm volume, motility, concentrations and abnormal sperm rate of rams were performed according to the method described by Hafez (12).

Statistical analyses

The mean and standard error of mean (SEM) values with the differences between the control and time groups were identified by independent Student's t-test. The correlation was made to examine the relationship between blood serum and semen hyaluronidase activities with sperm characteristics. The statistical significance of differences between the blood hyaluronidase activities of ewes and rams, and semen hyaluronidase activity was calculated using one way analysis of variance. All statistical analyses were carried out using the SPSS software (Win 6.0).

Results

The hyaluronidase activities of serum and semen

The hyaluronidase activities of the control and ivermectine groups with the differences between ewe serum, ram serum and semen are shown in Table 1. There was a significant difference ($p<0.05$) between the hyaluronidase activities of serum and semen in sheep at all times except the 216th hour (Table 1). The serum hyaluronidase activities of rams were determined to increase significantly ($p<0.001$) when compared with the control group between the first and 144th hours but these levels were estimated to decrease rather significantly ($p<0.001$) at the 264th hour. Furthermore, the serum hyaluronidase activities of ewes were demonstrated to increase highly significantly ($p<0.001$) in comparison with the control group between the first and the 216th hours and a significant ($p<0.05$) decrease was observed at the 264th hour. In addition, the semen hyaluronidase activities increased highly significantly ($p<0.001$) when compared with the control group between the first and the 120th hour, but the same values were observed to decrease highly significantly ($p<0.01$) at the 144th and 168th hours (Table 1).

The values of sperm concentration, volume, motility and morphological abnormality

The values of semen concentration were established to decrease highly significantly ($p<0.001$) when compared with the control group (Table 2). Furthermore, the levels of semen volume were determined to increase significantly ($p<0.01$) in comparison with the control group at the 1th and 72nd hours but a significant ($p<0.01$) decline was observed in semen volumes at the 24th, 72nd, 96th and 120th hours (Table 2). The sperm motility values

Table 1. The hyaluronidase activities of blood serum and semen in sheep, after the administration of ivermectin at a dose of 0.2mg/kg (s.c). The hyaluronidase activity was expressed as the mean (±SEM) µmol NAG/min/L.

Time (hour)	Serum Hyaluronidase activities of rams, (n=6)	Serum Hyaluronidase activities of ewes (n=6)	Semen Hyaluronidase activities, (n=6)
Control	^{a}62.26 ± 4.48	^{b}48.64 ± 2.73	^{c}44.16 ± 3.83
1	^{a}104.70 ± 4.29***	^{b}139.30 ± 6.20***	^{c}68.78 ± 1.17***
2	^{a}101.61 ± 6.36***	^{b}173.34 ± 2.29***	^{c}68.12 ± 1.23***
4	^{a}169.25 ± 2.3***	^{a}170.68 ± 7.21***	^{b}68.54 ± 1.88***
24	^{a}161.47 ± 4.11***	^{b}180.70 ± 4.84***	^{c}74.66 ± 3.95***
48	^{a}99.04 ± 2.13***	^{a}97.83 ± 3.40***	^{b}85.31 ± 1.31***
72	^{a}117.23 ± 6.81***	^{b}154.87 ± 3.93***	^{c}56.42 ± 1.48**
96	^{a}175.04 ± 2.33***	^{a}175.61 ± 4.99***	^{b}81.91 ± 0.66***
120	^{a}145.40 ± 2.94***	^{a}129.85 ± 4.21***	^{b}72.88 ± 3.81***
144	^{a}158.58 ± 1.04***	^{b}75.49 ± 5.19***	^{c}28.31 ± 1.57**
168	^{a}78.17 ± 2.71**	^{a}73.90 ± 2.46***	^{b}23.92 ± 0.88**
216	^{a}80.17 ± 2.55**	^{b}137.84 ± 2.79***	84.77 ± 1.30***
264	^{a}43.36 ± 1.21***	^{a}57.52 ± 7.01*	^{b}102.82 ± 3.53***

^{a,b,c} Different letters within a line showed significant ($p<0.05$) differences between serum and semen hyaluronidase activities

Significantly different from control groups, *$p<0.05$, **$p<0.01$, ***$p<0.001$

Table 2. The values of semen volume and concentration in rams, after the aplication of ivermectin at a dose of 0.2mg/kg, s.c. (n=6). The results are expressed as average values (± SEM) of semen concentration and volume.

Time (hour)	Sperm concentration $x10^9$	Semen volume (ml)
Control	2.42 ± 0.05	0.65 ± 0.03
1	1.78 ± 0.05***	0.90 ± 0.02**
24	1.68 ± 0.02***	0.50 ± 0.02**
48	1.10 ± 0.03***	0.71 ± 0.09
72	1.81 ± 0.04***	0.88 ± 0.04**
96	1.11 ± 0.09***	0.48 ± 0.03**
120	1.42 ± 0.04***	0.50 ± 0.02**
144	1.47 ± 0.08***	0.66 ± 0.08
168	1.47 ± 0.05***	0.33 ± 0.03**
216	1.09 ± 0.07***	0.60 ± 0.03
264	0.84 ± 0.09***	0.66 ± 0.09

Significantly different from control groups, *p<0.05, **p<0.01, ***p<0.001

were established to decrease at significant (p<0.01) rates when compared with the control group at all times except the first hour (Figure). The rates of morphological abnormality did not show significant differences when compared with the control group (Figure). The correlation test was calculated between serum and semen hyaluronidase activities with sperm motility, volume, concentration and abnormal spermatozoid rates. The results indicate that there is no significant relationship (p>0.05) between these parameters. Furthermore, one way analyses of variance indicated that there was no significant difference between sperm characteristics.

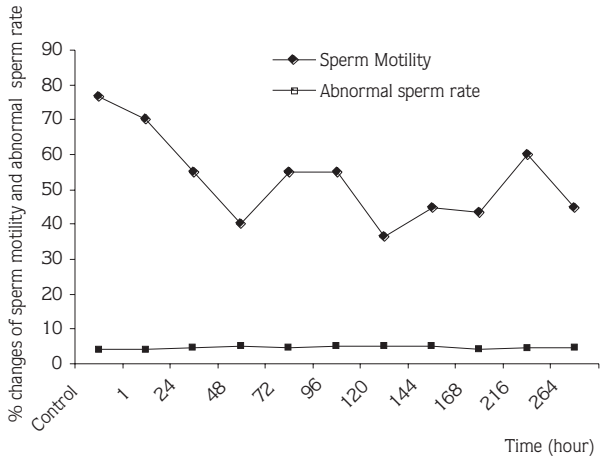

Figure. Effect of ivermectin at a dose of 0.2 mg/kg (s.c) on means (±SEM) of sperm motility and morphological abnormality values in rams (n=6).

In this study, all values of the control groups were determined according to time. But these values did not change according to time. Therefore, we used the average (mean±SEM) values of the control groups.

Discussion

Hyaluronidase is one of the lysosomal enzymes and this enzyme has an important role during the penetration of sperm into oocyte (13). Ivermectin is a drug used for nematodes and the half-life is 5-6 days (14). In this study, ivermectin was determined to increase the hyaluronidase activities of serum and semen highly significantly (p<0.001) in sheep. The elevation of hyaluronidase activities in serum can be explained by the fact that ivermectin increases the release of hyaluronidase enzyme from lysosomes in blood.

Cholesterol is secreted in the seminal plasma by the prostate to protect spermatozoa against environmental shock (15). While the sperm is in the male reproductive tract, cholesterol accumulates on the acrosomal membrane to protect the efflux of acrosomal contents from the plasma membrane to the seminal plasma. After the removal of spermatozoa from the male reproductive tract, cholesterol separates from the acrosomal membrane and the acrosomal content is released into the seminal plasma (8). In this study, semen hyaluronidase activities were established to increase highly significantly (p<0.001) when compared with control groups at all times except the 144^{th} and 168th hours. The rise in semen hyaluronidase activity can be manifested by the diffusion of cholesterol from the acrosomal membrane to the seminal plasma and then by the release of hyaluronidase enzyme into the seminal plasma.

Some investigators commented that females had a slightly higher mean hyaluronidase activity in blood serum than males and serum hyaluronidase activity is unlikely to originate from testicular tissue in humans and no significant differences were found (p>0.05) between hyaluronidase activity in females and males (5). In this study, a significant difference (p<0.001) was established between serum hyaluronidase activities in ewes and rams

in the control groups. Our findings showed that the mean serum hyaluronidase activity of rams (62.26±4.48) was significantly ($p<0.05$) higher than the mean hyaluronidase activity in ewes (48.64±2.73). As a result, it can be suggested that hyaluronidase enzyme in ram serum probably originates from lysosomes and testicular tissue in sheep.

It was reported that ivermectin had no harmful effects on the reproductive potential of rams. This was observed after the oral administration of ivermectin at dose of 400mg/kg to 10 merino rams (14). In this study, the values of sperm motility and concentration were observed to decrease significantly ($p<0.01$) when compared with the control group. There was discrepancy between our results and other investigator's findings. The cause of this discrepancy can be explained by the use of different breeds of sheep and by the application of ivermectin at the dose of 0.2mg/kg subcutaneously in this study.

The existence of a significant correlation was reported between acrosomal hyaluronidase activities and sperm concentration in male infertility (9). In this study, a significant correlation was not observed between semen hyaluronidase activities and sperm characteristics. The cause of this difference may be due to the measurement of hyaluronidase activity of semen samples but not sperm in this study. The elevation of semen hyaluronidase activity does not show the degree of fertility in rams, because semen hyaluronidase activity does not affect sperm characteristics directly. In conclusion, the use of ivermectin is not advisable, due to the reduction of sperm motility and concentration, during ramming season and in rams used for breeding.

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