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Effects of levamisole on hyaluronidase activity and sperm characteristics in rams

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Abstract

The aim of this study was to determine the effects of levamisole on sperm characteristics and hyaluronidase activity of blood serum and semen. For this purpose, 12 Akkaraman rams (2–3 years old) were used. Levamisole hydrochloride was administered orally at a dose of 7.5 mg/kg body weights once daily for 2 days. Serum and semen samples were collected from the rams at post-treatment 1, 2, 4, 24, 48, 72, 96, 120, 144, 216, 288 and 384 h and examined for sperm characteristics and hyaluronidase activity. The results showed that the use of levamisole caused significant ($P < 0.01$) increase in serum hyaluronidase activity at all times except the 72 h, and in semen hyaluronidase activity at 1, 2, 4, 24, 72, 96 and 120 h compared to the control group. In addition, the levamisole caused significant ($P < 0.05$) decreases in semen volume, sperm motility, concentration and total sperm number at all times. There was no correlation between semen hyaluronidase activity and the sperm characteristics. In conclusion, levamisole did not have any deleterious effect on hyaluronidase enzyme. However, the use of this drug in rams during the breeding season is harmful due to the decrease of sperm characteristics.

Keywords: Levamisole; Hyaluronidase; Sperm; Rams

1. Introduction

Levamisole is highly acceptable anti-nematodal drug because of its broad range of activity in a large number of hosts (sheep, cattle, pig, horse, chicken, dog) [1]. It is known

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that this drug stimulates cells mediated immune reactivity by potentiating the rate of T-lymphocyte differentiation and proliferation of macrophages [2]. Furthermore, levamisole is used to treat the chronic infection, inflammatory disease and some malignancies [3]. It has been reported that alkaline phosphatase inhibitor levamisole decreases the production of dihydrotestosterone in fibroblasts [4–6].

Hyaluronic acid is a component of extracellular matrix that holds the follicular cells together and degraded by hyaluronidase enzyme released from sperm head during acrosomal reaction [7–9]. It is also synthesized in the cellular plasma membrane of leukocytes, neutrophiles and fibroblasts and rapidly degraded in the lysosomes by hyaluronidase to lactate and acetate [10]. It was reported that hyaluronic acid levels in serum are elevated during infections and this elevation may be a clinically useful marker of the increased inflammatory activity [11]. The deficiency in hyaluronidase activity of serum causes the elevation of hyaluronic acid in serum 38–90 folds over normal and it can be fatal [12].

Semen hyaluronidase activity could be an index of fertilization ability [13] and there is no published report on the effects of levamisole on sperm characteristics and hyaluronidase activity of serum and semen. Levamisole are commonly in veterinary practice in Turkey. The objective of this study was to evaluate the effects of levamisole on sperm characteristics and hyaluronidase activity of serum and semen in rams, and to determine whether there is a relationship between semen hyaluronidase activity and sperm characteristics.

2. Materials and methods

2.1. Chemicals and drugs

Levamisole hydrochloride (Nilverm Fort poudre, Sanofi, İstanbul) was used. The other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals, drugs administration and sample collection

Twelve Akkaraman rams (2–3 years old) were used in the present study. The rams were fed on grass supplemented with lucerne hay, and drinking water was provided adlibitum. The rams were randomly divided into two groups. These groups were assigned as a control ($n = 4$) and treatment ($n = 8$). Serum and semen hyaluronidase activity and sperm characteristics of all rams in each group were determined prior to drug injection. Levamisole hydrochloride was dissolved in 20 ml distilled water and administered orally at a dose of 7.5 mg/kg body weight, in morning once daily for 2 days in breeding season. This oral dose is recommended against nematodes in sheep. Twenty milliliters of distilled water was administered orally to the control rams.

After the last drug administration blood samples were collected from jugular vein and semen samples were taken by using artificial vagina from all rams at 1, 2, 4, 24, 48, 72, 96, 120, 144, 216, 288 and 384 h. The mean residence time of levamisole in sheep has been found to be about 216 h [1].

2.3. Assays

Semen volume was determined by direct reading the graduations of collection tubes (from 0.1 to 10 ml). Sperm concentration was determined with a heamocytometer. Semen samples were decimally diluted with isotonic sodium citrate solution at 37 °C (3%, w/v, dissolved in distilled water) at the rate of 1:10. A slide was placed on phase contrast microscope and allowed to warm up to 37 °C, and then a small droplet of diluted semen was placed on the slide and percent motility was evaluated visually at a magnification of 400×. Motility estimations were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score [14].

Hyaluronidase activity of fresh whole semen was measured using the methods described by Tanyıldızı and Bozkurt [15] and Joyce et al. [16]. The serum and semen samples were diluted 1:5 with 0.15 mol/l sodium chloride before assay. One milliliter of diluted samples was added to 0.1 ml acetate buffer (0.3 mol/l, contained 0.45 mol/l sodium chloride) and 0.1 ml hyaluronic acid substrate (4 mg hyaluronic acid was dissolved in 1 l water) was added to these mixtures and then incubated for 24 h at 37 °C in a temperature controlled room. After the reaction mixtures were taken, 60 μl potassium tetraborate (0.8 mol/l in water, pH = 10) was added, and reaction was terminated by heating block for 5 min. Then, the mixtures were cooled in an ice-water bath before adding 2 ml of *p*-dimethylamino-benzaldehyde (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 read at 582 nm within 30 min using a spectrophotometer. *N*-Acetylglucosamine was used as a standard and was reacted with *p*-dimethylaminobenzaldehyde as describe above.

2.4. Statistical analysis

The data are presented as the mean ± S.E.M. Chi-square analysis was used to determine differences in the sperm motilities between control and treatment groups. Non-parametric Mann–Whitney *U* test was applied to determine statistically significant differences between control and treatment groups. To determine the differences between time points post hoc comparisons were made with Duncan's multiple comparison test. Spearman rank correlation test was used to determine the relationship between the hyaluronidase activity of semen and sperm characteristics. All analyses were carried out by SPSS statistical program (Win 6.0).

3. Results

The differences in sperm characteristics and hyaluronidase activity of serum and semen before the administration of levamisole between animals in control and treatment groups were not significant ($P > 0.05$) (Table 1).

The differences in serum hyaluronidase activity between control and treatment groups were shown in Table 1. Levamisole increased significantly ($P < 0.01$) the serum

Table 1
Hyaluronidase activities of serum and semen in rams treated with levamisole

Time (h)	Groups			
	Serum control ($n = 4$)	Serum treatment ($n = 8$)	Semen control ($n = 4$)	Semen treatment ($n = 8$)
Pre-treatment values	59.0 ± 4.2	51.6 ± 3.8	21.1 ± 3.5	26.2 ± 2.5
1	68.7 ± 3.2^{A}	$144.8 \pm 4.7^{a,B}$	20.2 ± 1.4^{A}	$48.2 \pm 2.7^{a,B}$
2	65.2 ± 2.8^{A}	$169.4 \pm 5.3^{b,B}$	21.4 ± 1.3^{A}	$50.1 \pm 3.5^{a,B}$
4	67.1 ± 2.1^{A}	$170.5 \pm 4.3^{b,B}$	23.8 ± 1.9^{A}	$55.7 \pm 3.2^{a,B}$
24	71.0 ± 2.5^{A}	$176.2 \pm 5.6^{b,B}$	19.4 ± 1.8^{A}	$60.1 \pm 3.9^{b,B}$
48	65.2 ± 2.3^{A}	$149.4 \pm 4.9^{A,b}$	20.8 ± 1.9	25.1 ± 1.1^{c}
72	66.1 ± 2.4^{A}	$38.8 \pm 2.1^{d,B}$	23.8 ± 2.2^{A}	$51.5 \pm 2.7^{a,B}$
96	68.3 ± 2.8^{A}	$138.1 \pm 5.5^{a,B}$	22.4 ± 1.2^{A}	$67.3 \pm 3.2^{b,B}$
120	63.0 ± 2.5^{A}	$122.2 \pm 4.3^{a,B}$	20.3 ± 1.1^{A}	$50.1 \pm 2.9^{a,B}$
144	69.1 ± 2.1^{A}	$135.1 \pm 3.6^{a,B}$	21.1 ± 1.5	27.5 ± 1.2^{c}
216	70.2 ± 2.9^{A}	$102.2 \pm 3.4^{c,B}$	22.2 ± 1.8	24.9 ± 1.9^{c}
288	68.1 ± 2.3^{A}	$109.6 \pm 3.3^{c,B}$	21.1 ± 1.5	26.6 ± 1.4^{c}
384	67.3 ± 2.3^{A}	$107.3 \pm 3.6^{c,B}$	22.2 ± 1.9	25.1 ± 1.5^{c}
Mean	67.4 ± 0.6^{A}	$130.3 \pm 11.0^{a,B}$	21.6 ± 0.4^{A}	$42.7 \pm 4.5^{a,B}$

Values with different lower case (a, b, c and d) in the same column differ significantly ($P < 0.05$).
Different upper case letters (A and B) within same line showed significant ($P < 0.01$) differences control and treatment groups.
The activity was expressed as the mean (±S.E.M.) μmol NAG/min/l.

hyaluronidase activity at all times except the 72 h (Table 1). A significant ($P < 0.01$) decrease was observed at 72 h. The differences in semen hyaluronidase activity between control and treatment groups were shown in Table 1. The administration of levamisole caused significant ($P < 0.01$) increases in the semen hyaluronidase activity at 1, 2, 4, 24, 72, 96 and 120 h (Table 1). However, levamisole therapy caused significant ($P < 0.05$) decreases in semen volume, sperm motility, sperm concentration and total sperm number (Table 2) at all times when compared with the control group. There was no correlation between semen hyaluronidase activity and sperm characteristics.

4. Discussion

Levamisole is effective against nematodes of the lungs and gastro-intestinal tract. It was reported that testosterone production of leydig cells is inhibited by levamisole [17]. Testosterone is required physiologically for the completion of meiosis and spermiogenesis in testes [18]. Additionally, fluid secretion by the sertoli cells also commences into the tubular lumen and is regulated by testosterone [19,20]. The findings of this study revealed that treatment of levamisole caused significant ($P < 0.01$) decreases in the sperm concentration and semen volume in rams. These decreases may be occurs from the effect of this drug to epididymal contractile elements and accessory glands.

Kavanagh et al. [21] reported that levamisole inhibited sperm motility due to it is a potent inhibitor of seminal diamino oxidase. The results of this study showed that treatment

Table 2
Semen volumes (ml), sperm concentrations ($\times 10^9$/ml) sperm motilities (%) and total sperm numbers ($\times 10^9$) of rams treated with levamisole

Sperm parameters	Pre-treatment Values	Hours post-treatment									
		24	48	72	96	120	144	216	288	384	Mean
Semen volume (ml)											
Control ($n = 4$)	0.80 ± 0.03	$0.92^{a,A} \pm 0.04$	$0.88^{a,b,A} \pm 0.05$	$0.93^{a,A} \pm 0.04$	$0.81^{b,A} \pm 0.08$	$0.83^{b,A} \pm 0.05$	$0.90^{a,A} \pm 0.07$	$0.82^{b,A} \pm 0.03$	$0.87^{a,b,A} \pm 0.05$	$0.93^{a,A} \pm 0.02$	$0.88^{a,b,A} \pm 0.01$
Treatment ($n = 8$)	0.83 ± 0.04	$0.68^{a,B} \pm 0.02$	$0.58^{b,c,B} \pm 0.03$	$0.57^{b,B} \pm 0.03$	$0.66^{a,B} \pm 0.06$	$0.64^{a,c,B} \pm 0.06$	$0.60^{b,B} \pm 0.03$	$0.68^{a,B} \pm 0.05$	$0.61^{b,B} \pm 0.01$	$0.56^{b,B} \pm 0.07$	$0.61^{b,B} \pm 0.01$
Sperm concentration ($\times 10^9$/ml)											
Control ($n = 4$)	2.52 ± 0.02	$2.42^{a,A} \pm 0.01$	$2.19^{b,c,d,A} \pm 0.03$	$2.10^{b,c,e,A} \pm 0.02$	$2.24^{b,c,d,A} \pm 0.05$	$2.34^{a,d,A} \pm 0.05$	$2.09^{c,e,A} \pm 0.03$	$2.00^{e,A} \pm 0.02$	$2.11^{b,e,A} \pm 0.01$	$2.07^{b,e,A} \pm 0.04$	$2.21^{b,c,d,A} \pm 0.04$
Treatment ($n = 8$	2.30 ± 0.03	$1.68^{a,e,B} \pm 0.01$	$1.66^{a,e,B} \pm 0.05$	$1.81^{a,B} \pm 0.01$	$1.25^{b,c,B} \pm 0.03$	$1.42^{b,B} \pm 0.03$	$1.35^{b,d,B} \pm 0.02$	$1.09^{c,B} \pm 0.06$	$1.28^{b,c,B} \pm 0.05$	$1.16^{c,d,B} \pm 0.01$	$1.56^{e,B} \pm 0.10$
Total sperm number ($\times 10^9$)											
Control ($n = 4$)	2.01 ± 0.02	$2.22^{a,A} \pm 0.04$	$1.92^{b,A} \pm 0.03$	$1.95^{a,A} \pm 0.02$	$1.81^{b,c,A} \pm 0.03$	$1.94^{b,A} \pm 0.05$	$1.88^{b,A} \pm 0.01$	$1.64^{c,A} \pm 0.02$	$1.83^{b,c,A} \pm 0.01$	$1.92^{b,A} \pm 0.02$	$1.90^{b,A} \pm 0.05$
Treatment ($n = 8$)	1.90 ± 0.01	$1.14^{a,B} \pm 0.03$	$0.96^{b,c,B} \pm 0.02$	$1.03^{a,b,B} \pm 0.03$	$0.82^{c,B} \pm 0.04$	$0.90^{b,c,B} \pm 0.02$	$0.81^{c,B} \pm 0.05$	$0.74^{c,d,B} \pm 0.01$	$0.78^{c,d,B} \pm 0.07$	$0.64^{d,B} \pm 0.03$	$0.86^{c,B} \pm 0.05$
Sperm motility (%)											
Control ($n = 4$)	76.1 ± 1.1	$75.4^{A} \pm 1.6$	$72.6^{A} \pm 2.3$	$74.6^{A} \pm 4.1$	$78.3^{A} \pm 1.7$	$75.5^{A} \pm 2.0$	$80.9^{A} \pm 2.8$	$80.7^{A} \pm 1.5$	$82.8^{A} \pm 2.6$	$81.9^{A} \pm 1.5$	$77.7^{A} \pm 0.9$
Treatment ($n = 8$)	75.3 ± 1.0	$65.0^{B} \pm 1.6$	$60.2^{B} \pm 3.0$	$63.2^{B} \pm 2.7$	$70.8^{B} \pm 3.8$	$65.0^{B} \pm 2.1$	$70.0^{B} \pm 1.4$	$70.3^{B} \pm 3.5$	$71.1^{B} \pm 1.7$	$69.4^{B} \pm 2.5$	$65.8^{B} \pm 1.2$

Hours post-treatment.

Different lower case letters (a, b, c, d and e) within same line indicate significant ($P < 0.05$) differences.

Different upper case letters (A and B) within same column showed significant ($P < 0.05$) differences between control and treatment groups.

Data are expressed as the mean (±S.E.M.).

with levamisole of rams caused significant ($P < 0.05$) decreases in the sperm motility. The inhibition of sperm motility showed that levamisole could cause a decrease in the sperm motility depending on the inhibition of diamino oxidase in seminal plasma.

Acrosomal enzymes play an essential role in fertilization and hyaluronidase being among the acrosomal enzymes is particularly important in dispersing cumulus cells [5]. It was also reported that the low hyaluronidase activity causes a decrease in fertilizing capability of sperm [22]. The findings of this study indicate that levamisole caused significant ($P < 0.001$) increases in the semen hyaluronidase activity of rams at 1, 2, 4, 24, 72, 96 and 120 h. The elevation of semen hyaluronidase activity may be explained with the movement of hyaluronidase from serum to seminal plasma. However, the hyaluronidase activity of semen at 48, 144, 216, 288 and 384 h did not change compared to the control group due to 6 h of the biological half life of levamisole in plasma and mean residence time of this drug in body about 216 h [1]. The cause of the decrease of semen hyaluronidase activity in treatment group at 48 h is not known. Further studies are required for explanation of this decrease.

It was reported that levamisole increases the release of hyaluronidase from macrophages and neutrophiles [23,24]. The results of this study showed that levamisole caused significant ($P < 0.01$) increases in the serum hyaluronidase activity of rams at all times except the 72 h. The cause of the decrease of serum hyaluronidase activity at 72 h is unknown. Further studies are required for explanation of this decrease at this time. This elevation may indicate that levamisole increases serum hyaluronidase activity in sheep depending on the increase of hyaluronidase releasing by macrophages and neutrophiles. Additionally, levamisole is used during chronic bacterial disease and inflammatory disease [2] and hyaluronic acid levels in serum are increased during infections [8]. A deficiency in hyaluronidase activity of serum causes to the elevation of hyaluronic acid in serum 38–90 fold over normal and it is incompatible with life [12]. After treatment with levamisole of rams, the elevation of hyaluronidase activity in serum is a beneficial effect for body due to the degradation of hyaluronic acid by serum hyaluronidase.

In conclusion, the findings of this study indicate that levamisole increases hyaluronidase activity in serum and semen. Besides, it causes significant decreases in semen volume, sperm motility, concentration and total sperm number in rams. Despite some reported benefits of elevated hyaluronidase activity, our observation, indicate that levamisole was found to be clearly detrimental to semen quality. For this reason, the use of levamisole is not suitable during breeding season in rams.

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