

The effect of L-glutamine and trehalose on dog sperm cryopreservation

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Abstract

This study aimed to test different doses of L-glutamine and trehalose in the canine semen diluent while determining their protective effects on spermatological and biochemical indices of the thawed samples. Semen samples were collected from three fertile dogs using the digital manipulation method. The mixed ejaculates were divided into five portions at 37 °C and diluted with additives. Five study groups were formed with L-glutamine (10 and 20 mM), trehalose (25 and 50 mM), and no additives (control). After the dilution, the semen samples were cooled for 1.5 h at 5 °C and frozen (-110 to -120 °C) in liquid nitrogen vapor. Then, they were stored at -196 °C. For spermatological evaluations, samples were thawed at 38 °C for 30 s. L-glutamine (20 mM) was found to be significantly different ($P < 0.05$) and led to higher percentages of motility, membrane integrity, and acrosome integrity compared to the control group. Considering the total oxidant status (TOS) assay, the lower values were determined in all the antioxidant groups compared to the control group ($P < 0.05$). Supplementing the semen extender with L-glutamine showed a higher total antioxidant status (TAS) concentration compared to the control group ($P < 0.05$). As a result of this study, a higher protective effect was found in all the spermatological evaluations after thawing the frozen semen samples, especially in the group containing L-glutamine (20 mM).

Acrosome integrity, membrane integrity, TAS, TOS

Dog breeders have shown increasing interest in reproductive biotechnology, but the assisted breeding techniques for dogs are limited to artificial insemination and semen protection (Gobello and Corrada 2003). Sperm cryopreservation decreases sperm motility and fertility by causing the formation of reactive oxygen species (ROS). However, identifying the important stages in this process has contributed to the prevention of oxidative stress (Lucio et al. 2016a). Lipid peroxidation (LPO) in the freeze-thawed semen samples caused higher concentrations of intracellular hydrogen peroxide along with DNA fragmentation compared to fresh semen in dogs (Kim et al. 2010). Many researchers have focused on using antioxidants as preservatives to reduce the negative effects of ROS on sperm cells (Neagu et al. 2010; Lucio et al. 2016b).

The cytoplasm of mammalian sperm cells decreased during the maturation phase of spermatogenesis. Due to the predominantly cytoplasmic nature of the antioxidant defense systems, the cells that are deprived of antioxidant reserves are vulnerable to the harmful effects of lipid peroxidation during cryopreservation (Iqbal et al. 2016). Hence, to reduce the negative effects of oxidative stress on semen and to protect the spermatological indices, many studies have documented that adding antioxidants to the extender could be beneficial in preserving the sperm indices after thawing in animals such as rams (Gungor et al. 2018), bulls (Büyükleblebici et al. 2014), and canines (Caturla-Sánchez et al. 2018).

Trehalose, a disaccharide sugar, has antioxidative properties at low doses, and when used in the diluent, it could protect spermatozoa during cryopreservation by reducing the LPO (Büyükleblebici et al. 2014). Trehalose protected the sperm cells from osmotic stress caused by the freezing process. Also, its cryoprotective effect is attributed to the increased sperm membrane fluidity before the freezing process occurs (Aboagla and Terada 2003;

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Zhang et al. 2016). The post-thaw semen analysis determined that trehalose preserved motility, vitality, and membrane integrity in different species while also reducing oxidative stress (Iqbal et al. 2016; Park et al. 2018; Keskin et al. 2020).

Glutamine has an alpha-amino acid structure and is one of the components of glutathione, which has a significant role in protecting the sperm cells against ROS (Topraggaleh et al. 2014). It is also a major free amino acid found in mammalian semen, mainly in seminal plasma (Setchell et al. 1967). Organisms are known to utilize amino acids to resist cold shock. For example, in the ram, glutamine acted at the extracellular level after the dissolution to improve the semen motility and oxidative stress indicators (Bucak et al. 2009). The addition of glutamine to semen was reported to be beneficial towards the motility and oxidative stress indicators in boar sperm during short-term storage (Wang et al. 2018) and ram semen after the thawing of the frozen sample (Bucak et al. 2009).

This study aimed to test different doses of L-glutamine and trehalose in the canine semen diluent while determining their protective effects on spermatological and biochemical indices of the thawed samples.

Materials and Methods

Chemicals

The chemicals used in the study were procured from Sigma-Aldrich Chemical (St Louis, MO, USA).

Experimental design

Three adult fertile dogs were used in this study. The animals were provided by a private breeder under the guidance of the dog protection association. The Ethics Committee permission was obtained as well (Ethics Committee of Mehmet Akif Ersoy University, No 2016/217).

Collection and preparation of semen

The study was continued for four weeks and one ejaculate per week was collected. From the collected semen, the ones with more than 75% motility and a concentration of 200×10^6 spermatozoa/ml were used. The tris base extender (TBE) was used to dilute the semen (tris 297.58 mM, citric acid 96.32 mM, fructose 82.66 mM, 20% egg yolk, 6% glycerol). The five groups formed based on the additives were as follows:

| | |
|-------------------|-------|
| TBE + L-glutamine | 10 mM |
| TBE + L-glutamine | 20 mM |
| TBE + trehalose | 25 mM |
| TBE + trehalose | 50 mM |

No additives (control)

The samples were loaded onto 0.25 ml straws and equilibrated for 1.5 h at 5 °C. The loaded straws were frozen in liquid nitrogen vapor (15 min) and stored in liquid nitrogen (-196 °C). After 48 h of freezing, the straws were thawed in a water bath for 30 s at 38 °C for spermatological and biochemical evaluations.

Sperm motility

The motility of spermatozoa was determined using a phase-contrast microscope. For the subjective evaluation, at least five microscopic areas were scanned at $\times 400$ magnification, and the mean score was determined.

Plasma membrane integrity

Semen samples were diluted with the tris stock solution at a ratio of 1:3 using a modified staining protocol of Garner and Johnson (1995). Thirty μ l of the diluted sample were mixed with 6 μ l of SYBR-14 and 2.5 μ l of propidium iodide (PI) and then incubated at 37 °C in the dark for 20 min. The process was completed by adding 10 μ l of Hancock solution.

Acrosome integrity

The method by Nagy et al. (2003) was used to determine the integrity of the acrosome. The semen was diluted with the tris diluent at a ratio of 1: 3. Then, 60 μ l of semen were mixed with 10 μ l of FITC-PNA and 2.5 μ l of PI and incubated at 37 °C in the dark for 20 min. The process was completed by adding 10 μ l of Hancock solution (Schafer and Holzmann 2000).

Semen preparation for enzymatic assays

To separate the spermatozoa, the ejaculate was centrifuged at 800 g for 15 min at 4 °C and then washed thrice with phosphate buffered saline (PBS: pH 7.2-7.6; P4417). After the last centrifugation, 0.5 ml of PBS was added

to the suspension. For homogenization, the sperm suspension was placed in a 2 ml ice water chamber and then subjected to 8 repetitions of sonication for 4 s each.

Analysis of the total antioxidant status of semen

The antioxidative status (TAS) of sperm samples was determined using the method developed by Ereli (2004). It is important to perform these tests in various biological fluids with high reliability and speed. In this method, the colour of the sample changes into a dark blue-green colour, which is measured as the absorbance change. The calibration analysis at 660 nm was performed with a stable antioxidant standard solution.

Analysis of the total oxidant status of semen

The total oxidant levels (TOS) of semen samples were determined by the measurement method specified by Ereli (2005). The oxidants present in the samples at acidic conditions could oxidize the ferrous ion-chelator complex to ferric ion. The colour intensity determined using the spectrophotometric method was proportional to the total amount of oxidant molecules in the sample. Test results were expressed as $\mu\text{mol H}_2\text{O}_2$ equivalent per litre at 530 nm.

Statistical analysis

Normality and homogeneity of variances were verified using Shapiro-Wilk test. The results of thawed sperm samples were expressed as mean \pm standard deviation by one-way analysis of variance (ANOVA). Duncan's post hoc test was used to determine the differences between the groups. Differences with the values of $P < 0.05$ were considered significant. Analyses were made using the SPSS 21 program.

Results

As shown in Table 1, the diluent group that contained 20 mM glutamine showed a higher protective effect on the membrane integrity, motility, and acrosome integrity of the sperms compared to the control group ($P < 0.05$). The highest TAS concentrations were reached in the 20 mM glutamine group, which showed a significant difference compared to the control group. The highest TOS value was reached in the control group, which showed a significant difference compared to all the other groups. We found the 50 mM trehalose group to be useful in the TOS value (Table 2) and it showed a significant difference compared to the control group ($P < 0.05$).

Table 1. The values (mean \pm SD) of the membrane integrity (%), motility (%), and acrosome integrity (%) in the frozen-thawed dog semen samples.

| Groups | Membrane integrity | Motility | Acrosome integrity |
|-------------------|--------------------------------|--------------------------------|-------------------------------|
| L-glutamine 10 mM | 56.22 \pm 5.84 ^{ab} | 52.50 \pm 5.00 ^a | 61.35 \pm 2.76 ^a |
| L-glutamine 20 mM | 61.80 \pm 5.31 ^a | 56.25 \pm 2.50 ^a | 62.00 \pm 4.78 ^a |
| Trehalose 25 mM | 54.78 \pm 6.11 ^{ab} | 48.75 \pm 6.29 ^{ab} | 55.68 \pm 1.87 ^b |
| Trehalose 50 mM | 59.08 \pm 4.12 ^{ab} | 55.00 \pm 4.08 ^a | 60.88 \pm 3.78 ^a |
| Control | 50.68 \pm 4.29 ^b | 43.75 \pm 4.79 ^b | 53.00 \pm 3.53 ^b |

^{a,b} values with different superscripts within columns are significantly different ($P < 0.05$)

Table 2. The values (mean \pm SD) of total oxidant status (TOS), and total antioxidant status (TAS) in the frozen-thawed dog semen samples.

| Groups | TAS (mmol/l) | TOS ($\mu\text{mol/l}$) |
|-------------------|-------------------------------|-------------------------------|
| L-glutamine 10 mM | 0.30 \pm 1.71 ^b | 5.2 \pm 1.31 ^b |
| L-glutamine 20 mM | 0.60 \pm 2.16 ^a | 3.76 \pm 0.90 ^b |
| Trehalose 25 mM | 0.36 \pm 1.63 ^b | 5.18 \pm 1.52 ^b |
| Trehalose 50 mM | 0.43 \pm 2.78 ^{ab} | 3.23 \pm 1.29 ^b |
| Control | 0.25 \pm 2.86 ^b | 12.29 \pm 2.87 ^a |

^{a,b} values with different superscripts within columns are significantly different ($P < 0.05$)

Discussion

Commercial diluents have been developed for domesticated animal species and are used in most production stations as standard diluents. However, such comprehensive field fertility results are not available for dogs. Also, only a little incentive was available to develop standardized semen extenders (Farstad 2009). Although successful results were obtained in sperm cryopreservation, the routine procedure caused serious harmful effects on sperm functions. On the other hand, the additives with antioxidant properties were reported to reduce the effect of ROS and cold shock damages (Amidi et al. 2016).

This study was aimed to determine the effects of antioxidant application on the spermatological indices and oxidative stress while defining a useful diluent for dog sperms. The sperm motility, membrane integrity, acrosome integrity, TAS, and TOS levels were examined after thawing the frozen semen samples. Glycerol and glutamine were used in the semen extender to provide a synergistic cryoprotective role during cryopreservation (Khelifaoui et al. 2005). The testicular damage caused by the chemicals could be prevented by oral glutamine consumption due to its free radical neutralizing properties (Chinoy and Mehta 1999). Many researchers have stated that glutamine is beneficial for the motility of post-thawed sperm, its acrosome, and membrane integrity (Renard et al. 1996; Amirat-Briand et al. 2009; Bucak et al. 2009). Despite similar results, Bucak et al. (2009) reported that glutamine (2.5 and 5 mM) could not provide a preventive effect on the oxidative stress in ram semen, which could be attributed to the fact that glutamine has a toxic effect at a concentration above 20 mM (Wang et al. 2018). Glutamine is biphasic and could not demonstrate sufficient protection at the doses of 5 mM or less. Similarly, our study also showed that 20 mM glutamine had a positive effect on sperm quality compared to the 10 mM glutamine group, which indicates that TAS and TOS levels are important factors too in affecting the quality of the dog sperm after being thawed. The motility and the membrane and acrosome integrity results obtained in our study support this effect. Our results were also in line with the studies conducted by other researchers on the semen of bulls (Dawra et al. 2015) and stallions (Khelifaoui et al. 2005).

Trehalose showed its cryoprotective effect by interacting with the membrane phospholipids to provide stability or by binding to extracellular ice crystals, which was similar to the action of glycerol (Bakas and Disalvo 1991; Gao and Critser 2000). Trehalose is stated to have antioxidant properties to protect the sperm cells from ROS damage (Tuncer et al. 2013). In our study, the addition of trehalose to the canine semen decreased the TOS production after the thawing of the frozen sample. These results were consistent with studies conducted on rabbits (Zhu et al. 2017) and Karan Fries (Chhillar et al. 2012) as well. Plasma membrane integrity and acrosome integrity is important in the evaluation of semen quality (Garner and Johnson 1995). We obtained higher values of motility, membrane integrity, and acrosome integrity in the 50 mM trehalose group compared to the 25 mM trehalose and the control groups, which was consistent with the study on buffaloes by Kumar and Atreja (2012) and Reddy et al. (2010) who showed the benefit of using higher amounts of trehalose. During the cryopreservation, oxidative stress, osmotic stress, or ice crystal formation caused acrosome damage in the cells (Holt 2000). The use of trehalose in the semen extender provided a protective effect on the acrosome integrity of the semen of rams (Bucak et al. 2007) and goats (Quan et al. 2012). Aisen et al. (2005) stated that trehalose added to the diluent preserved the integrity of sperm plasma and acrosomal membranes. This contributed to the organelle protection during the freezing process by intracellular dehydration due to hypertonicity of the diluents and reduced formation of ice crystals. Similar to the above-mentioned studies, our study also showed the cryoprotective effects of trehalose on semen samples.

In conclusion, L-glutamine (20 mM) used in the canine semen extender showed a protective effect on the evaluations of motility, membrane and acrosome integrity after the thawing process. A similar protective effect was achieved in the group with corresponding TAS and TOS measurements. However, upon the usage of low doses of the antioxidants in our study, the protective effect was not observed at the desired level.

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