

# The effects of diminazene aceturate and ceftriaxone on ram sperm

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## Abstract

Effects of diminazene aceturate and ceftriaxone disodium were evaluated on sperm quality of rams. Daily intramuscular injections of diminazene (6 mg/kg) or ceftriaxone (28.5 mg/kg) were given to each of seven Akkaraman rams assigned per drug for two days. Semen samples were collected from the rams at post-treatment 1, 4, 24, 48, 72, 144, 288 and 336 h and examined for sperm characteristics and hyaluronidase activity. Results showed that use of ceftriaxone and diminazene caused significant ( $P < 0.01$ ) decreases in sperm concentration, volume and motility compared to control group within 288 h post-treatment. In addition, hyaluronidase activity increased significantly ( $P < 0.01$ ) in semen of rams treated with ceftriaxone while remained unchanged in those received diminazene. In conclusion, diminazene aceturate and ceftriaxone disodium did not have any deleterious effect on hyaluronidase enzyme. However, both drugs caused impairment of sperm in rams during the 288 h.

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**Keywords:** Hyaluronidase; Sperm characteristics; Diminazene; Ceftriaxone

## 1. Introduction

Diminazene aceturate is an antiprotozoan drug used to treat the infections of babesia and trypanosoma in animals [1–3]. The half life of this drug is approximately 1.3 h for the initial phase and approximately 188 h for the terminal phase. However, sheep have been shown to display a triphasic elimination curve which is indicative of a three-compartment model [4].

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Ceftriaxone is one of the cephalosporin derivatives and is effective against *Brucella melitensis* [6] and *Streptococcus faecalis* [7]. It is used in the treatment of respiratory infections and gonorrhoe in humans [8–10]. It has been reported that the mean values of terminal half life, steady state volume of distribution, renal clearance and total body clearance for ceftriaxone in merino ewes are 1.7 h, 0.3 l/kg, 0.08 l/h/kg and 0.22 l/h/kg, respectively [5].

Hyaluronidase enzyme degrades hyaluronic acid, a glycosaminoglycan present in the extracellular matrix of ovum. This enzyme is released from the head of sperm during acrosomal reaction and it function in the penetration of sperm through the cumulus oophorus matrix during the fertilization [11,12]. The measurement of semen hyaluronidase activity provides presumptive information about acrosomal function and fertilizing capability of sperm [13] and there is no published report about the effects of diminazene aceturate and ceftriaxone disodium on sperm characteristics and hyaluronidase activity. Ceftriaxone disodium and diminazene acetate are commonly used in veterinary practice in Turkey. Objective of this study was to evaluate the effects of these drugs on sperm characteristics and hyaluronidase activity of ram semen, and to determine whether there is a relationship between semen hyaluronidase activity and sperm characteristics.

## 2. Materials and methods

### 2.1. Chemicals and drugs

Piroid (Diminazene acetate powder, Farmaceutici gellini S.p.A., Aprilia, Italy), Rocephin flacon (Ceftriaxone disodium 1 g, F. Hoffmann-La Roche Ltd., Basel, Switzerland) and other chemicals were purchased from Sigma–Aldrich Co.

### 2.2. Animals, administration of drugs and sample collection

Twenty-one healthy Akkaraman rams, between age of 2 and 3, were used in the present study. The rams were fed on grass supplemented with alfalfa hay, and drinking water was provided ad libitum. The rams were randomly divided into three groups of seven. These groups were assigned as control, diminazene, and ceftriaxone regimens. Semen hyaluronidase activity and sperm characteristics of all rams in each group were determined prior to drug injections. Ceftriaxone disodium (Rocephin flacon, 1 g) was dissolved in 3.5 ml distilled water and injected intramuscularly at a dose of  $28.5 \text{ mg kg}^{-1}$  body weights, once daily for two days to the ceftriaxone group [5]. At the same time, diminazene acetate (Piroid, 5 g) was dissolved in 100 ml distilled water and injected intramuscularly at a dose of  $6 \text{ mg kg}^{-1}$  body weight, once daily for two days to the diminazene group [14]. Four milliliters of distilled water was injected intramuscularly once daily for two days to the control rams. After the last administration of both drugs, semen samples were collected using artificial vagina from all animals at 1, 4, 24, 48, 72, 144, 288 and 336 h post-treatment. All samples were analyzed for determination of sperm characteristics and semen hyaluronidase activity. The mean residence times of these drugs in sheep have been found to be about 168 h [4,5].

### 2.3. Assays

Sperm samples were decimally diluted with isotonic sodium citrate solution (3%, w/v dissolved in distilled water). A slide was placed on phase contrast microscope and allowed to warm to a temperature of 37 °C, and then a small droplet of diluted semen was placed on the slide and % motility was evaluated visually at a magnification of 400× [15]. Motility estimations were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score. Sperm concentration was determined with a hemocytometer. The percentage of morphologically abnormal sperm was determined from slides prepared with an Indian ink. A total of 300 sperm cells were counted on each slide under a phase-contrast microscope at 100× magnification [16]. Semen volume was determined by direct reading the graduations of collection tubes.

Hyaluronidase activity of whole semen was measured using the methods described by Joyce and Mack [17] and Tanyıldızı and Bozkurt [18]. The semen samples were diluted to 1:5 with 0.15 mol/l sodium chloride before the assay. One milliliter 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 (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*-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 in a 37 °C 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 analyses

The data are presented as the mean ± S.E.M. Both hyaluronidase activity and sperm characteristics (motility, concentration and volume) were compared between treatments and between time points by one-way analysis of variance (ANOVA). 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 before the injection of drugs between animals in control and treatment groups were not significant (Table 1).

The differences in hyaluronidase activity between control and treatment groups were shown in Table 2. There was no significant difference in the hyaluronidase activity of diminazene group compared to control group (Table 2). However, ceftriaxone therapy

Table 1

Pre-treatment values of control and experiment groups

| Groups             | Hyalurotildase activity<br>( $\mu\text{mol NAG/min/l}$ ) | Sperm motility<br>(%) | Sperm concentration<br>( $\times 10^9 \text{ ml}^{-1}$ ) | Semen volume<br>(ml) | Sperm abnormality<br>(%) |
|--------------------|----------------------------------------------------------|-----------------------|----------------------------------------------------------|----------------------|--------------------------|
| Control $n = 7$    | $42.1 \pm 5.2$                                           | $73.6 \pm 1.0$        | $2.32 \pm 0.15$                                          | $0.96 \pm 0.08$      | $6.92 \pm 0.02$          |
| Treatment $n = 14$ | $45.0 \pm 4.6$                                           | $75.3 \pm 0.8$        | $2.89 \pm 0.84$                                          | $1.08 \pm 0.12$      | $7.63 \pm 0.90$          |

Data were expressed as the mean  $\pm$  S.E.M.

Table 2

The hyaluronidase activity in control group and rams treated with diminazene aceturate and ceftriaxone disodium

| Groups              | Hours post-treatment |                |                |                |                |                |                |                |                |
|---------------------|----------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                     | 1                    | 4              | 24             | 48             | 72             | 144            | 288            | 336            | Mean           |
| Control ( $n = 7$ ) |                      |                |                |                |                |                |                |                |                |
| Control             | $43.4 \pm 3.2$       | $45.3 \pm 4.1$ | $45.2 \pm 1.8$ | $41.9 \pm 2.6$ | $43.7 \pm 1.7$ | $39.4 \pm 2.1$ | $40.6 \pm 3.0$ | $38.2 \pm 4.0$ | $42.2 \pm 0.9$ |
| Diminazene          | $56.8 \pm 1.3$       | $53.7 \pm 1.3$ | $42.7 \pm 1.0$ | $38.6 \pm 0.6$ | $58.7 \pm 1.1$ | $40.2 \pm 3.5$ | $36.5 \pm 1.4$ | $44.2 \pm 3.0$ | $46.4 \pm 3.0$ |
| Ceftriaxone         | $67.4 \pm 5.7$       | $71.7 \pm 1.7$ | $63.0 \pm 6.8$ | $74.3 \pm 2.7$ | $66.3 \pm 3.2$ | $61.2 \pm 6.1$ | $71.7 \pm 3.1$ | $43.1 \pm 4.2$ | $64.8 \pm 3.4$ |

The hyaluronidase activity was expressed as the mean ( $\pm$ S.E.M.)  $\mu\text{mol NAG/min/l}$ .

Table 3

Percentages of spermatozoa motility in control group and rams treated with diminazene acetate and ceftriaxone disodium

| Groups              | Hours post-treatment |                |                |                |                |                |                |                |                |
|---------------------|----------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                     | 1                    | 4              | 24             | 48             | 72             | 144            | 288            | 336            | Mean           |
| Control ( $n = 7$ ) |                      |                |                |                |                |                |                |                |                |
| Control             | $80.4 \pm 3.4$       | $74.1 \pm 4.6$ | $77.8 \pm 3.0$ | $75.7 \pm 4.1$ | $75.1 \pm 1.5$ | $85.3 \pm 5.1$ | $75.4 \pm 2.1$ | $78.9 \pm 5.6$ | $77.8 \pm 1.3$ |
| Diminazene          | $64.1 \pm 3.1$       | $62.0 \pm 5.9$ | $50.2 \pm 2.6$ | $60.3 \pm 4.1$ | $52.4 \pm 3.4$ | $65.6 \pm 4.0$ | $58.8 \pm 4.1$ | $75.5 \pm 4.9$ | $61.1 \pm 2.7$ |
| Ceftriaxone         | $65.7 \pm 3.5$       | $58.9 \pm 3.6$ | $55.0 \pm 5.7$ | $63.1 \pm 3.4$ | $63.3 \pm 3.4$ | $65.4 \pm 4.2$ | $61.0 \pm 3.9$ | $73.4 \pm 4.2$ | $63.2 \pm 1.9$ |

Data are expressed as mean (%)  $\pm$  S.E.M.

increased significantly ( $P < 0.01$ ) hyaluronidase activity in ram semen at all times except the 336 h (Table 2). Furthermore, both drugs caused a significant ( $P < 0.01$ ) decrease in motility (Table 3), concentration (Table 4) and volume (Table 5) at all times except the 336 h in comparison with the control group. Data were analyzed for presence of any correlation between hyaluronidase activity and sperm characteristics. Results showed that there was no significant relationship between these parameters.

The mean ( $\pm$ S.E.M.) percentage of abnormal sperm was not affected by treatments with diminazene or ceftriaxone. These values were ranged between  $5.0 \pm 0.2\%$  and  $7.5 \pm 1.2\%$

Table 4

Sperm concentration of control group and rams treated with diminazene acetate and ceftriaxone disodium

| Groups              | Hours post-treatment |                 |                 |                 |                 |                 |                 |                 |                 |
|---------------------|----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                     | 1                    | 4               | 24              | 48              | 72              | 144             | 288             | 336             | Mean            |
| Control ( $n = 7$ ) |                      |                 |                 |                 |                 |                 |                 |                 |                 |
| Control             | $3.06 \pm 0.02$      | $3.21 \pm 0.03$ | $3.03 \pm 0.05$ | $2.85 \pm 0.03$ | $2.79 \pm 0.01$ | $3.32 \pm 0.05$ | $3.11 \pm 0.02$ | $2.96 \pm 0.22$ | $3.04 \pm 0.06$ |
| Diminazene          | $2.15 \pm 0.32$      | $1.60 \pm 0.45$ | $2.15 \pm 0.13$ | $0.70 \pm 0.09$ | $1.25 \pm 0.28$ | $1.28 \pm 0.16$ | $0.85 \pm 0.14$ | $3.02 \pm 0.15$ | $1.62 \pm 0.27$ |
| Ceftriaxone         | $2.95 \pm 0.54$      | $1.55 \pm 0.03$ | $2.11 \pm 0.18$ | $2.15 \pm 0.21$ | $1.85 \pm 0.14$ | $1.32 \pm 0.16$ | $1.49 \pm 0.22$ | $2.88 \pm 0.51$ | $2.03 \pm 0.21$ |

Data are expressed as mean ( $\times 10^9 \text{ ml}^{-1}$ )  $\pm$  S.E.M.

Table 5

Sperm volume in control group and rams treated with diminazene aceturate and ceftriaxone disodium

| Groups<br>(n = 7) | Hours post-treatment |             |             |             |             |             |             |             | Mean        |
|-------------------|----------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|                   | 1                    | 4           | 24          | 48          | 72          | 144         | 288         | 336         |             |
| Control           | 1.05 ± 0.09          | 1.00 ± 0.05 | 0.95 ± 0.07 | 1.02 ± 0.02 | 1.14 ± 0.04 | 0.93 ± 0.08 | 1.12 ± 0.11 | 1.15 ± 0.11 | 1.04 ± 0.03 |
| Diminazene        | 0.54 ± 0.04          | 0.46 ± 0.01 | 0.44 ± 0.06 | 0.47 ± 0.01 | 0.68 ± 0.09 | 0.45 ± 0.07 | 0.72 ± 0.13 | 1.16 ± 0.23 | 0.61 ± 0.08 |
| Ceftriaxone       | 0.63 ± 0.06          | 0.62 ± 0.03 | 0.54 ± 0.05 | 0.59 ± 0.07 | 0.57 ± 0.02 | 0.56 ± 0.11 | 0.81 ± 0.14 | 1.06 ± 0.08 | 0.67 ± 0.06 |

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

in the rams of diminazene group and between  $6.3 \pm 0.2\%$  and  $8.1 \pm 0.3\%$  in the rams of ceftriaxone group. Mean ( $\pm$ S.E.M.) percentage of abnormal sperm of control rams was  $6.7 \pm 0.4\%$ . There was no significant difference between the control and treatment groups and values were in normal ranges for ram sperm.

#### 4. Discussion

Babesia and trypanosomes depend on host glucose reserves for aerobic glycolysis and diminazene causes hypoglisemia in the host organism. The antiprotozoal action of diminazene seems to be produced by the inhibition of aerobic glycolysis and the denaturation of nucleoproteins of the parasite [3]. Results of this study showed that, diminazene causes a significant decrease in the percentage of sperm motility of rams. Fructose, sorbitol and glycerylphosphorylcholine are found in seminal plasma and are used by spermatozoa as energy substrates [15]. The decrease in sperm motility could be due to the lower production of energy substrates depending on hypoglycemia in rams treated with diminazene. Our findings, in addition, indicate that diminazene caused a significant decrease in semen volume and sperm concentration. The cause of this effect is unknown as there is no published data about the subject.

It has been previously reported that cephalosporins have toxic effects on testicular tissue and decrease sperm production in rats [19,20]. Our findings indicate that ceftriaxone caused significant decreases in sperm concentration and semen volume in rams over the period up to 288 h. Ceftriaxone may play an important role in decrease of sperm concentration and semen volume by its preventive effects on meiosis and mitosis of the seminal cells, as reported in rats [21].

Newly released eggs are surrounded by the cumulus oophorus and zona pellucida, and spermatozoa have to penetrate these barriers before fertilization. Acrosomal enzymes, especially hyaluronidase, play an important role in supporting sperm penetration into the cumulus oophorus matrix [22]. The results of our experiments indicate that ceftriaxone at a dose of  $28.5 \text{ mg kg}^{-1}$  significantly, increased the hyaluronidase activity of semen in rams, in contrast to diminazene that did not significantly affect on hyaluronidase activity. The elevation of hyaluronidase activity in ram semen treated with ceftriaxone may indicate that it causes subtle sperm damage resulting in enzyme leakage from cells or causes an increase in the production of hyaluronidase enzyme in testicular tissue. This conclusion would be supported by our earlier findings [18], which suggested that the hyaluronidase enzyme in semen might have been originated from testicular tissue.

Hirayama et al. [23] and Abdul-aziz et al. [13] suggested that there is a significant correlation between the hyaluronidase activity of individual sperm and sperm concentration in infertile men. Similarly, it was reported that the increase of semen hyaluronidase activity is a beneficial effect for fertilizing ability of sperm [23]. In the present study, however, no correlation was found between semen hyaluronidase activities and sperm characteristics. In other words, our findings showed that the elevation of semen hyaluronidase activity did not increase the degree of fertilizing ability of ram sperm. This difference can be explained by the measurement of hyaluronidase activity in semen samples rather than each spermatozoa and by the use of fertile rams in this study. Nevertheless, action mechanism of these drugs on sperm characteristics and hyaluronidase activity requires further research.

In conclusion, our data revealed that ceftriaxone disodium and diminazene aceturate may cause infertility problem within first 288 h of injection in rams unless certain time is allowed to pass to reduce the adverse effects of these drugs on sperm. More specifically, at least 336 h post-drug application is needed for breeding rams for fertile semen. Our results may help breeders and veterinary practitioners to reduce potential infertility problems in rams that may have been caused by these drugs.

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