Influence of ascorbic acid addition to the extender on dog sperm motility, viability and acrosomal integrity during cooled storage

A. CEYLAN1*, I. SERIN2

1 Department of Reproduction and Artificial Insemination, Adnan Menderes University, Faculty of Veterinary Medicine, Batıkampusı, PK 17, 09016, Aydın, TURKEY
2 Department of Reproduction and Artificial Insemination, University of Adnan Menderes, Faculty of Veterinary Medicine, 09016 Aydın, TURKEY

SUMMARY

The objective of this study was to investigate the effect of addition of different concentrations of a water-soluble antioxidant ascorbic acid to the extender on dog spermatozoa during storage at 5°C for 72 h. Semen samples were collected from six sexually mature dogs twice a week. Different concentrations of ascorbic acid (0.22, 0.45 and 0.90 mg/mL) were added to the extender. No antioxidant was added to the extender in the control group. Sperm motility, viability and acrosomal integrity in extended semen were investigated immediately after dilution and after 24, 48, and 72 h at 5°C, respectively. Ascorbic acid at concentrations of 0.22, 0.45 and 0.90 mg/ml significantly increased the percentage of viable and acrosome intact spermatozoa at 48 and 72 h when compared with the controls. However, the addition of ascorbic acid to the extender did not improve the percentage of motile spermatozoa during the investigated period. In conclusion, the addition of ascorbic acid to the extended dog semen improved sperm survival and acrosomal integrity of spermatozoa, however not the rate of sperm motility during cooled storage of semen for 72 h at 5°C.

Keywords : Ascorbic acid, semen storage, dog, spermatozoa.

RÉSUMÉ

Influence d’addition d’acide ascorbique au milieu de dilution sur la motilité de sperme de chien, la viabilité et l’intégrité acrosomale pendant le stockage au froid

L’objectif de cette étude était d’évaluer l’effet de l’addition d’acide ascorbique à différentes concentrations dans le milieu de dilution du sperme de chien au cours du stockage à 5°C pendant 72 h. Les échantillons de sperme ont été obtenus à partir de 6 chiens collectés 2 fois par semaine. L’acide ascorbique a été ajouté au milieu de dilution aux concentrations de 0.22, 0.45 et 0.90 mg/mL. Aucun antioxydant n’a été ajouté au milieu de dilution des échantillons contrôle. La motilité des spermatozoïdes, la viabilité et l’intégrité de l’acrosome des échantillons de sperme ont été évalués immédiatement après la dilution et 24, 48, et 72 h après dilution et conservation à 5°C. L’acide ascorbique aux concentrations de 0.22, 0.45 et 0.90 mg/mL a augmenté significativement le pourcentage de spermatozoïdes viables et celui des spermatozoïdes à acrosome intact. Cependant, l’addition d’acide ascorbique au milieu de dilution n’a pas augmenté le pourcentage de spermatozoïdes motiles au cours de la période de l’étude. En conclusion, l’addition d’acide ascorbique au milieu de dilution du sperme de chien améliore la survie et l’intégrité de l’acrosome des spermatozoïdes mais pas le pourcentage de spermatozoïdes motiles au cours de la conservation du sperme pendant 72 h à 5°C.

Mots-clés : Acide ascorbique, conservation du sperme, chien, spermatozoïdes.

Introduction

Cooled-stored semen is useful when a chosen male is geographically distant from the bitch to be bred or has a work or show schedule requiring frequent travel, or when dogs cannot be shipped because of extreme environmental temperatures [22]. Maintaining the motility and fertility of spermatozoa during short-term storage at 5°C is an important consideration in the use of cooled semen in dogs. Despite its positive effects on the longevity of spermatozoa, semen dilution may contribute to losses of motility and fertilizing ability. An important reason for the decrease in fertility during storage of semen is the formation of lipid peroxides in the presence of oxygen radicals [7]. Lipid peroxidation of sperm membrane lipids is a major cause for the loss of motility and fertilizing ability of human and mammalian spermatozoa [4, 21].

The susceptibility of spermatozoa to oxidative damage is attributed to the high concentration of unsaturated fatty acids in membrane phospholipids, the limited antioxidant capacity of spermatozoa as well as the ability of spermatozoa to generate reactive oxygen species [2]. The sperm plasma membrane contains a high amount of unsaturated fatty acids. Therefore, it is particularly susceptible to peroxidative damage, with subsequent loss of membrane integrity, impaired cell function and decreased motility of spermatozoa [18]. The uncontrolled production of reactive oxygen species (ROS) by defective spermatozoa can have detrimental effect on sperm function [9].

Semen contains antioxidants, including ascorbic acid, tocopherol, catalase, glutathione peroxidase and superoxide dismutase, that balance lipid peroxidation and prevent excessive peroxide formation [6, 10, 18, 20]. However, the endogenous antioxidative capacity of semen may be insufficient during prolonged storage.
Results on the effects of antioxidants added to diluted semen are contradictory, and positive as well as negative effects have been reported in rams, humans, equine, cattle and poultry [7, 8, 13, 14, 23]. However, no reports have addressed their application in preservation of dog semen.

In this study, the effect of different concentrations of ascorbic acid, supplemented to a Tris-citric acid-fructose extender with 20% egg yolk, on sperm motility, viability and acrosome integrity were studied during storage at 5°C for 72 h.

Materials and Methods

SEMEN COLLECTION

Six sexually mature dogs of different breeds between 3 to 7 years old (weight 26 to 32 kg) were used. The animals were fed a dry commercial food once a day with free access to water. All dogs had been on a regular semen collection regimen once a week for more than 2 months before the study. Semen samples were collected by digital manipulation into warm, sterile glass collection tubes [17]. Only the second (sperm-rich) fraction of the ejaculate was collected and semen volume recorded. After collection, the ejaculates were immediately placed in a water bath at 37°C. 15 ejaculates per dog were obtained during the study at twice a week.

SEMEN EVALUATION

Immediately after collection, the semen was analyzed to determine its volume, motility and sperm concentration, as well as sperm viability and acrosomal status. Motility was evaluated subjectively under a phase-contrast microscope (magnification 200x) on a warm (37°C) stage in samples diluted two-fold in Tris buffer. Motility was expressed as the percentage of motile spermatozoa with moderate to vigorous linear progressive movement in a given sample. Sperm concentration was determined by using standard hemocytometer methods (1:200). Sperm viability was assessed in smears by eosin-nigrosin staining. The proportions of live and dead spermatozoa were then evaluated under oil immersion (magnification 1250x) using a phase-contrast microscope; 200 cells were counted [16]. The percentage of sperm cells with acrosomal abnormality or damage was determined by evaluation of sperm smears stained with nigrosin-eosin under phase contrast microscope at x1250 magnification [15]. The acrosomal integrity was assessed in 200 spermatozoa per sample. Thickened, vesiculated, detached and absent acrosomes were classified as damaged during examinations.

EXPERIMENTAL PROCEDURES

Only semen samples exhibiting individual motility more than 80% and dead sperm rates lower than 10% were used in trials. As sperm diluent, a Tris-citric acid-fructose extender (Merck, Darmstadt, G) with 20% egg yolk (v/v) was used. The Tris-citric acid-fructose extender was made by dissolving 2.90 g Tris-hydroxymethyl-aminomethane, 1.32 g monohydrated citric acid and 1.25 g D-fructose in 100 mL of distilled water. The extender prepared without antioxidant was divided into 4 parts that were supplemented with different concentrations of L-ascorbic acid (0.22, 0.45, 0.90 mg/mL; Sigma, Deisenhofen, G). Concentrations of L-ascorbic acid described by AURICH et al. (7) No antioxidant was added to the extender in the control group. The ejaculate was divided into 4 equal portions which were diluted 1:3 with Tris-citric acid-fructose based extenders containing different concentrations of L-ascorbic acid at room temperature. After incubation in the dark for 1 h at room temperature, semen samples were packaged into 0.25-ml straws and stored at 5°C for 3 d. Motility, viability and acrosomal integrity of spermatozoa were evaluated immediately after dilution, 24, 48 and 72 h of storage at 5°C.

Data were subjected to analysis of variance (one-way ANOVA); comparisons between individual means were performed with Duncan’s multiple range test. Differences were considered statistically significant when P<0.05 against control group. All values are presented as mean ± SEM.

Results

Motility, live sperm and damaged acrosome rates did not differ among groups following dilution with extenders containing different concentrations of ascorbic acid (0.22, 0.45 and 0.90 mg/mL) and no ascorbic acid (control group). The percentage of motile spermatozoa immediately after dilution was 83.7 ± 1.2% when no ascorbic acid was added, 83.2 ± 1.1% with 0.22 mg/mL, 83.0 ± 1.2% with 0.45 mg/mL and 83.3 ± 1.4% with 0.90 mg/mL ascorbic acid. During storage at 5°C for 72 h, the loss in the percentage of motile spermatozoa increased irrespective of the addition of ascorbic acid. Adding different concentrations of this water-soluble antioxidant did not significantly improve the maintenance of motility during cooled storage at 5°C for 72h (Figure 1).

The percentage of viable spermatozoa in diluted semen containing 0.22, 0.45, 0.90 mg/mL or no ascorbic acid did not differ between treatments immediately after dilution and during 24 h stored at 5°C. However, the percentage of live sperm in semen samples containing 0.45 and 0.90 mg/mL ascorbic acid at 48 h and 72 h was significantly (P<0.05 and P<0.01) higher than in the respective control values. At 48 and 72h, the percentage of live sperm in semen samples containing 0.45 mg/ml ascorbic acid (78 ± 3.1% and 73.9 ± 3.5% at 48 and 72h) and 0.90 mg/mL ascorbic acid (78.9 ± 3.5% and 75.3 ± 3.5% at 48 h and 72 h) was significantly higher than respective control values (74.5 ± 3.2% and 67.8 ± 2.9%). The percentage of live sperm in samples containing 0.22 mg/mL

![FIGURE 1. The effect of addition of different antioxidant concentrations to the extender on motility of dog spermatozoa during storage at 5°C for 24, 48 and 72 h (no significant differences between groups)](image)

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ascorbic acid after 48 h and 72 h was significantly (P<0.05) different from that of controls, and at 72h it was significantly (P<0.05) different from samples containing 0.45 and 0.90 mg/mL ascorbic acid (Figure 2).

The loss in the percentage of intact acrosomes during storage did not differ significantly between each of the three concentrations of ascorbic acid and increased continuously over time. However, the percentage of damaged acrosomes in samples containing 0.22, 0.45 and 0.90 mg/mL ascorbic acid was significantly lower at 48 h (13.4 ± 1.8%, 12.2 ± 1.8% and 12.4 ± 1.7%) and 72 h (16.3 ± 1.7%, 15.2 ± 1.8% and 15.7 ± 1.9%) than in corresponding control samples without ascorbic acid (15.5 ± 1.7% and 18.0 ± 1.6% at 48 and 72h, (P<0.05, Figure 3).

**Discussion**

In the present study, the effects of three different concentrations of ascorbic acid on motility, viability and acrosomal integrity of diluted dog semen during storage for 72 h at 5°C were investigated. After 24 h of storage with the extenders containing different concentrations of ascorbic acid, motility, viability and percentages of damaged acrosomes were not significantly different between samples. However, motility, live sperm and intact acrosomes decreased in all samples during the 72 h storage period. Although the percentage of motility was different among groups, none of the supplemented concentrations of ascorbic acid improved motility in comparison to the control group.

Motility, viability and acrosomal integrity are critical in enabling the sperm to ascend the female reproductive tract to the site of fertilization and also are necessary to achieve fertilization [2, 19]. Several observations in mammals have demonstrated that the formation of peroxides of sperm membrane lipids during in vitro storage of semen will damage the sperm plasma membrane and result in loss of motility and/or fertilizing capacity [3, 4, 5, 12]. Therefore, sperm membranes must be protected by a highly efficient antioxidant system to prevent peroxidative damage during in vitro and in vivo storage. It was reported that the beneficial effect of antioxidant Vitamin C in improving fertilization was possibly due to a reduction in lipid peroxidation [24]. The beneficial effects of ascorbic acid on maintenance of membrane integrity noted in the present study can be attributed to the antioxidant effects of this vitamin. Similarly, in the horse, addition of antioxidants such as ascorbic acid resulted in better maintenance of membrane integrity or motility of spermatozoa [7].

Ascorbic acid increased the percentage of live, acrosome-intact spermatozoa during storage at 5°C. This indicates that ascorbic acid does inhibit peroxidation of membrane lipids during storage and thus has protective effects on sperm membranes. This finding is similar to the results with horse and ram, in which ascorbic acid adding reduced reactive oxygen species generation and did improve the maintenance of sperm membrane integrity during storage at 5°C [7, 8, 23]. The beneficial influences of ascorbic acid can be attributed to the fact that ascorbic acid is a very efficient antioxidant, and a scavenger of oxygen free radicals which are toxic products of many metabolic processes [11]. Besides, it protects the activity of superoxide dismutase and regenerates other antioxidative systems [10]. However, in another study, the addition of ascorbic acid did not improve maintenance of viability during cooled storage of turkey semen [14].

In the present study, addition of water-soluble antioxidant ascorbic acid to extended dog semen, did not improve the maintenance of motility of cooled dog spermatozoa during the 72 h storage period. Adding ascorbic acid to stored semen in other studies has resulted in variable success in improving the maintenance of motility. Our result is consistent with the observations of AURICH et al. (1997) and BALL et al. (2001) who also did not detect a positive effect after addition of Vitamin C to cooled, extended equine spermatozoa. Likewise, in turkeys, another study demonstrated that maintenance of sperm motility was no effect of addition of ascorbic acid [14]. The antioxidant may have decreased the motility of spermatozoa by a reduction in pH value. A low pH does induce reversible reduction in the motility of spermatozoa [1], and thus the beneficial effects of ascorbic acid on motility may have been masked. In the opposite to our result, one study [23] reported about a positive effect on the maintenance of motility during cooled storage of ram semen after addition of ascorbic acid.

In conclusion, the present study showed that the addition of ascorbic acid to extenders caused significant increase in the percentage of live and acrosome-intact spermatozoa during cooled-storage of semen for a period of 72 hours at 5°C. Ho-
never, antioxidants added to the cooled-storage extender in this study did not significantly improve maintenance of sperm motility during liquid semen storage for a period of 72 hours.

References

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