The effect of ascorbic acid on semen hyaluronidase activity and sperm characteristics in rams

M. SÖNMEZ1, S. TANYILDIZI2

1 Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Fırat University, 23119 Elazig – TURKEY
2 Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Fırat University 23119 Elazig – TURKEY

*Corresponding author: E-mail: msonmezvet@hotmail.com

Introduction

L-isomer of ascorbic acid, known as Vitamin C, is a water-soluble vitamin which is required for normal function of the body. It is widely used for the scavenging of free radicals, strengthening the immune system and prevention of chemical carcinogenesis induced by a number of xenobiotics [16]. It has also been reported that ascorbic acid has direct antiviral properties and stimulates interferon production by virus-infected cells in culture [13].

Ascorbic acid has been associated with fertility for many years and may have evolutionary significance, but its precise physiological role in reproduction has been uncertain [25]. Ascorbic acid has antioxidant activity and, it is present at a high concentration in the epididymal fluid and seminal plasma [11]. High level of ascorbic acid in semen may play an important role in protecting sperm cells against reactive oxygen species (ROS) and in maintaining the genetic integrity of sperm cells by preventing of sperm DNA damage [14, 30]. In addition, ascorbic acid supplementation can improve sperm count and motility [2, 33]. However, it was reported that both deficiency [9] and excessive intake [27] of ascorbic acid can cause the decrease in reproductive performance of male.

Hyaluronidase is one of the acrosomal enzymes and it is required for the penetration of sperm through the cumulus oophorus matrix during the fertilization [24, 34]. This enzyme is released from the head of sperm during acrosomal reaction and degrades hyaluronic acid, a glycosaminoglycan present in the extracellular matrix of ovum [19]. The measurement of semen hyaluronidase activity can provide presumptive information about acrosomal function and fertilizing capability of sperm [1, 35] and there is no published record about the effect of ascorbic acid on semen hyaluronidase activity. This study was conducted to investigate the effect of ascorbic acid on semen hyaluronidase activity and sperm characteristics in rams.

SUMMARY

The effects of ascorbic acid on semen hyaluronidase activity and sperm characteristics in rams were investigated in this study. For this purpose, twelve rams were used. The animals were randomly divided into two groups of 6 as control and treatment groups. Ascorbic acid was intravenously administered at a dose of 10 mg/kg body weight once daily for three days. Semen samples were taken at 1, 2, 4, 8, 12, 24, 48 and 72 h after treatment and examined for hyaluronidase activity, level of ascorbic acid and sperm characteristics. The results showed that the use of ascorbic acid caused the significant increase in semen ascorbic acid level and decrease in hyaluronidase activity within 8 hours after treatment when compared to the control group. However, there was no significant difference in the sperm characteristics between the control and treated animals. In conclusion, although short-term administration of ascorbic acid did not have any effect on sperm characteristics, it caused a significant decrease in the semen hyaluronidase activity.

Keywords: Ram, Semen, Ascorbic acid, Hyaluronidase, Sperm.

RéSUMÉ

Les effets de l’acide ascorbique sur l’activité de la hyaluronidase spermatique et sur les caractéristiques du sperme de bélier

Les effets de l’acide ascorbique sur l’activité de la hyaluronidase spermatique et sur les caractéristiques des spermatozoïdes de béliers ont été évalués . Pour cela, 12 béliers ont été utilisés. Les animaux ont été aléatoirement répartis en 2 groupes de 6, un groupe contrôle et un groupe traité. L’acide ascorbique a été administré par voie intraveineuse à la dose de 10 mg/kg une fois par jour pendant 3 jours. Les concentrations en acide ascorbique, le niveau de l’activité de la hyaluronidase et les caractéristiques des spermatozoïdes ont été évalués dans les échantillons de sperme collectés aux temps 1, 2, 4, 8, 12, 24, 48 et 72 h après le traitement. Les résultats montrent que l’utilisation d’acide ascorbique augmente significativement les concentrations spermatiques en acide ascorbique et diminue le niveau de l’activité de la hyaluronidase spermatique pendant les 8h qui suivent le traitement quand ces valeurs sont comparées à celles du groupe contrôle. Cependant, aucune différence n’a été observée entre les caractéristiques des spermatozoïdes des animaux traités et contrôles. En conclusion, bien qu’un traitement à court terme avec de l’acide ascorbique n’affecte pas les caractéristiques des spermatozoïdes, il diminue de façon significative l’activité de la hyaluronidase spermatique.

Mots-clés : Bélier, sperme, acide ascorbique, hyaluronidase, spermatozoïdes.
Material and Methods

CHEMICALS

L-ascorbic acid (Redoxon®) was obtained from Roche (Roche Inc., Istanbul, TURKEY) and the other chemicals were purchased from Sigma-Aldrich Co.

ANIMALS AND TREATMENT

Twelve healthy Akkaraman rams at the 2-3 years old were used in the study. The rams were fed on grass supplemented with alfalfa hay and drinking water was provided ad libitum. The rams were randomly divided into two groups. These groups were assigned as control (n=6) and treatment (n=6). The semen hyaluronidase activity, semen ascorbic acid level and sperm characteristics of all rams in each group were determined prior to treatment. Ascorbic acid was treated intravenously at a dose of 10 mg/kg body weight in the morning once daily for three days. This dose is recommended for ruminants [7]. At the same time, 5 mL of physiological saline (0.9% NaCl) was treated intravenously to the control rams. After the last drug administration, semen samples were collected by artificial vagina from all rams at 1, 2, 4, 8, 12, 24, 48 and 72 h. All samples were examined for sperm characteristics. Then, they were analyzed for hyaluronidase activity and ascorbic acid level of semen.

SEmen EVALUATION

Semen volume was calculated by direct reading the graduations of collection tubes (from 0.1 to 5.0 mL). Sperm concentration was determined with a hemocytometer [3]. The percentage of progressive sperm motility was evaluated using a light microscope with heater table. A slide was placed on microscope stage and, allowed to warm a temperature of 35°C by heater table. Several droplets of sodium citrate solution (3%, w/v, dissolved in distilled water) were dropped on this solution and, a very small droplet of semen sample with a pipette was added on this solution and mixed by a cover-slip. The percentage of progressive sperm motility was visually evaluated using a score ranging from 0 to 100% at a magnification of 400x [4]. Motility estimations were performed from three different fields in each sample. The mean value of three successive estimations was used as the final motility score.

HYALURONIDASE ACTIVITY

Hyaluronidase activity of semen was measured using the methods described by TANYILDIZI and BOZKURT [34] and JOYCE et al. [19]. For this process, one volume of semen sample (approximately containing 500x10⁶ sperm cells) was slowly added to a centrifuge tube. One volume of the semen sample (approximately containing 500x10⁶ sperm cells) was slowly added to this solution. The mix was allowed to stand for 30 min at room temperature, and then centrifuged at 3000 rpm for 15 min. The blue-coloured supernatant was placed in a spectrophotometer cuvette and read at 700 nm against a blank. The concentration of ascorbic acid was expressed as mg/100 mL.

ASCORBIC ACID

The level of ascorbic acid in semen was determined at spectrophotometrically at 700 nm using acid phosphotungstic acid [20]. Briefly, a mixture of 20 g of sodium tungstate and 10 g of disodium hydrogen phosphate was suspended in 30 mL of water and warmed to dissolve (Solution A). Then, 5 mL of sulphuric acid was added to 15 mL of water (Solution B). Solution B was poured slowly into warm Solution A. The content was boiled gently for 2 h under reflux, and then this solution was cooled and stored at room temperature as colour reagent. One volume of colour reagent was put in a centrifuge tube. One volume of the semen sample (approximately containing 500x10⁶ sperm cells) was slowly added to this solution. The mix was allowed to stand for 30 min at room temperature, and then centrifuged at 3000 rpm for 15 min. The blue-coloured supernatant was placed in a spectrophotometer cuvette and read at 700 nm against a blank. The concentration of ascorbic acid was expressed as µmol NAG/min/L.

STATISTICAL ANALYSIS

The data are presented as the mean ± SEM. The differences were considered to be significant for P<0.05. Mann-Whitney U Test was used to compare the volume, sperm concentration, hyaluronidase activity and ascorbic acid level of semen between the control and treatment groups. Chi-square analysis was used to compare the sperm motility between control and treatment groups. All data were analyzed using SPSS (Version 10.0) software package program.

Results

The semen samples were obtained from all rams prior to initiation of treatment and post treatment. These values are given in Table I. There was no significant (P>0.05) difference in sperm characteristics between the control and treatment groups. Ascorbic acid therapy did not affect sperm characteristics along the 72 h after treatment.

The differences in semen hyaluronidase activity between control and treatment groups are shown in Figure 1. There was no significant (P>0.05) difference in semen hyaluronidase activity between the control and treatment groups. Ascorbic acid therapy did not affect the semen hyaluronidase activity of rams.
was no significant (P>0.05) difference in semen hyaluronidase activity of treatment group when compared to control group before administration of ascorbic acid. However, ascorbic acid therapy caused a significant (P<0.05) decrease in hyaluronidase activity of semen at 1, 2, 4 and 8 h after treatment.

The differences in ascorbic acid level of semen between control and treatment groups are present in Figure 2. There was no significant (P>0.05) difference in ascorbic acid level of treatment group when compared to control group before treatment. However, administration of ascorbic acid caused a significant (P<0.05) increase in ascorbic acid level of semen within 12 h after treatment.

![Figure 1: The hyaluronidase activity of semen in control and treatment groups (mean ± SEM). Each bar represents mean of six values. * P<0.05.](image1)

![Figure 2: Ascorbic acid level of semen in control and treatment groups (mean ± SEM). Each bar represents mean of six values. * P<0.05.](image2)

<table>
<thead>
<tr>
<th>Sperm Parameters</th>
<th>Pre-Treatment</th>
<th>Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 4 8 12 24 48 72</td>
<td></td>
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<tr>
<td><strong>Semen Volume (mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.10±0.09</td>
<td>1.03±0.09</td>
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<tr>
<td>Treatment</td>
<td>1.08±0.07</td>
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<tr>
<td><strong>Sperm Concentration (x10^9/mL)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>2.37±0.08</td>
<td>2.28±0.07</td>
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<tr>
<td>Treatment</td>
<td>2.42±0.07</td>
<td>2.35±0.05</td>
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<tr>
<td><strong>Sperm Motility (%)</strong></td>
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<tr>
<td>Control</td>
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<tr>
<td>Treatment</td>
<td>77.2±2.0</td>
<td>72.8±1.6</td>
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**Table 1:** Semen volumes (mL), sperm concentrations (x10^9/mL) and sperm motilities (%) of control and treatment group. Data are expressed as the mean ± SEM.

**Discussion**

Ascorbic acid is present at a high concentration in the epididymal fluid and seminal plasma compared to blood plasma [11, 18]. There is a relationship concentration of ascorbic acid and genetic integrity of sperm cells. Ascorbic acid plays an important role in protecting sperm DNA from the oxidative damage induced by ROS [14, 30]. In the other hand, Testes and seminal plasma are extremely sensitive to a decrease in blood plasma levels of ascorbic acid [9]. FRAGA et al. [14] reported that the decrease in ascorbic acid intake from 250 mg/day to 5 mg/day caused a 50% decline concentration of ascorbic acid of semen in healthy men. EBESUNUN et al. [12] indicated that semen ascorbate level may play a significant role in male fertility, and the decrease in concentration of ascorbic acid of semen caused to reduce sperm characteristics. Similarly, some early studies demonstrated that the deficiency of ascorbic acid caused degeneration of the testicular germinal epithelium [15], reduced sperm concentration and motility [10] and, associated with poor breeding performance [29]. Furthermore, male fertility would be improved by an increased dietary ascorbic acid intake [11]. It is reported that ascorbic acid supplementation...
can improve sperm count and motility [2, 33]. Our previous study [32] found that ascorbic acid supplementation increased levels of ascorbic acid in testes tissue and it caused an improvement in the reproductive ability of male rats. Similarly, YOUSEF et al. [36] reported that ascorbic acid supplementation to drinking water for 12 weeks increased sperm concentration of male rabbits. On the contrary, our previous study [31] indicated that although short-term administration of the ascorbic acid increased ascorbic acid level of semen within 24 h after treatment, it did not affect the sperm characteristics. However, the administration of ascorbic acid along 30 days caused an improvement in semen quality of rams. Similarly, the results of present study found that administration of ascorbic acid for three days increased ascorbic acid level of semen although it did not affect spermatozoal parameters. The reason of this result can be explained by the short-term administration of ascorbic acid in this study.

The administration of ascorbic acid caused a significant increase in ascorbic acid level of semen within 12 h after treatment. However, there was no significant difference in ascorbic acid level of treatment group when compared to control group between 12 to 72 h after treatment in this study. This situation may be associated with pharmacokinetic characteristics of ascorbic acid. BLACK and HIDIROGLU [6] indicate that ascorbic acid is eliminated very rapidly from blood after IV administration and it reverses to its basal level within 6 to 8 h after treatment.

Hyaluronidase is one of the acrosomal enzymes. This enzyme is released from the head of sperm during acrosomal reaction. The hyaluronidase is required for penetration of sperm through cumulus oophorus matrix during fertilization [24, 34]. ABDUL-AZIZ et al. [1] reported that the inhibition of hyaluronidase activity could be a simple mechanical block to sperm penetration and might reduce fertilization rate. LI et al. [22] indicated that some flavonoids such as kaempferol, quercetin and apigenin inhibited the sperm hyaluronidase activity and sperm penetration. Similarly, it was reported that the antifertility effects of some drugs such as sodium aurothiomalate [28] and gossypol [37] might be due to the inhibition of hyaluronidase.

The results of the present study indicated that the administration of ascorbic acid for three days decreased significantly (P<0.05) hyaluronidase activity of semen within 8 h after treatment. This result is supported by findings of OKORUKWU and VERCUYSSSE [26] who suggested that L-ascorbic acid inhibited testicular hyaluronidase. LI et al. [23] reported that ascorbic acid inhibited the hyaluronan degradation by hyaluronidase. It is structurally similar to one of the sugar units of hyaluronan. Ascorbic acid binds to active site of hyaluronidase and, competes with the binding of the hyaluronan substrate. In this study, hyaluronidase activity of semen was measured using the methods described by TANYILDIZI and BOZKURT [34] and JOYCE et al. [19]. According to this method, semen hyaluronidase enzyme digests hyaluronic acid substrate to liberate N-acetylglucosamine (NAG), and NAG is quantified using the colorimetric method. The findings of this study indicated that L-ascorbic acid caused a significant decrease in semen hyaluronidase activity. This decrease may be explained by the binding of L-ascorbic acid to active site of semen hyaluronidase and the decrease of production of NAG.

Hyaluronic acid is present in basement membrane and extracellular matrix to regulate a variety of normal physiological functions [21]. It is important for maintaining the integrity of tissue architecture and provides a barrier for tumor invasion [5]. Hyaluronidase enzyme is required for degradation of hyaluronic acid, allowing penetration of tumor cells thorough the basement membrane and entry into systemic circulation [33]. CAMERON et al. [8] suggested that ascorbic acid could have anti-cancer action by inhibiting hyaluronidase and thereby preventing cancer spread. Similarly, HEAD [16] reported that the use of high dose of ascorbic acid has an important role in prevention of various types of cancer. The one of proposed mechanisms of ascorbic acid in the prevention and treatment of cancer is inhibition of hyaluronidase which keeps the ground substance around the tumor intact and preventing metastasis.

Hyaluronidase enzyme is considered to be involved in many physiological and pathological processes like fertilization, tumor growth and metastasis. Bacterial hyaluronidases contribute to the spreading of microorganism in tissue. In this respect, ascorbic acid could be useful as pharmacological drug to prevent certain functions of hyaluronidase. However, hyaluronidase which localizes in the acrosomal region of sperm plays an important role at fertilization and, the reduction of its activity in the acrosome may cause to decrease in fertilizing capability of sperm [17].

In conclusion, the results of this study indicated that the administration of ascorbic acid decreased significantly hyaluronidase activity within 8 h after treatment. Hyaluronidase is required for the penetration of sperm through the cumulus oophorus matrix during the fertilization. We hypothesize that the reduction and inhibition of hyaluronidase activity by ascorbic acid may cause to decrease sperm penetration capacity into cumulus complex. However, further studies are required to examine whether there is relationship between ascorbic acid and sperm penetration capacity.

References
