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Morus nigra Fruit Extract Safeguards Frozen Thawed Bovine Sperm Parameters

Research Article

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ABSTRACT

This study explored the effect of *Morus nigra* fruit extract (MFE) on microanatomical and physiological parameters of cryopreserved bovine sperms. Three ejaculates were collected on weekly basis, from five fertile bulls. Each semen sample was mixed with dilution medium (DM) (1:2 v/v), centrifuged (32 g) for 10 minutes to pore away the upper-half of the mixture. The remaining material was then extended (1:4 ratios) with standard cryopreservation extender (SCE). Three aliquots (0.3 mL) from this extended sample were further extended with 0.7 mL pure SCE (control group), 0.7 mL SCE containing 3% MFE (MFE-3 group) and 0.7 mL SCE containing 6% MFE (MFE-6 group) respectively to attain final dilutions (25 times dilution of fresh semen ejaculate, containing approximately 1836.5 ± 85.23 million sperms per mL). From each final dilution (0.1 mL) sample was directly analyzed for semen quality parameters (SQPs) and rest of the material was placed in liquid nitrogen for 24 hrs for post thaw study of the SQPs and in-vitro fertilizability. Results revealed substantial improvement in sperm membrane integrity, motility, and fertilizability in MFE-3 and MFE-6 against SCE group. Likewise, significantly high mean percent number of progressively motile sperms and sperms showing 20 µ/sec or above velocity both before and after cryopreservation, were observed in MFE-6 and MFE-3 groups as compared to SCE. These findings show protective effects of MFE for bovine spermatozoa against cryoinjuries and the post thawed oxidative stress.

KEY WORDS cryo-injuries, cryopreservation, *Morus nigra,* oxidative stress, semen quality.

INTRODUCTION

Semen cryopreservation gives advantages of infinite storage time and worldwide transportation of high quality semen for artificial insemination (Grötter *et al.* 2019; Sharma and Sood, 2019). The major disadvantage of this technology is the wastage of large number of sperms that may lose membrane integrity and/or fertilizability in the freeze thaw processes essential to it (Waberski *et al.* 2019).

Additionally, the cryo-injuries of spermatozoa may include mitochondrial and nuclear DNA damage (Zhang *et al.* 2021); while the abrupt resurrection of metabolic activities of the frozen spermatozoa in thawing process may inflict extreme oxidative stress that logically cause oxidative damage and a resultant compromised fertility (Gadani *et al.* 2017). Nevertheless, moderate availability of oxygen to the spermatozoa is vital for normal physiological process (including sperm capacitation, acrosomal reaction and chemo-

tactic responses) to ensure successful fertilization (Aitken, 2017). Supplementation of mammalian semen cryopreservation media with exogenous antioxidants has been found to be helpful in conservation of the attributes of the frozen thawed sperms (Shah *et al.* 2017). Moreover, it has been shown that the supplementation of different plant extracts containing phyto-medicinal antioxidants in the semen cryopreservation extenders may help to protect sperm quality parameters of the frozen thawed semen (Saxena *et al.* 2019; Suleman *et al.* 2021). The medicinal plants are naturally enriched with various phyto-medicinal agents, such as phytosterols, poly-phenols, flavonoids and anthocyanins specifically known for their ameliorative and curative potentials against toxicological insults. The curative activity of the phytosterols is particularly executed through the protection of the integrity of membranous organelles and the plasma membrane in freeze thaw processes. Moreover, the natural phyto-medicinal antioxidants provide excellent cover against instantaneous increase in the reactive oxygen species on the vital body cells particularly deficient in endogenous antioxidants (the stem and germ cells) (Bibi *et al.* 2017; Suleman *et al.* 2021).

Morus nigra (family: Moraceae) is widespread plant species throughout Afghanistan, Iraq, Iran, India and Pakistan (Lim and Choi, 2019). Its fruits are mostly consumed afresh or in the form of jams and juices. Different parts of *Morus nigra* are traditionally being used for medicinal purposes and have been reported to contain important bioactive compounds and phytochemicals in root, stem, leaves and fruit extracts. Its fruit is rich in phenol, flavonoid (Quercetin 3-*O*-rutinoside, Quercetin 3-*O*-glucoside, Kaempferol 3-*O*-rutinoside), anthocyanin (Cyanidin 3-*O*-glucoside, Cyanidin 3-*O*-rutinoside, Pelargonidin 3-*O*-glucoside, Pelargonidin 3-*O*-rutinoside) benzoic acid derivatives (Protocatechuic acid, *p*-Hydroxybenzoic acid, Vanillic acid), cinnamic acid derivatives (Caffeic acid, Ferulic acid, Chlorogenic acid) and ascorbic acid (Koyuncu *et al.* 2014; Sánchez-Salcedo *et al.* 2015). Anthocyanin is an important component of *Morus nigra* fruit which give it a characteristic purple color. The anthocyanin contained in the berries and red fruits are reported to have various health benefits in coronary heart disease, stroke, cancer and aging (Krishna *et al.* 2018). The present study was aimed to explore the curative capacities of MFE against freeze-thaw stresses on the bovine spermatozoa in routine cryopreservation and reactivation protocols.

MATERIALS AND METHODS

Process for ethical approval

This research paper is a part of the results obtained from a project (UOS/ORIC/2016/63; Titled: Role of jambul and strawberry fruit pulp extract on bovine semen quality) funded by University of Sargodha, Punjab, Pakistan. The project was duly endorsed by the "ethical committee for Life and Pharmacological Science, University of Sargodha".

Chemicals

Chemicals and reagents used in this study were obtained from Sigma-Aldrich except: Estradiol and Heparin (Fluka); Adrenaline, Penicillamine and Eosin (Alfa Aesar, Germany) and HEPES (Scharlau, Spain).

Preparation of MFE

Fully ripped fruits were collected from five selected *Morus nigra* trees located on the main campus, University of Sargodha. The delicate fruits were washed in boiled cooled water, blow dried and crushed in an electric juicer to obtain dark violet suspension. The crude suspension was centrifuged (32 g) for half an hour and the crystal clear dark violet MFE so obtained was vacuum dried at 25 ˚C for 24 hrs. The dried concentrate of MFE was weighed to obtain the amount of total dissolved solid per 100 mL of MFE. Weight of resultant MFE concentrate was measured for assessment of the "total dissolved solids". Total 'anthocyanins', 'polyphenols' and 'flavonoids' estimations were made separately from each MFE concentrate using "the pH-differential method", "modified colorimetric Folin-Ciocalteu method" and the "aluminum chloride colorimetric assay" respectively (Ghosh *et al.* 2017; De Carvalho Tavares *et al.* 2020). The MFE concentrate was stored in a sealed dark glass bottle at-20 ˚C for experimental usage.

Semen collection and processing

Five bulls with an excellent fertility record were selected for semen collection from the farmhouse in the suburbs of Agriculture College University of Sargodha. A sum total of 15 ejaculates (three from each bull-with a refectory period of seven days between two successive ejaculates from each bull) were collected using Artificial Vagina (AV). Each freshly ejaculated semen sample was readily mixed with DM (Sodium citrate (1.65 g), Glucose (1 g), Gentamicin (2 mL), and egg yolk plasma (30 mL) in normal saline to make the volume 100 mL in 1:2 ratio. A small fraction of diluted semen was tested for sperm quality parameters (viability, motility, and velocity-results represented in bar graphs under title SDM) while the remaining part was centrifuged at 32 g for 10 minutes. The top 1/2 volume of each sample was discarded readily to get rid of harmful components of seminal plasma. The remaining part mainly containing spermatozoa was re-diluted with SCE (12% glycerol (v/v) in SDM) in 1:4 ratios. Each SCE extended semen sample was distributed into three aliquots (0.3 mL each) for further dilution with group specific {vis: SCE, MFE-3 (3%

MFE added into SCE) and MFE-6 (6% MFE added into SCE)} extenders in 3:7 ratios before cryopreservation. The final semen dilutions were slowly cooled (3-4 ˚C. 10 minutes^{-1}) to 4 ˚C in an hour approximately and kept at 4 ˚C for the next two hours. From each of these semen samples 0.1ml only was used for quality parametric analyses while rest of it was packed in properly labeled semen straws for storage. Each straw was properly sealed and gradually cooled (5 ˚C. 15minutes^{-1}) to achieve -20 ˚C in a digitally controlled convertible fridge – freezer, and were thereafter shifted to liquid nitrogen (LN) for cryopreservation and storage. After a minimum 24 hours storage in LN one straw from each semen sample was gradually warmed in water bath at 37 ˚C to analyze quality parameters.

Parameters for analysis of semen quality

Velocity test

Sperm velocity was estimated as the distance travelled by the sperms in one second. On the basis of their estimated velocity the spermatozoa were categorized as A, B or C type (A; ≥20 µm/sec, B: 10-19 µm/sec, C: 1-9 µm/sec).

Motility test

To estimate percent motile sperms 50 µL of sample was examined under a dark field microscope (400×) from five randomly selected areas. Moreover, the sperms were further categorized on the basis of the type of movement shown such as progressive, twitching, and whirling to estimate the percent spermatozoa showing progressive as well as non-progressive motility.

These estimations were carried-out using Corel Video Studio 2020 and corelDRAW2019. Individual frames from a 5 second movie (30 frames sec.$^{-1}$) of sperm -captured on a 7.2 megapixcels digital camera (Sony DSC-W35) mounted on a (converted to dark field) trinocular microscope (Lobomid CXR2) - were separated by Corel Video Studio 2020 and imported in corelDRAW2019 where the distance travelled by a given spermatozoon was estimated in 30 successive frames using a pre-calibrated grid application (Suleman *et al*. 2021).

Viability test

Eosin-Nigrosine stain was used to detect membrane integrity - used as an indicator for lysed or live sperms.

Fertilizability test

The success of *in vitro* fertilization was considered as a test for sperm fertilizability. For each group, 45 + 45 IVFs (5 for each sample) were performed separately for fresh and frozen thawed sperms.

Oocyte aspiration and media treatments

One ml of fertilization medium ($CaCl_2.2H_2O$, $MgCl_2.6H_2O$, KH_2PO_4, KCl, NaCl, $NaHCO_3$, Na-Lactate, BSA, Na pyruvate, Heparin, Caffeine, Adrenaline, and Gentamicin) was poured into disposable plastic fertilization plates containing twelve fertilization wells and incubated at 38.5°C and ambient 5% CO_2 for 2 hrs.

Ovaries of slaughtered cows were obtained from the slaughterhouse situated at Hyderabad Town, Sargodha. The cumulus-oocyte complexes were aspired from antral follicles from these ovaries. The oocytes were placed into oocyte maturation media (containing NaCl, KCl, $MgCl_2$, KH_2PO_4, $NaHCO_3$, Na-lactate, Na-pyruvate, Glucose, bovine serum albumin (BSA), HEPES, amino acids, bovine steer serum (BSS), reduced glutathione, human menopausal gonadotrophin, estradiol, and gentamycin) in a CO_2 incubator (38.5 ˚C and 5% CO_2) 24 hrs. The oocytes after maturation were washed in pre-warmed wash media (containing HEPES, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, KH_2PO_4, KCl, NaCl, $NaHCO_3$, Na-lactate, Na-pyruvate and gentamicin) and loaded (5 each) into the fertilization wells.

IVF protocol

The frozen semen was thawed at 38 ˚C for 20 sec and vortexed with wash media (1-3 v/v) followed by centrifugation (500rpm for 5 min) to discard half of the supernatant. The remaining material was again incubated (37 ˚C for 20 min) to obtain the maximum number of live sperms that swims up during the incubation. The unfrozen samples of the SCE added semen from each group were also treated in the same way to obtain the live sperms. Live spermatozoa contained in 200 µL of these samples were transferred in each fertilization well already containing mature oocytes immersed in the fertilization medium. Fertilization plates were kept at 38 ˚C and 5% ambient CO_2 for 48 hrs in the incubator. After incubation the number of 2-4 celled embryos was counted under the dark field microscope that represented the number of successful IVFs.

Statistical analysis

The data for specified sperm quality parameters and *in vitro* fertilizability test (both before and after cryopreservation) were statistically evaluated using one-way ANOVA and the Tukey Multiple Range Test (TMRT). Additionally, data obtained from post-thaw sperm analysis was subjected to ANCOVA and LSD (least significance difference test for multiple comparisons) to confirm MFE potential against detrimental outcomes of freeze-thaw processes on sperm parameters. All statistical analyses were carried out employing IBM SPSS statistics 20 software (SPSS, 2011).

RESULTS AND DISCUSSION

Antioxidant estimations of MFE

Mean weight estimated for total dissolved solids in 25 mL MFE was 3.75 ± 0.50 g (i.e. 15g.100 mL^{-1}). While means differential weights for anthocyanins, flavonoids and polyphenols in 100 g of total dissolved solids were 169.20 ± 3.14 mg, 231.00 ± 1.20 mg and 155.28 ± 5.40 mg respectively.

Plasma membrane integrity

At both stages (before and after 24 hrs of cryopreservation) percent sperm plasma membrane integrity in MFE-6 (90.00±1.30, 80.40±1.26) and MFE-3 (89.70±1.32, 75.33±1.50) remained significantly high (P<0.05, N=100) as compared to SCE group (72.00±1.30, 63.50±1.22). ANCOVA based analysis of the data, using mean percentile values of the sperm membrane integrity of the unfrozen semen as covariate, revealed MFE concentration dependent improvement in sperm membrane integrity (Figure 1).

Sperm motility

Mean percent number of motile sperm without and after 24hrs of cryopreservation was significantly high (P<0.05, N=100) in MFE-3 (81.00±1.41; 67.36±1.73) and MFE-6 (82.33±1.60; 70.9±1.3) as compared to SCE (62.80±0.92; 55.00±1.13).

Furthermore, ANCOVA based analysis of the data -using mean percentile values of motile sperms before cryopreservation as covariate- also revealed similar results (Figure 2).

Differential percentile sperm motility

Statistical analysis (ANOVA as well as ANCOVA) indicated significance difference (P≤0.001, N=100) among groups in mean percent number of sperm exhibiting progressive, twitching/shaking or whirling motility at both stages (before and after 24 hrs of cryopreservation). The highest percent progressive sperm motility was seen in MFE-6 (84.67±1.03; 71.60±1.15) followed by MFE-3 (81.44±1.50; 69.66±1.35) and SCE (69.78±0.97; 55.20±3.47) groups whereas the order of arrangement was inversed for the mean percent twitching / shaking and whirling spermatozoa (Figure 3).

Differential percentile sperm velocity

Significant variation (P≤0.05) was seen in differential percentile values of sperm velocity [ranked as A (≥20 µm/sec), B (10-19 µm/sec) and C (1-9 µm/sec)] among the groups. The percentage of "A" type spermatozoa was significantly higher in MFE-6 and MFE-3 groups than that of the SCE (Figure 4).

***In vitro* fertilizability**

The mean percentage of IVF embryos was significantly higher in MFE-3 and MFE-6 group to that of the SCE group at both stages (before and after 24 hrs of cryopreservation).

A further TMR analysis revealed significantly higher mean successful IVFs in MFE-6 (3.55±0.12, 3.11±0.07) and MFE-3 (3.11±0.12; 2.78±0.13) as compared to SCE (2.89±0.15; 2.30±0.14) group (Figure 5).

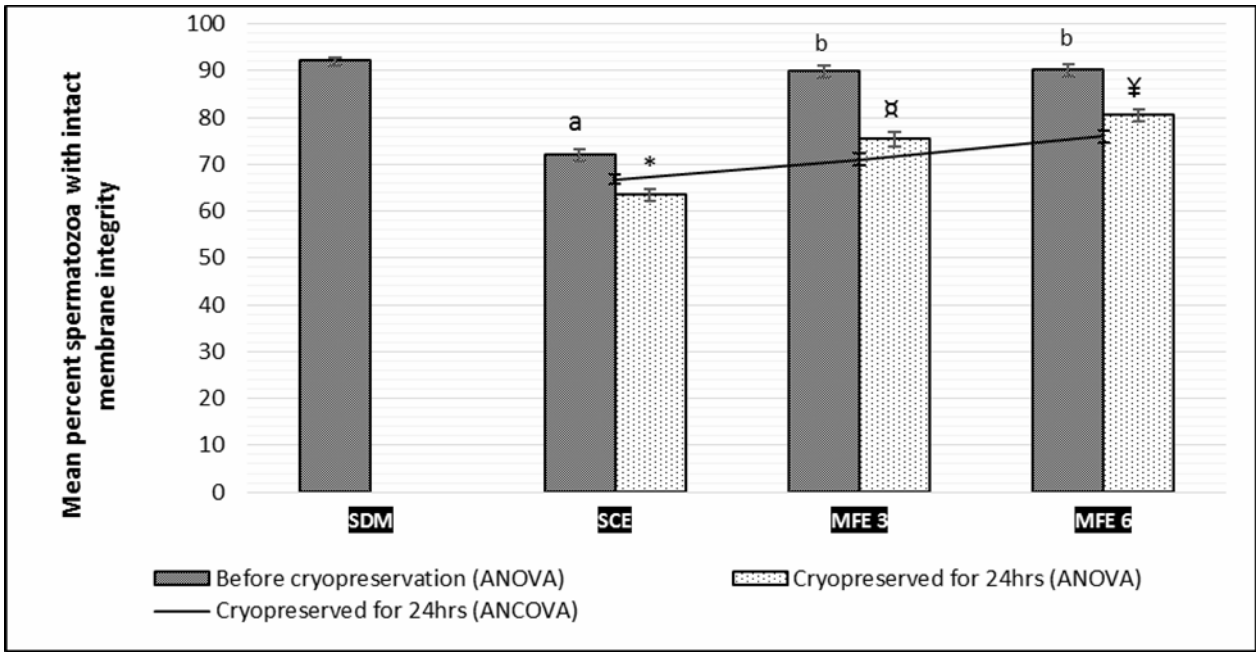

Figure 1 Mean percent spermatozoa with intact membrane integrity in all groups, N=100, ± bars indicate SEM, statistical analysis (ANOVA/ANCOVA). Any two groups not sharing a common lowercase superscript letter (^{abc}) or symbol ($^{*¤¥}$) differ significantly (P≤0.05) with each other (TMRT)

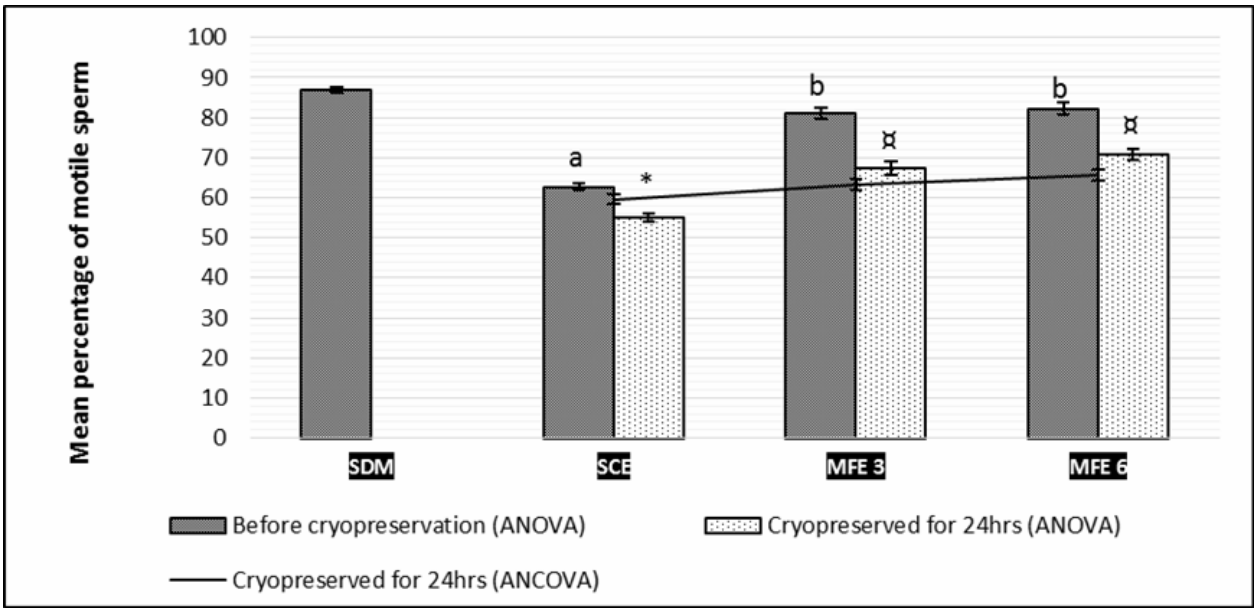

Figure 2 Mean percentage of motile sperm in all groups, N=100, ± bars indicate SEM, statistical analysis (ANOVA/ANCOVA). Any two groups not sharing a common lowercase superscript letter (^{abc}) or symbol ($^{*¤¥}$) differ significantly (P≤0.05) with each other (TMRT)

Sperm cryopreservation has revolutionized the field of assisted reproduction (Sharma and Sood, 2019). In last two decades' new advancements have been made in cryopreservation protocols and various new tools and techniques have been introduced (Zhang *et al.* 2019). Despite these advancements oxidative stress (OS) is still one of the major problems associated with cryopreservation that may result in marked reduction of post-thaw sperm quality (Malo *et al.* 2019). The vulnerability of spermatozoa to OS is high due to the insufficient amount of antioxidants in their cytoplasm (Aitken and Drevet, 2020) and enormously high level of polyunsaturated fatty acids (PUFA) contents in their plasma membranes (Craig *et al.* 2019). Sperms contain many mitochondria in middle piece that empower sperm flagellum during active propulsive movements by oxidative turnover of ATP (Gu *et al.* 2019). Even with such metabolic significance mitochondria are also highlighted as a source of prooxidant species (Moraes and Meyers, 2018). During oxidative phosphorylation in mitochondria reactive oxygen species (ROS) are produced that may act as an important trigger for various reproductive physiological mechanisms including sperm capacitation, acrosomal reaction and chemotactic responses (Aitken, 2017). However, their high level may be extremely harmful to the spermatozoa (Bollwein and Bittner, 2018). On thawing the metabolism of cryopreserved spermatozoa suddenly accelerate and very active oxidative phosphorylation begins in mitochondria that intern may accelerate production of reactive oxygen species (Len *et al.* 2019). This simply means that the abrupt metabolic activation of spermatozoa in freshly thawed semen opens a wide window of oxidative stress.

This rapid increase in ROS production may cause peroxidation of membranous lipid contents which may breach sperm membranous integrity ultimately causing lysis of spermatozoa (Bui *et al.* 2018). Thus in freshly thawed semen the plasma membrane and mitochondria emerge as the most susceptible sites for reactive oxygen species (ROS) induced injuries in spermatozoa (Sabeti *et al.* 2016). Spermatozoa are incapable of surviving such oxidative damages due to inadequate intrinsic defense systems.

Seminal plasma is naturally complemented with different antioxidant enzymes including glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase. Therefore, under *in vivo* settings, the seminal plasma may provide efficient protection to sperm against OS (Soni *et al.* 2019). However, cryopreservation procedure has resulted in progressive reduction in antioxidant enzymes concentration and activity from fresh to post thaw stag (Lone *et al.* 2016). During semen extension, the concentration of semen antioxidant enzymes also significantly reduce due to dilution effect of semen extender (Salmon *et al.* 2017). Particularly the concentrations of superoxide dismutase and glutathione deteriorate during equilibration time cryopreservation further deteriorates the activities of catalase, superoxide dismutase and glutathione activities (Iqbal *et al.* 2019).

In recent years these apprehensions are being resolved by supplementation of exogenous antioxidants in semen extenders. Now it has become a routine practice to add exogenous antioxidants (glutathione, α-tocopherol, superoxide dismutase, catalase, glutathione peroxidase, vitamin C, resveratrol) in semen extenders (Del Prete *et al.* 2019; Ullah *et al.* 2019).

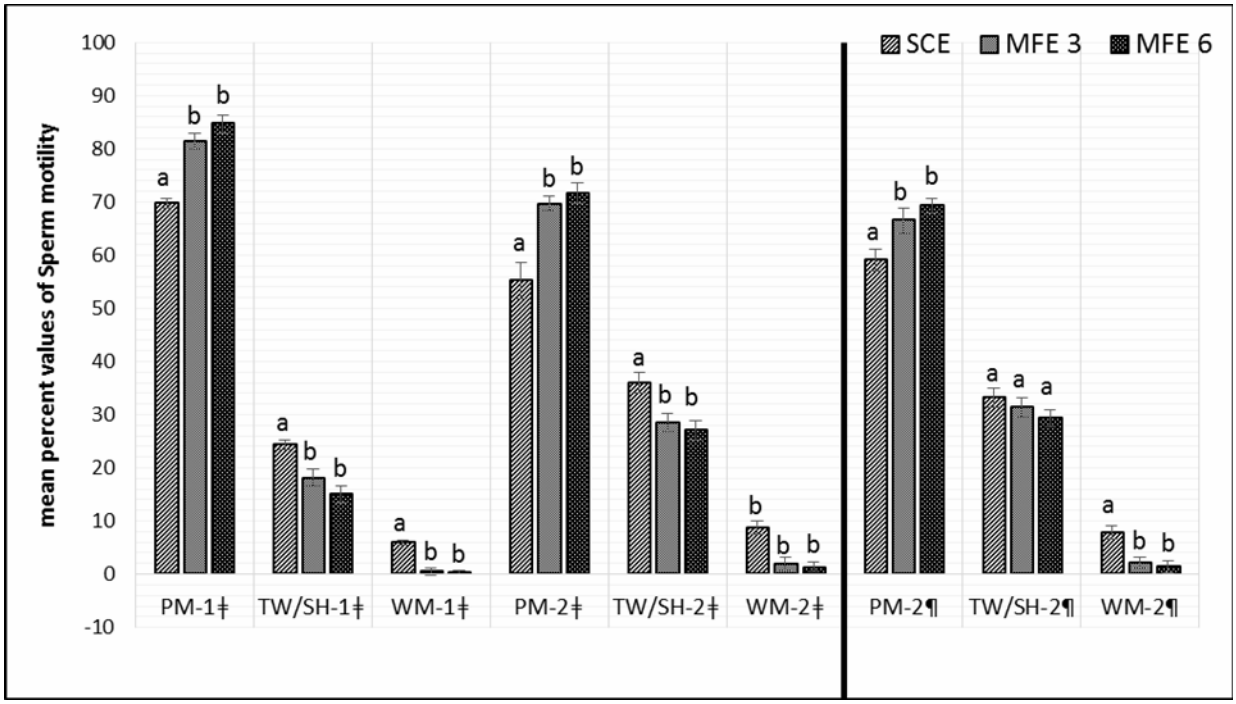

Figure 3 Mean percent values of three modes {progressive (PM), twitching/shaking (TW/SH) and whirling (WM)} of sperm motility before cryopreservation (PM-1,TW/SH-1,WM-1) and cryopreserved for 24 h (PM-2,TW/SH-2,WM-2), ± bars indicate SEM. [(abc)] indicate a significant difference between the groups not sharing a common lower case letter. ǂ analyzed by ANOVA; ¶ ANCOVA based estimated percent post thaw mean values ± SEM of the sperm velocity employing mean percent values before cryopreservation as co-variant (P<0.05, N=100)

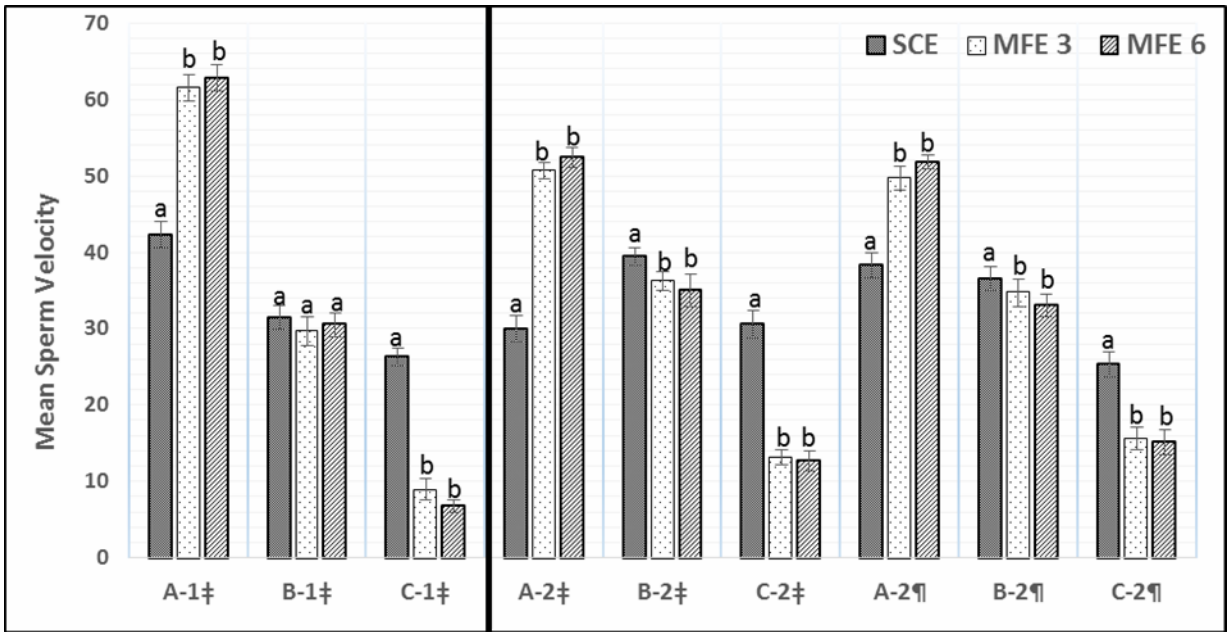

Figure 4 Differential percentile of sperm velocity (A=20 µm/sec or above, B=10-19 µm/sec, C=1-9 µm/sec) before cryopreservation (A-1,B-1,C-1) and cryopreserved for 24 h (A-2,B-2,C-2), ± bars indicate SEM. [(abc)] indicate a significant difference (P<0.05) between the groups not sharing a common lower case letter. ǂ analyzed by ANOVA; ¶ ANCOVA based estimated percent post thaw mean values ± SEM of the sperm velocity employing mean percent values before cryopreservation as co-variant (P<0.05, N=100)

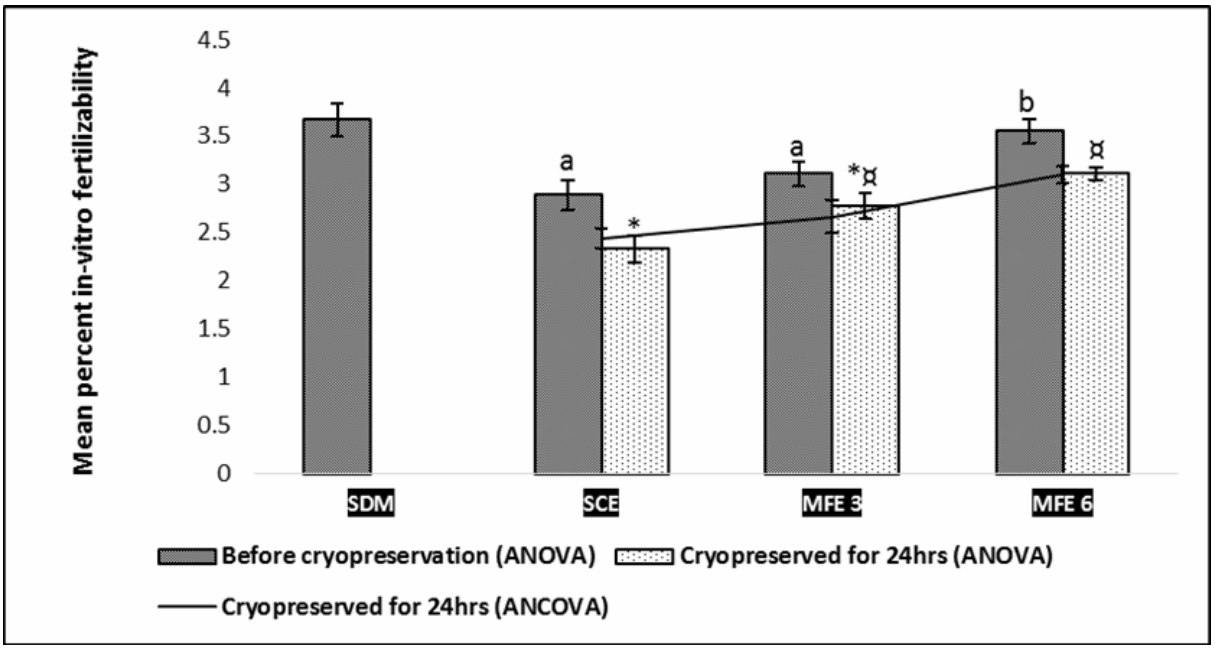

Figure 5 Mean No. of successful IVFs out of 5 in all groups, ± bars indicate SEM, statistical analysis (ANOVA/ANCOVA). Any two groups not sharing a common lowercase superscript letter (^{abc}) or symbol ($^{*¤¥}$) differ significantly (P≤0.05, N=100) with each other (TMRT)

Medicinal plants are laden with a variety of precious phytochemicals including polyphenols, anthocyanins, flavonoids and phytosterols that are reported to exhibit excellent antioxidant properties (Lee *et al.* 2017) and are being used as a natural replacement of synthetic antioxidant in semen extender to combat and counteract the damaging effects of thawing induced OS (Saxena *et al.* 2019).

Morus nigra fruit is rich in large number of phenolics, flavanoids, anthocyanins, alkaloids, cardiac glycosides, saponins, tannins and reducing sugars which are all biologically active components (Lim and Choi, 2019). These phytochemicals harbor exceptional antioxidant potentials because of the presence of multiple hydroxyl groups, conjugated double bonds and electron releasing capacity (Pervaiz *et al.* 2017; Latos-Brozio and Masek, 2019). The major ingredients in *Morus nigra* fruit, other than antioxidants, include reducing and non-reducing sugars and various amino acids (Koyuncu *et al.* 2014). These components harbor excellent cryo-protective capacity that further helps against cryogenic injuries (Arando *et al.* 2019). Our results reveal substantial improvement in sperm membrane integrity, motility, and fertilizability in MFE-3 and MFE-6 as compared to SCE group. Also, considerably higher percentage of progressively motile sperms at the expense of the twitching shaking and whirling spermatozoa, were found in MFE-3 and MFE-6 than that of the SCE group. Moreover, the mean percentage of progressively motile spermatozoa (showing 20 μ sec^{-1} or above velocity) was also significantly higher in MFE-3 and MFE-6 than that of the SCE-group. On the whole this research reveals significant impact of MFE for the improvement of various quality parameters of the cryopreserved bovine semen. Results of present study would reveal significant effect of MFE on the plasma membrane integrity, progressive motility and fertilizability of the bovine frozen-thawed spermatozoa that must be attributable to its variety of anti-oxidative inclusion.

CONCLUSION

In conclusion, the addition of MFE (3% and 6%) significantly improves sperm survival rate, progressive motility, and *in vitro* fertilizability without any harmful consequences. Further studies of a similar nature may aid to remodel the semen extender recipes and cryopreservation protocols.

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