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Original Article

Supplementation of Plant-Based Cryopreservation Medium with Folic Acid Conserves the Quality of Bulk Post-Thawed Spermatozoa

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Abstract

The current study evaluated the effects 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 bulls 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, and 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 the results, FA0.05 presented higher ($p \leq 0.05$) total motility, progressive motility, membrane integrity, and 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

La Supplémentation du Milieu de Cryoconservation à Base de Plantes Avec de l'acide Folique Préserve la Qualité des Spermatozoïdes en Vrac Après Décongélation

Résumé: La présente étude a évalué les effets de la supplémentation en milieu de cryoconservation avec de l'acide folique en tant qu'antioxydant sur la qualité du sperme après décongélation en vrac. Des échantillons de sperme ont été prélevés sur quatre taureaux Mahabadi iraniens éprouvés et dilué dans un extenseur contenant 1.5% de lécithine de soja. Le sperme dilué a été réparti en six parties et complété par différentes doses d'acide folique comme suit: FA0 (extenseur sans acide folique), FA0.05, FA0.1, FA0.2, FA0.4 et FA0.8 (extenseurs contenant 0.05, 0.1, 0.2, 0.4 et 0.8 mM d'acide folique, respectivement). Ensuite, les échantillons de sperme ont été cryoconservés dans de l'azote liquide. Les paramètres de motilité et de vitesse des spermatozoïdes, l'intégrité de la membrane, la morphologie anormale, la viabilité et la peroxydation lipidique ont été évalués après décongélation. Dans les résultats, FA0.05 a présenté une motilité totale plus élevée ($p \leq 0.05$), une motilité progressive, une intégrité de la membrane et une viabilité et une peroxydation lipidique plus faible par rapport aux autres groupes. La morphologie anormale n'a pas été affectée ($p > 0.05$) par les traitements. En conclusion, la supplémentation du milieu de cryoconservation avec 0.05 mM d'acide folique est une méthode utile pour conserver la qualité du sperme après décongélation en vrac.

Mots-clés: vrac, cryoconservation, acide folique, sperme, lécithine de soja

1. Introduction

During the cryopreservation process, the production of reactive oxygen species (ROS) causes adverse effects on the sperm membrane, DNA, and physiological processes and thereby affects sperm quality. The sperm plasma membrane contains significant amounts of polyunsaturated fatty acids and is highly susceptible to ROS (1). The quantity of adenosine triphosphate (ATP) is drastically reduced by increasing lipid peroxidation, which is associated with axonemal damage and increased midpiece and tail morphological defects, leading to impaired motility and decreased sperm viability (2).

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 (3). 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 ruminants. Goat sperm cells also contain a large number of PUFAs and are sensitive to temperature changes (4).

Under normal circumstances, to counteract the damaging effects of ROS, the sperm and semen plasma contains 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: 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. 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 (5).

The folic acid coenzyme catalyzes reactions related to the synthesis of nucleic acids and proteins. It is a B-group vitamin that is 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, it can protect biological compounds such as cell membranes or DNAs against free radical damage (6).

To the best of our knowledge, no research has yet been conducted on goat spermatozoa to control oxidative stress in the freezing process by folic acid, which at optimal levels is expected to be effective in increasing goat sperm quality parameters after freezing and thawing. Therefore, the present study 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 the cryopreservation process.

2. Material and Methods

2.1. Chemicals

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

2.2. 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.

2.3. 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 and then immersed into liquid nitrogen at -196 °C and stored. For post-thawing sperm evaluation, the frozen straws were thawed individually at 37 ℃ for 30 seconds in a water bath.

2.4. 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).

2.5. Sperm Lipid Peroxidation

The rate of lipid peroxidation in the semen samples was measured by determining the MDA concentration (7). 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.

2.6. Plasma Membrane Integrity

The hypoosmotic swelling (HOS) test was used to assess the plasma membrane integrity of spermatozoa after the freeze-thawing process (8). 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.

2.7. Abnormal Morphology

Hancock solution was used to evaluate post-thawed sperm abnormal morphology (9). Therefore, a drop of thawed sample was placed in an Eppendorf tube containing 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 was recorded.

2.8. Sperm Viability

Sperm viability was determined by Eosin Y 0.5%-Nigrosin 0.1% staining (10). 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 damaged membranes of spermatozoa, sperm cells that absorbed the eosin-nigrosin solution were recorded as dead sperm cells.

2.9. Statistical Analysis

The normal distribution of data was examined using univariate and Shapiro-Wilk tests. Data was evaluated

using the general linear model (GLM) in the Statistical Analysis System software. 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 $p \leq 0.05$.

3. Results

3.1. 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 ($p \leq 0.05$) in AF0.05 compared with the other groups. Groups AF0.1 and AF0.2 showed higher ($p \leq 0.05$) TM and PM than groups AF0, AF0.4, and AF0.8. There was no significant difference ($p > 0.05$) among the AF0, AF0.4, and AF0.8 groups in cases of TM and PM. No significant difference ($p \leq 0.05$) was observed among treatment groups for VAP, VSL, VCL, or ALH.

MDA concentration was lower in the AF0.05 group ($p \leq 0.05$) than in the other groups. Groups AF0.1 and AF0.2 showed lower ($p \leq 0.05$) MDA concentrations than groups AF0, AF0.4, and AF0.8. There was no significant difference ($p > 0.05$) in MDA concentration among groups AF0, AF0.4, and AF0.8.

3.2. Membrane Integrity, Abnormal Morphology, and Viability

Figure 1 presents the effects 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 the AF0.05 group compared to the other groups. Groups AF0.1 and AF0.2 showed higher ($p \leq 0.05$) membrane integrity than the 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 to be higher ($p \leq 0.05$) in the AF0.05, AF0.1, and AF0.2 groups compared to the other groups.

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

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

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 malondialdehyde concentration (MDA) were assessed after thawing. Different letters showed significant differences at $p \leq 0.05$ among groups.

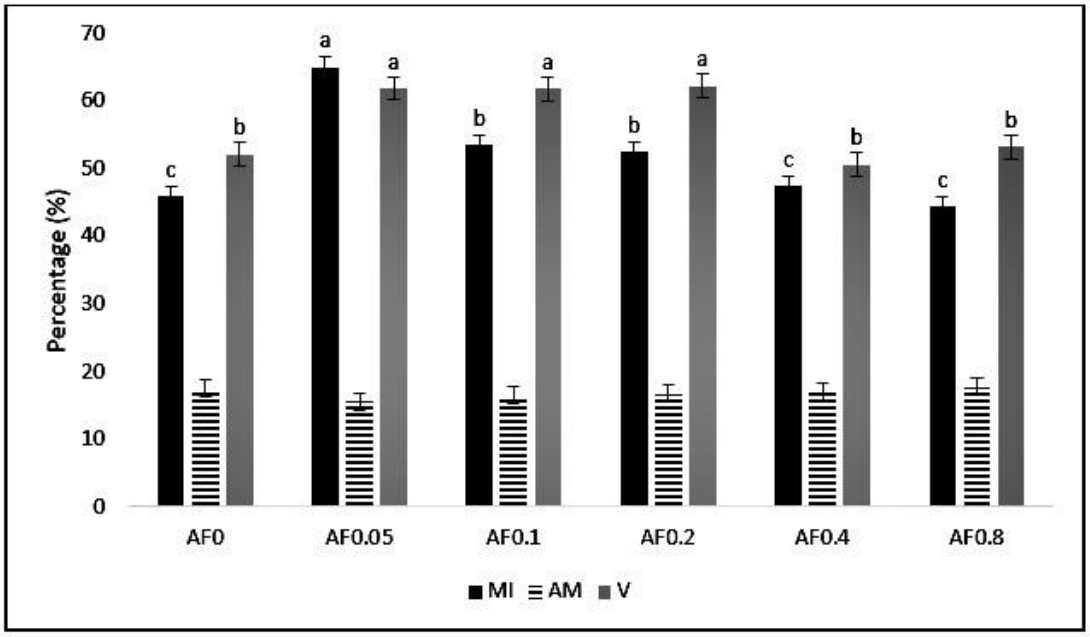

Figure 1. Percentage of membrane integrity (MI), abnormal morphology (AM), and viability (V) of bulk spermatozoa after freezing-thawing in cryopreservation medium. Different letters within the same column show significant differences among the groups ($p \leq 0.05$).

4. Discussion

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, ultimately resulting in sperm dysfunctions, such as impaired motility, sperm membrane integrity, sperm fertility, intracellular enzymes, and sperm DNA damage through oxidative stress (11).

Folic acid, as an antioxidant, normally neutralizes oxidized free radicals. Although this antioxidant is known as a water-soluble vitamin, it has been 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 (6). 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 (12, 13).

The current study aimed to investigate the antioxidant effect of folic acid on the post-cryopreservation and lipid oxidation characteristics. It was 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 (14). Although there is no previous study on the effects of oral folic acid on the mammalian

frozen sperm, according to the literature, the oral consumption of folic acid is associated with semen parameters. One study showed that oral administration of 500 mg of folic acid per liter for four weeks in rabbits significantly increased all physical characteristics of semen, including semen volume, sperm concentration, mass sperm motility, and individual motility percentage, and it reduced the sperm motility index, number of dead and abnormal sperm cells, and acrosome sperm damage. These results showed the beneficial effects of antioxidants and folic acid on semen parameters (15).

Furthermore, the results of a previous study showed that the addition of folic acid to the diet of New Zealand male rabbits improved semen characteristics and reduced the level of free radicals (16). 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 sperm quality after thawing. It was found that adding 50 nM folic acid as an antioxidant to a sperm cryopreservation medium could improve 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 (17).

Another study showed that supplementation of human semen samples with 50 nmol/l of folic acid before freezing increased semen quality after freezing compared to the control group in terms of the percentages of sperm motility, progressive motility, and live sperm (18). These results suggest that folic acid can act as an antioxidant in sperm cryopreservation medium and were in agreement with the results of the current study.

5. Conclusion

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

Authors' Contribution

Study concept and design: S. Z.
Acquisition of data: Z. A.
Analysis and interpretation of data: Z. A.
Drafting of the manuscript: Z. A.
Critical revision of the manuscript for important intellectual content: Z. A.
Statistical analysis: Z. A.
Administrative, technical, and material support: S. Z., M. Zh. and A. T.

Ethics

All the procedures and animal handling were approved by the animal Ethics Committee at the University of Tehran, Karaj, Iran under the project number of 2020-2587464-5

Conflict of Interest

The authors declare that they have no conflict of interest.

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