The impact of addition of ascorbic acid to cryopreservation medium on dog epididymal spermatozoa

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1. Abstract

The objective of this study was to assess the effect of the addition of different concentrations of ascorbic acid to the extender on frozen-thawed epididymal dog spermatozoa. Epididymides from 17 castrated dogs were minced and incubated in a Tris buffer. The retrieved spermatozoa were diluted with Tris based-egg yolk-glycerol extender supplemented with different concentrations of ascorbic acid (0.45 mg/ml and 0.90 mg/ml) and the control (0.0 mg/ml). Diluted samples were equilibrated at 5°C for 2 h, packaged in 0.25 ml straws, and stored in liquid nitrogen (-196°C). After thawing (37°C for 30 s), sperm motility, viability, membrane integrity, acrosome integrity, DNA integrity, and lipid peroxidation by malondialdehyde (MDA) concentration were evaluated. The results were expressed as mean ± SE. Adding 0.90 mg/ml ascorbic acid to the cryopreservation medium significantly (P<0.05) improved motility, viability, membrane and acrosome integrity compared to the control. MDA concentration was significantly (P<0.05) reduced at 0.90 mg/ml related to the control. Percent of DNA damage was significantly ($P<0.05$) reduced in 0.45 mg/ml and 0.90 mg/ml ascorbic acid compared to the control. In conclusion, addition of ascorbic acid (0.90 mg/ml) to TCF extender resulted in a significant increase in the percentage of motility, viability, membrane intact, and acrosome-intact canine epididymal sperm, as well as the maintenance of DNA integrity and the reduction of lipid peroxidation at the membrane level.

Key words: *Dog, Epididymal sperm, Cryopreservation, Ascorbic acid, oxidative stress.*

2. Introduction

In recent years, there has been a significant surge in interest in commercial breeding dogs **[1],** especially in terms of genetic improvement and reproduction of dogs with high zootechnical and economic importance **[2].** Domestic dogs, too, are not only excellent companions but also ideal experimental models due to their reproductive physiology's resemblance to that of wild species and humans. Thus, studies on epididymal sperm in dogs may provide valuable information not only for improving canine reproductive management but also for conserving wild animal health and human reproduction, particularly in males with obstructive azoospermia and oligospermia **[3].**

Canine reproduction may benefit from the retrieval and cryopreservation of the spermatozoa from the cauda epididymides **[4].** This procedure allows for the recovery of viable cells after the death of a valuable dog or an endangered species individual. However, cryopreservation may cause irreversible damage to the sperm membrane, impairing viability. Sperm cells are exposed to a variety of stressors, including thermal shock, the creation of intracellular ice crystals, osmotic shock, and the generation of reactive oxygen species (ROS) **[5, 6].** Due to several intra- and extra-cellular changes during sperm

maturation **[7, 8],** such as the loss of a large amount of cytoplasm (resulting in low antioxidant content) and an increase in plasma membrane polyunsaturated fatty acid proportion, epididymal spermatozoa are highly susceptible to oxidative imbalance **[9, 10].** Furthermore, lipid peroxidation products such as malondialdehyde (MDA) can be as harmful as ROS, producing DNA fragmentation and decreased fertilization potential **[11].**

To counteract the negative effects of ROS, the inclusion of various antioxidants in the freezing extenders increases the semen quality after thawing. Antioxidants are the most important defense mechanisms against free radical-induced oxidative stress **[12].** Antioxidants work as chelators or binding proteins, and their three major jobs are to prevent ROS from being produced and to remove ROS that is already present **[13].** Ascorbic acid is a water-soluble chain-breaking antioxidant with high effectiveness and low toxicity that can scavenge oxygen radicals **[14].** The extender supplemented with ascorbic acid improved bovine sperm motility, acrosome, and membrane integrity, and resulted in a decrease in GSH-Px (glutathione peroxidase) in contrast to an increase in GSH (glutathione) [**15].** Similarly, the addition of ascorbic acid before cryopreservation reduced DNA damages exclusively in infertile males **[16],** presumably due to larger DNA damages in infertile men than fertile men **[17].**

Since epididymal spermatozoa are more susceptible to ROS than ejaculated spermatozoa**.** It's worth looking into whether ascorbic acid can protect epididymal spermatozoa against cryoinjury. Therefore, the present study aimed to scrutinize the effect of ascorbic acid on canine epididymal spermatozoa motility, viability, membrane integrity, acrosome integrity, DNA integrity, and membrane lipid peroxidation levels after cryopreservation and thawing.

3. Materials and Methods

All experimental procedures were approved by the Ethics Committee for Animal Use at the Faculty of Veterinary Medicine, Cairo University, Egypt (approval number: Vet CU28/04/2021/300). This study was carried out at Theriogenology Department, Faculty of Veterinary Medicine, Cairo University and at the Artificial Insemination Department, Animal Reproduction Research Institute, Agricultural Research Centre at Giza. Unless otherwise stated, chemicals were purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA).

3.1. Animals

Testes and their epididymides were obtained from 17 clinically healthy dogs of various breeds (age range 2-5 years, bodyweight 25-45 kg) after a routine castration at the dog shelter of the Egyptian Society of Mercy to Animals (ESMA). Clinical examination of their genital organs showed no obvious abnormalities and revealed their soundness. Dogs were housed in individual boxes of concrete floor with an outdoor and covered shelter that avoided direct sunlight. Diets offered to dogs consisted of commercial dry food (Royal Canin®) once daily and water offered *ad libitum*.

3.2. Harvesting epididymal sperm

Following castration, each dog's testis, epididymis, and surrounding tunics were placed in a thermos flask containing sterile 0.9 % NaCl and kept at 4ºC for 12 hours before being brought to the laboratory. The tunics were removed from each testicle for processing, with special care taken not to harm the epididymis where it connects to the testis. Using a curved scissor, the blood arteries leading to the testis and the vas deferens were clipped away along the natural curve of the testicle. The cauda epididymis was detached from each testis and minced with a scalpel blade on a clean, dry Petri plate with 4 ml of Tris buffer **[4].**

The minced epididymides were incubated for 30 minutes at 37 °C in Tris buffer [3.025 g Tris, 1.7 g citric acid, 1.25 g fructose, Na-benzyl-penicillin (100,000 IU), and dihydrostreptomycin sulphate (100 mg)] in 100 ml distilled water **[18].** Tissue pieces were removed by filtration through 80 µm metal meshes and the sperm suspensions were collected into a calibrated plastic tube. The recovered spermatozoa from the left and right cauda epididymis were examined for motility and the percentage of live spermatozoa. Samples showing more than 70% motility and 75% live sperm were selected for processing **[19].**

3.3. Sperm processing

The collected sperm suspension was diluted with Tris-Citric acid-Fructose extender (TCF) to a final concentration of 300×10^6 sperm/ml. TCF extender was composed of Tris (3.025 g), monohydrated citric acid (1.7 g) and D-fructose (1.25g) in 100 ml of ultrapure distilled water. Egg yolk (20%), glycerol (7%) and antibiotics [Na-benzyl-penicillin (100,000 IU) and dihydrostreptomycin sulphate (100 mg)] were added to the composed extender and the pH of the solution was adjusted to 6.74 **[20].** The diluted spermatozoa were divided into three aliquots and L-Ascorbic acid (VWR international bvba, Belgium) was added as follow: 0.0 mg/ml (the control), 0.45 mg/ml and 0.90 mg/ml **[21].** Then, the diluted spermatozoa were cooled to 5ºC gradually and maintained at this temperature for 2 hours to equilibrate **[22],** packaged in 0.25 ml straws (IMV, L'Aigle, France) and frozen horizontally 5 cm above liquid nitrogen vapor for 10 min in a foam box filled with the coolant **[23].** Straws were then rapidly plunged into the liquid nitrogen, transferred to a storage tank and left there for at least one week before thawing. Thawing was performed at 37 ºC for 30 seconds **[24].**

3.4. Evaluation of frozen-thawed spermatozoa

3.4.1. Sperm progressive motility

For motility assessment, a small drop of frozen-thawed epididymal spermatozoa was placed onto a pre-warmed glass slide, covered with a coverslip, and examined under a hot stage microscope (38 ºC) at 40x objective. Progressive motile spermatozoa were examined subjectively in at least five microscopy fields and expressed as a percentage.

3.4.2. Sperm viability

The viability of sperm was determined using the Eosin-Nigrosin staining technique **[25].** The stain was made up of Eosin-y (1.67 g) and Nigrosin (10 g) in 100 ml of 2.90% sodium citrate buffer. Two hundred spermatozoa per slide of live sperm (non-stained head) and dead sperm (red head) were evaluated by a light microscope with a 40x objective.

3.4.3. Plasma membrane integrity

The plasma membrane integrity of canine epididymal spermatozoa was assessed by the hypo-osmotic swelling (HOS) test **[26].** The solution of HOS consisted of sodium citrate (0.73 g) and fructose (1.35 g), dissolved in 100 ml of distilled water. The assay was carried out by mixing 50 µl of frozen-thawed canine epididymal sperm sample with 500 µl of HOS solution and incubating it for 40 minutes at 37 ˚C. After incubation, a drop of the sample was examined under a light microscope with a 40x objective. Two hundred spermatozoa were counted for their swelling. Spermatozoa with coiled or swollen tails had functional intact membranes.

3.4.4. Sperm acrosome integrity

The acrosome integrity of canine epididymal spermatozoa was assessed by Trypan Blue-Giemsa (TBG) stain: Staining was performed as described by **[27].** One drop of trypan blue and one drop of the sample were mixed on a glass slide and distributed over the entire surface with another slide. Slides were air-dried vertically, then placed in a jar of fixative for two minutes before being rinsed with tap

and distilled water. Slides were placed in jars containing Giemsa staining solution and left at room temperature overnight (16 to 20 hours). Slides were washed again with tap and distilled water before being air-dried vertically. Sperm cells were examined under a microscope at 100x oil immersion magnification. Spermatozoa were evaluated as acrosome intact (light purple) and damaged/lost acrosome (unstained or blue acrosome).

3.4.5. Evaluation of lipid peroxidation

MDA concentration as a lipid peroxidation marker was measured as described previously **[28]** in canine epididymal sperm samples. Using commercial kits (Biodiagnostic, Egypt), thiobarbituric acid (TBA) interacts with MDA in an acidic medium for 30 minutes at 95°C to create a thiobarbituric acid reactive product. Using a spectrophotometer (UV-1800PC, Shanghai Mapada Instruments Co., Ltd, China), the absorbance of the pink product was measured at 534 nm The MDA concentrations in the samples were measured in nmol/ml.

3.4.6. Sperm DNA integrity (Comet assay)

Single-cell gel electrophoresis (alkaline comet test) was used to examine the DNA integrity of frozen-thawed canine epididymal spermatozoa, according to **[29].** Sperm samples were washed with phosphate buffer saline (PBS) mixed with 50 µl of 1% low melting point agarose and placed onto frosted microscope slides pre-coated with 50 µl of 1% normal melting point agarose. The slides were lysed for 1 hour in lysis buffer. Electrophoresis was performed on the lysed cells. Following neutralization in Tris solution, the slides were stained with ethidium bromide (pH 7). A fluorescent microscope (Olympus, Japan) was used to study 200 sperm cells at a magnification of 400x. The amount of DNA present was expected to be proportional to the intensity of the stain in the comet tail area. Based on measurements of the DNA % in the tail, tail length, and olive tail moment, image analysis software was utilized to evaluate DNA damage (Comet-Score program). Spermatozoa with fragmented DNA had a higher rate of DNA migration from the nucleus to the anode, but spermatozoa with intact DNA did not produce a "comet" **[30].**

3.5. Statistical analysis

All values were expressed as mean ± standard error (SE) of five replicates. Statistical analyses were performed using SPSS, (ver. 25.0; SPSS Inc., Chicago, IL). Comparisons between the sperm quality parameters were done using simple one way ANOVA followed by Duncan's comparison test. Significance was set at least at $P<0.05$. All statistical analysis were carried out according to **[31].**

4. Results

4.1. Sperm characteristics and MDA concentration in freeze-thawed epididymal dog sperm

Table 1 showed the post-thaw epididymal dog sperm motility, viability, membrane integrity, and acrosome integrity, as well as MDA content at two ascorbic acid doses (0.45 and 0.90 mg/ml) in TCF extender and the control.

Supplementing TCF extender with 0.90 mg/ml ascorbic acid significantly ($P<0.05$) enhanced post-thaw motility, viability, and membrane integrity as compared to the control. There were no significant variations in post-thaw motility, viability, or membrane integrity between spermatozoa supplemented with 0.45 mg/ml ascorbic acid and control. On the other hand, the percentage of sperm acrosome integrity was significantly ($P<0.05$) higher in 0.90 mg/ml and 0.45 mg/ml ascorbic acid containing extenders than the control.

MDA generation was much lower at 0.90 mg/ml ascorbic acid and was significantly ($P<0.05$) different from the control group implying that 0.90 mg/ml ascorbic acid prevented lipid peroxidation at the membrane level. The difference between

ascorbic acid 0.45 mg/ml and the control is not statistically significant.

4.2. DNA integrity

Images of frozen-thawed dog epididymal spermatozoa tested for DNA integrity using the alkaline comet assay are shown in Figure 1. The results of the comet assay data analysis (percentage of DNA damage, DNA in tail, tail length, tail moment and olive tail moment) were presented in table 2. Supplementing TCF extender with 0.90 mg/ml and 0.45 mg/ml ascorbic acid significantly ($P<0.05$) reduced the percentages of DNA damage compared to the control. The percentage of DNA in tail was significantly ($P<0.05$) lower at 0.90 mg/ml ascorbic acid than the control. Whereas, tail length was significantly ($P<0.05$) higher at 0.90 mg/ml ascorbic acid than the control. Tail moment, and olive tail moment were significantly ($P<0.05$) lower at 0.90 mg/ml ascorbic acid than at 0.45 mg/ml ascorbic acid and the control.

5. Discussion

It is widely assumed that a significant proportion of spermatozoa were destroyed during the freezing and thawing processes **[32].** Although the mechanisms causing these impairments are assumed to be complex, increased production of ROS has been suggested as a major contributor. Spermatozoa have a unique cell shape and plasma membrane, as well as a large number of mitochondria, little cytoplasm, and low antioxidant levels in their cytoplasm, making them potentially sensitive to free radical damage **[33].** Lipid peroxidation occurs when partially reduced oxygen molecules, such as superoxide, hydrogen peroxide, and hydroxyl radicals, oxidize membrane lipids, making mammalian spermatozoa extremely vulnerable. The structure of the lipid matrix is destroyed by spontaneous lipid peroxidation of the membranes of mammalian spermatozoa. Through oxidative stress and the generation of cytotoxic aldehydes, these attacks eventually compromise sperm function, such as motility, functional membrane integrity, and fertility, as well as leakage of intracellular enzymes and damage to sperm DNA **[34, 35, 36].** In an attempt to offset the cellular damage produced by cryopreservation, a number of cryoprotective media, most of which are fortified with antioxidants, have been used**.** Antioxidants are the most important defense mechanisms against free radical-induced oxidative damage **[12].** Ascorbic acid has been used as an antioxidant addition and has been demonstrated to have protective properties when added to an extender due to its low toxicity and good water solubility. In the present study, the effects of two concentrations of ascorbic acid (0.45 and 0.90 mg/ml) in TCF extender on motility, viability, membrane integrity, acrosome integrity and DNA integrity of dog epididymal sperm were investigated. The results revealed a considerable improvement in progressive motility with the addition of 0.90 mg/ml of ascorbic acid. This results were consistent with the findings of other authors who found that supplementing ascorbic acid at the time of extension increased progressive sperm motility in canine sperm **[37],** Awssi ram sperm **[38],** and bull sperm **[39].** In this study, the percentage of viable spermatozoa in 0.90 mg/ml ascorbic acid treated samples were likewise significantly higher than control which is in agreement with **[37]** who reported that canine semen treated with 0.5 mM ascorbic acid and polyphenol showed enhanced sperm viability and motility. Furthermore, ascorbic acid improved plasma membrane integrity of epididymal dog sperm in our study, which is consistent with the findings of **[40]** who reported that ascorbic acid protects sperm membrane integrity in diluted stallion sperm. Also supplementing TCF extender with ascorbic acid (0.90 mg/ml) greatly enhanced the percentage of intact acrosomes in dog epididymal spermatozoa, indicating that ascorbic acid protected the

acrosome and acrosomal membrane during freezing. In agreement with **[41]** who found a considerable improvement in the percentage of intact acrosomes in frozen-thawed bovine semen: Meanwhile, **[42]** found a significant improvement in buffalo semen after adding vitamin C to the semen extender. Furthermore, **[21]** revealed that utilizing ascorbic acid significantly boosted the percentage of live and acrosome intact dog spermatozoa at 48 and 72 hours when compared to controls. On the other hand, the latter authors stated that adding ascorbic acid to the extender did not increase the proportion of motile spermatozoa during the study period.

The positive effects of ascorbic acid can be ascribed to the fact that it is a very powerful antioxidant and a scavenger of oxygen free radicals, which are hazardous byproducts of numerous metabolic processes **[43].** Besides, it protects superoxide dismutase activity and regenerates other antioxidative systems **[41].** Vitamin C protects lipoproteins against detectable peroxidative degradation by scavenging superoxide anions and singlet oxygen **[44].** This could explain why ascorbic acid (0.90 mg/ml) in TCF extender had a beneficial effect on lipid peroxidation in the current study. The current study results showed that ascorbic acid reduced MDA content, which is consistent with earlier research on dog semen **[45],** bull semen **[46],** stallion semen **[40],** boar semen [**47]** and ram semen **[48].** These results are similarly consistent with those of **[41]** and **[49, 50],** who found that adding vitamin C and glucose to buffalo spermatozoa decreased MDA formation and enhanced percent motility and live spermatozoa, respectively. These findings could be explained by the fact that vitamin C protects spermatozoa against endogenous oxidative DNA and membrane damage. In agreement with the present findings, **[51]** found a substantial improvement in progressive sperm motility, viability percent, acrosomal integrity, and MDA in buffalo bull semen supplemented with vitamin C, which is consistent with the current findings. In contrast to the current findings, **[45]** found that adding the following doses of vitamin C (0.1, 0.5, 1 or 2.5 mM) to a Tris-glucose-egg yolk extender does not improve the quality of canine spermatozoa. This, however, could be due to discrepancies in the concentrations of vitamin C used and the manner in which the sperm cells were collected.

The quality and functional integrity of frozen-thawed sperm is determined by DNA integrity **[52].** In goats, a link between sperm DNA integrity and fertility has been described **[52].** Cryopreservation has been shown to damage sperm DNA integrity in rabbits **[53],** rams **[54],** boars **[55],** and humans **[56].** The harmful effects of freezing appear to have been caused by alterations in DNA chromatin structure **[57].** In dogs, it is not suggested to remove plasma from the second fraction of the ejaculate before cryopreservation since samples frozen with prostatic fluid demonstrated higher DNA stability because DNA breakage in cryopreserved spermatozoa may be induced by oxidative damage caused by an imbalance between ROS and antioxidants, the protective effect of seminal plasma on DNA may be connected to the presence of antioxidants in the plasma **[58, 59, 60].** Since epididymal spermatozoa do not benefit from the antioxidant action of seminal plasma, they may be prone to oxidative stress during cryopreservation. In the present study, the comet assay was used for the assessment of the DNA status of canine frozen-thawed epididymal spermatozoa and revealed that ascorbic acid 0.90 mg/ml significantly improved DNA integrity of epididymal dog sperm. In a human investigation, patients with low levels of seminal ascorbic acid had a much higher percentage of men with defective sperm DNA integrity than those with normal or high levels **[61].** The addition of ascorbic acid had no harmful effects on sperm DNA, as previously stated for human spermatozoa **[62].** The current study's findings indicate that the comet

assay can be utilized as a supplementary parameter for assessing the quality of epididymal dog after cryopreservation.

6. Conclusion

Addition of ascorbic acid (0.90 mg/ml) to TCF extender resulted in a significant increase in the percentage of motility, viability, membrane intact, and acrosome-intact of canine epididymal sperm, as well as the maintenance of DNA integrity and the reduction of lipid peroxidation at the membrane level.

7. References

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Table 1: Effects of the addition of ascorbic acid to the cryopreservative medium on sperm progressive motility, viability, membrane integrity and acrosome integrity, and MDA concentration of frozen-thawed epididymal dog sperm.

Groups	Sperm progressive motility (%)	Sperm viability (%)	Sperm membrane integrity (%)	Sperm acrosome integrity (%)	MDA concentration (nmol/ml)
Control (0.0 mg/ml)	20.00 ± 2.23 [b]	38.20 ± 1.28 [b]	36.40 ± 1.20 [b]	41.80 ± 2.15 [b]	3.70 ± 0.14 [b]
Ascorbic acid (0.45 mg/ml)	23.00 ± 3.39 [b]	41.40 ± 1.36 [b]	38.20 ± 1.28 [b]	49.40 ± 1.36 [a]	3.33 ± 0.09 [b]
Ascorbic acid (0.90 mg/ml)	34.00 ± 1.87 [a]	51.20 ± 2.17 [a]	50.80 ± 1.15 [a]	52.40 ± 1.28 [a]	2.46 ± 0.13 [a]

Data were expressed as mean ± SE; means with different superscripts (a, b) within the same column are significantly different at least at P<0.05.
Number of samples examined= 170.
MDA: Malondialdehyde.

Table 2: Effects of ascorbic acid on DNA integrity of frozen-thawed epididymal dog sperm.

Groups	DNA damage (%)	DNA in tail (%)	Tail length (px)	Tail moment	Olive tail moment
Control (0.0 mg/ml)	20.50 ± 0.70 [a]	10.00 ± 0.70 [a]	6.37 ± 0.62 [b]	1.15 ± 0.12 [a]	1.58 ± 0.08 [a]
Ascorbic acid (0.45 mg/ml)	16.27 ± 0.73 [b]	8.82 ± 0.86 [a]	8.66 ± 0.52 [ab]	1.07 ± 0.09 [a]	1.47 ± 0.05 [a]
Ascorbic acid (0.90 mg/ml)	16.30 ± 0.60 [b]	5.68 ± 0.85 [b]	10.36 ± 0.74 [a]	0.65 ± 0.06 [b]	1.06 ±0.07 [b]

Data were expressed as mean ± SE; means with different superscripts (a, b) within the same column are significantly different at least at P<0.05.
Number of samples examined= 170.

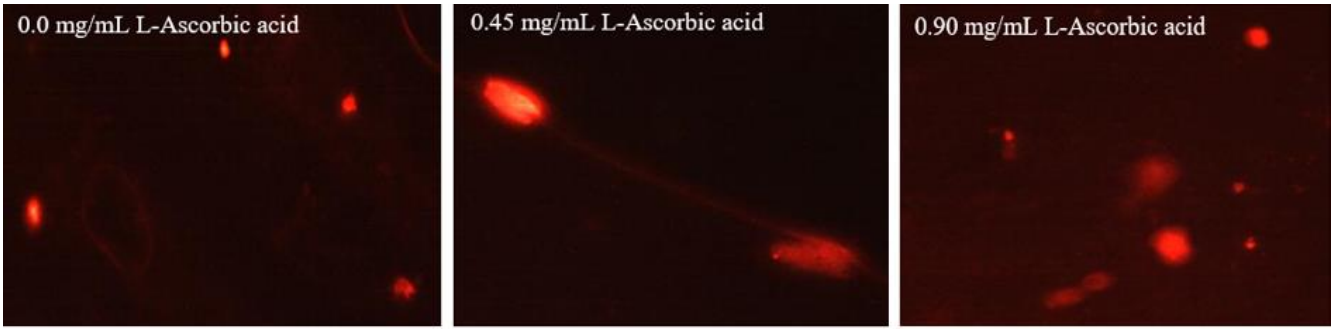

Fig .1: Images demonstrating the influence of L-Ascorbic acid addition to TCF extender on DNA integrity of frozen-thawed epididymal dog spermatozoa evaluated by comet assay (single-cell gel electrophoresis).