Supplementation of plant-based cryopreservation medium with folic acid conserves the quality of bulk post-thawed spermatozoa

Abdollahi, Z., Zeinoaldini, S.*, Zhandi, M., Towhidi, A., Baghshahi, H.

Department of Animal Science, Campus of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

Email: zeinoaldini@ut.ac.ir

Abstract

The aim of the current study was to evaluate the effect of cryopreservation medium supplementation with folic acid as an antioxidant on post-thawed semen quality in bulk. Semen samples were collected from four proved Iranian Mahabadi bulkes and diluted in extender containing 1.5 % soybean lecithin. The diluted semen was assigned into six parts and supplemented with different doses of folic acid as follows: FA0 (extender without folic acid), FA0.05, FA0.1, FA0.2, FA0.4 and FA0.8 (extenders containing 0.05, 0.1, 0.2, 0.4, 0.8 mM folic acid, respectively). Then, the semen samples were cryopreserved in liquid nitrogen. Sperm motility and velocity parameters, membrane integrity, abnormal morphology, viability and lipid peroxidation were evaluated after thawing. In results, FA0.05 presented higher (P≤0.05) total motility, progressive motility, membrane integrity, viability and lower lipid peroxidation compared to other groups. Abnormal morphology was not affected (P>0.05) by treatments. In conclusion, supplementation of cryopreservation medium with 0.05 mM folic acid is a helpful method to conserve the quality of post-thawed semen in bulk.

Keywords: Bulk, Cryopreservation, Folic acid, Sperm, Soybean lecithin.

Introduction
During cryopreservation process, production of reactive oxygen species (ROS) causes adverse effects on the sperm membrane, DNA, and physiological processes and thereby affects the sperm quality. The sperm plasma membrane contains significant amounts of polyunsaturated fatty acids and is highly susceptible to ROS (Bansal and Bilaspuri, 2011). The quantity of adenosine triphosphate (ATP) is drastically reduced by increasing lipid peroxidation, associated with axonemal damage and increased midpiece and tail morphological defects, leading to impaired motility and decreased sperm viability (Agarwal et al., 2014).

The specific structure of sperm cells and the plasma membrane, large numbers of mitochondria, and low cytoplasm and antioxidant levels in the sperm cytoplasm result in sperm exposure to free radical damage (Betteridge, 2000). The mechanism of ROS-induced damage to sperm cells involves an oxidative attack against sperm membrane lipids, which leads to the onset of a cascade of lipid peroxidation (LPO). The sensitivity of ruminant sperm to oxidative stress is a result of the abundance of polyunsaturated fatty acids (PUFAs) in the plasma membrane of sperm cells in the ruminants. The goat sperm cells also contain a large number of PUFAs and is sensitive to temperature changes (Ren et al., 2018).

Under normal circumstances, to counteract the damaging effects of ROS, the sperm and semen plasma contain a number of antioxidant systems that degrade ROS and prevent internal cell damage. Antioxidants are major defense agents against oxidative stress, induced by free radicals. There are two types of antioxidants, that is, enzymatic and non-enzymatic. Enzymatic antioxidants are also known as natural antioxidants and include glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), and catalase, all of which contribute to the natural sperm antioxidant defense. On the other hand, non-enzymatic antioxidants, also known as synthetic or complementary antioxidants, include glutathione (GSH), urate, ascorbic acid, vitamins, carotenoids (carotene), ubiquinones, taurine, selenium, zinc, and so forth (Amidi et al., 2016).
The folic acid coenzyme catalyzes reactions, related to the synthesis of nucleic acids and proteins. It is a B-group vitamin, transformed into another coenzyme (N5,N10-methylene-5,6,7,8-tetrahydrofolic acid) by a sequence of enzyme transformations in vivo. Although folic acid is significantly soluble in water, its lipid peroxidation inhibitory activity has been reported to be strong. Therefore, folic acid can protect biological compounds, such as cell membranes or DNAs against free radical damage (Joshi et al., 2001).

To the best of our knowledge, no research has been yet conducted on the goat spermatozoa to control oxidative stress in the freezing process by folic acid. Therefore, folic acid at optimal levels is expected to be effective in increasing the quality of goat sperm quality parameters after freezing and thawing. Therefore, the present study was aimed to investigate the effects of different concentrations of folic acid on the sperm quality parameters of Mahabadi goat, including sperm motility, membrane integrity, abnormal morphology, viability, and malondialdehyde (MDA) concentration after cryopreservation process.

Materials and Methods

Chemicals

All chemicals used in this study were obtained from Sigma–Aldrich® (USA).

Animals

Semen samples were collected from four mature Mahabadi goats with proven fertility (with fresh semen), showing a minimum of 80% sperm motility, morphologically normal spermatozoa, and a total sperm concentration of at least 4×10^9 spermatozoa/ml. All experimental animals were maintained under the same conditions with uniform feeding at the university of Tehran farm (37°47 N, 50°55 E). Semen samples were collected with an artificial vagina twice a week for four weeks during the breeding season. To eliminate individual differences, semen samples were pooled from the four goats.

Semen processing
Sperm samples were diluted using a soybean lecithin–based extender, composed of soy lecithin (1.5% w/v), citric acid (16.4 g/l), fructose (12.6 g/L), tris buffer (30.7 g/L), and glycerol (5% v/v). The osmolarity and pH were set at 320-325 mOsm and 6.5-7.5, respectively. Different levels of folic acid (0.8, 0.4, 0.2, 0.1, and 0.05 mM folic acid and witness group) were added to the basic extender. After dilution, the semen samples were inserted into 15-ml plastic Falcon tubes in a refrigerator at 4°C for two hours and then drawn into 0.25-ml French straws (IMV, L’aigle, F-61300, France). The straws were refrigerated at 4°C for two hours before filling. Subsequently, they were exposed to liquid nitrogen vapor (3 cm above liquid nitrogen) for 15 minutes. They were then immersed into liquid nitrogen at -196°C and stored. For post-thawing sperm evaluation, the frozen straws were thawed individually at 37°C for 30 seconds in a water bath.

**Sperm motility**

Motility characteristics were assessed via Sperm Class Analysis software (SCA, Version 5.1; Microptic, Barcelona, Spain) and the following items were recorded: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s) and amplitude of lateral head displacement (ALH, µm).

**Sperm lipid peroxidation**

The rate of lipid peroxidation in the semen samples was measured by determining the MDA concentration (Masoudi et al., 2019a). The lipid peroxidation product (MDA) was estimated in biochemical systems. To precipitate protein, 1 ml of semen and 2 ml of 20% trichloroacetic acid were blended and centrifuged at 900 g for 15 minutes. The supernatant (1 µl) was incubated with 1 ml of 0.67% thiobarbituric acid in boiling water for ten minutes. After cooling down to room temperature, absorbance was read with a spectrophotometer at 532 nm.

**Plasma membrane integrity**
The hypoosmotic swelling (HOS) test was used to assess the plasma membrane integrity of spermatozoa after the freeze-thawing process (Masoudi et al., 2019b). For this purpose, 4.9 g/l of sodium citrate dihydrate and 9 g/l of D-fructose were dissolved in 100 ml of distilled water (osmolarity, 100 mOsm/kg). The assay was carried out by adding 30 µl of semen to 300 µl of HOS solution, followed by incubation at 37°C for 20 minutes. Next, a volume of 10 µl was placed on a warm slide, mounted with a coverslip, and examined under a phase contrast microscope at 400× magnification. Two-hundred spermatozoa per slide were counted in five different microscopic fields, and the percentage of sperms, showing curled/swollen and non-swollen tails, was considered as having intact and damaged plasma membranes, respectively.

**Abnormal morphology**

Hancock solution was used to evaluate post-thawed sperm abnormal morphology (Masoudi et al., 2019c). Therefore, a drop of thawed sample was placed to eppendorf tube contained 1 ml of Hancock solution [150 ml sodium saline solution, 500 ml double-distilled water, 62.5 ml formalin (37%), and 150 ml buffer solution]. Then, 300 spermatozoa were counted under a microscope and the percentage of sperm cells with abnormal heads and/or tails were recorded.

**Sperm viability**

Sperm viability was determined by Eosin Y 0.5%-Nigrosin 0.1% staining (Masoudi and Davachi, 2020). To prepare the sperm smear, one drop (10 µl) of semen was mixed with two drops of the stain on a warm slide. After air-drying, at least 200 sperm cells were evaluated in four different microscopic fields on each slide with a phase microscope (400× magnification). Since the dye only penetrated into the damaged membrane of spermatozoa, sperm cells that absorb the eosin-nigrosin solution were recorded as dead sperm cells.

**Statistical analysis**

The normal distribution of data was examined, using univariate and Shapiro-Wilk tests. Data were evaluated, using the general linear model (GLM) in the Statistical Analysis System
software (SAS, 2003). The Tukey’s test was also used to examine differences between the mean concentrations. The results are described as the least squares mean and standard error of the mean (SEM). Differences were considered statistically significant at P ≤ 0.05.

Results

Motility characteristics and lipid peroxidation

Table 1 shows the effects of different concentrations of folic acid on motility parameters and MDA concentration of bulk post-thawed sperm cells. The TM and PM of bulk post-thawed sperm cells was higher (P ≤ 0.05) in AF0.05 compared with the other groups. The groups AF0.1 and AF0.2 showed higher (P ≤ 0.05) TM and PM than AF0, AF0.4 and AF0.8 groups. There was no significant difference (P > 0.05) among AF0, AF0.4 and AF0.8 groups in cases of TM and PM. No significant difference (P ≤ 0.05) was observed among treatment groups for items of VAP, VSL, VCL, and ALH.

The MDA concentration in the AF0.05 group was lower (P ≤ 0.05) than in the other groups. The groups AF0.1 and AF0.2 showed lower (P ≤ 0.05) MDA concentration than AF0, AF0.4 and AF0.8 groups. There was no significant difference (P > 0.05) in MDA concentration among AF0, AF0.4 and AF0.8 groups.

Table 1: Effects of different concentrations of folic acid (mM) on the motility parameters and malondialdehyde concentration of bulk frozen-thawed semen.

<table>
<thead>
<tr>
<th>Variables</th>
<th>AF0</th>
<th>AF0.05</th>
<th>AF0.1</th>
<th>AF0.2</th>
<th>AF0.4</th>
<th>AF0.8</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM (%)</td>
<td>50.2c</td>
<td>60.5a</td>
<td>55.7b</td>
<td>56.1b</td>
<td>51.3c</td>
<td>49.5c</td>
<td>1.3</td>
</tr>
<tr>
<td>PM (%)</td>
<td>25.8c</td>
<td>35.2a</td>
<td>31.5b</td>
<td>30.6b</td>
<td>26.7c</td>
<td>26.0c</td>
<td>1.5</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>85.5</td>
<td>87.0</td>
<td>86.3</td>
<td>86.0</td>
<td>84.7</td>
<td>85.4</td>
<td>2.0</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>70.4</td>
<td>71.7</td>
<td>70.6</td>
<td>69.2</td>
<td>70.6</td>
<td>70.0</td>
<td>1.1</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>162.5</td>
<td>164.2</td>
<td>163.7</td>
<td>163.0</td>
<td>161.5</td>
<td>162.4</td>
<td>2.1</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>7.6</td>
<td>8.0</td>
<td>7.8</td>
<td>7.5</td>
<td>7.1</td>
<td>7.0</td>
<td>0.7</td>
</tr>
<tr>
<td>MDA (nmol/ml)</td>
<td>2.72c</td>
<td>1.42a</td>
<td>1.62b</td>
<td>1.88b</td>
<td>2.39c</td>
<td>2.42c</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Means ± SEM of total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and MDA concentration for different folic acid concentrations.
Malondialdehyde concentration (MDA) were assessed after thawing. Different letters showed significant differences at \( P \leq 0.05 \) among groups.

**Membrane integrity, abnormal morphology and viability**

Figure 1 presents the effect of different concentrations of folic acid on membrane integrity, abnormal morphology and viability of bulk post-thawed sperm cells. Membrane integrity was higher \( (P \leq 0.05) \) in AF0.05 compared to the other groups. The groups AF0.1 and AF0.2 showed higher \( (P \leq 0.05) \) membrane integrity than AF0, AF0.4 and AF0.8 groups. The difference among AF0, AF0.4 and AF0.8 groups was not statistically significant \( (P > 0.05) \).

Supplementation of cryopreservation medium with different concentrations of folic acid did not show any significant effect \( (P > 0.05) \) on the rate of abnormal morphology in bulk post-thawed sperm cells.

The viability rate in bulk post-thawed sperm cells was found higher \( (P \leq 0.05) \) in the AF0.05, AF0.1 and AF0.2 compared to the other groups.

![Fig. 1. Percentage of membrane integrity (MI), abnormal morphology (AM) and viability (V) of bulk spermatozoa after freezing-thawing in the cryopreservation medium. Different letters within the same column show significant differences among the groups \( (P \leq 0.05) \).](image-url)


Discussion

The mammalian spermatozoa contain high levels of unsaturated fatty acids in their membrane layer, which increase their susceptibility to oxidative damage during freezing-thawing. Lipid peroxidation destroys the matrix structure of the sperm membrane, which ultimately results in sperm dysfunctions, such as impaired motility, sperm membrane integrity, sperm fertility, intracellular enzymes, and sperm DNA damage through oxidative stress (Zorn et al., 2003).

Folic acid, as an antioxidant, normally neutralizes oxidized free radicals. Although this antioxidant is known as a water-soluble vitamin, it is established that folic acid can reduce the peroxidation of lipids. Therefore, it can protect biological compounds, such as the cell membrane or DNA against free radical damage (Joshi et al., 2001). Considering the role of folic acid in the homocysteine-methionine cycle, it can be important in the production of energy, proteins, pyrimidines, and purines. Therefore, it plays an essential role in the construction, methylation, and repair of DNAs (Graulet., 2007; Huang et al., 2013).

The current study aimed to investigate the antioxidant effect of folic acid on the post-cryopreservation and lipid oxidation characteristics. We observed that folic acid samples, compared to the control group, could better improve the sperm parameters after freezing. However, a folic acid level of 0.05 (compared to other levels above 0.8, 0.4, 0.2, and 0.1 mM of folic acid) was associated with improved parameters, such as sperm motility, membrane integrity, and fat peroxidation.

It has been shown that appropriate amounts of ROS are required for many biological processes, such as production of adenosine triphosphate (ATP). In other words, very low levels of ROS can interfere with these essential processes (Bardaweel et al., 2018). Although there is no previous study on the effects of oral folic acid on the mammalian frozen sperm, according to the literature on the oral consumption of folic acid, acid folic is associated with semen parameters. In this regard, a study showed that oral administration of 500 mg of folic acid per
liter for four weeks in rabbits could significantly increase all physical characteristics of semen, including semen volume, sperm concentration, mass sperm motility, and individual motility percentage, and reduce the sperm motility index, dead and abnormal sperm cells, and acrosome sperm damage. These results showed the beneficial effects of antioxidants and folic acid on the semen parameters (El-Ratel, 2017).

Moreover, the results of a previous study showed that addition of folic acid to the diet of New Zealand male rabbits improved the semen characteristics and reduced the level of free radicals (Yousef et al., 2006). Another study aimed to investigate the association of chromatin motility and survival with sperm DNA before and after cryopreservation and to determine the effect of folic acid on the sperm quality after thawing. It was found that addition of 50 nM folic acid as an antioxidant to a sperm cryopreservation medium could improve the sperm motility and viability, compared to the control group. It also significantly increased the quality of sperm DNA and chromatin after thawing, compared to the control group (Rarani et al., 2019).

Another study showed that supplementation of human semen samples with 50 nmol/l of folic acid before freezing increased the semen quality after freezing, compared to the control group in terms of the percentage of sperm motility, percentage of progressive motility, and percentage of live sperm (Khamsuk et al., 2014). These results suggest that folic acid can act as an antioxidant in the sperm cryopreservation medium and were in agreement with the results of the current study.

**Conclusion**

Supplementation of bulk semen cryopreservation medium with optimum dose of folic acid preserved the quality parameters bulk sperm cells after cryopreservation process. Therefore, it could be concluded addition of folic acid to the cryopreservation extender is an efficient strategy to conserve bulk post-thawed semen quality for other goals.

**References**


