

QUALITY ASSESSMENT OF CRYOPRESERVED NEW ZEALAND WHITE RABBIT SPERMATOZOA IN INRA-82 EXTENDER CONTAINING DIFFERENT CRYOPROTECTANTS

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Abstract: The present study aimed to evaluate the effect of supplementation of INRA-82 semen extender with different cryoprotectants (dimethyl sulphoxide; DMSO vs. dimethyl formamide; DMF) on the quality of white New Zealand rabbit buck spermatozoa. We also investigated the possible association between the synergistic action of DMSO and DMF and their relation with INRA-82 extender composition. Semen was collected and pooled from 8 adult rabbit bucks. Pooled semen samples were diluted 1:1 with INRA-82 extender supplemented with DMSO 8%, DMF 8% or a combination of DMSO 4% and DMF 4%. The diluted semen samples were cryopreserved in 0.25 plastic straws. After thawing, progressive motility, sperm viability, sperm abnormalities, membrane integrity, acrosome status, viability index and DNA integrity were evaluated. The results showed that dilution of rabbit buck semen in INRA-82 supplemented with DMSO and DMF (4% each) before freezing significantly ($P < 0.05$) improved sperm motility (42.00%), percentage of live spermatozoa (45.30%), proportions of spermatozoa with intact acrosome (59.75%) and percentage of spermatozoa with non-fragmented DNA (86.04%), compared to those diluted in INRA-82 supplemented either with DMSO 8% (+9, +10, +5 and +7 percentage points, respectively) or with DMF 8% alone (+18 +18, +12 and +9 percentage points, respectively). In conclusion, dilution of rabbit buck semen before freezing with INRA-82 extender supplemented with a combination of DMSO 4% and DMF 4% improved quality of frozen-thawed New Zealand White rabbit spermatozoa. Furthermore, our results also suggest that supplementation of INRA-82 with DMSO or with DMF alone at higher concentrations deteriorates the sperm quality.

Key Words: rabbit buck, cryopreservation, INRA-82, DMSO, DMF.

INTRODUCTION

Sperm cryopreservation is one of the main tools for genetic preservation in many species. However, in rabbits the success rates and fertility outcomes of frozen-thawed semen are still low compared to those of other domestic species (Mocé and Vicente, 2009). It is well known that spermatozoa undergo various degrees of damage during cryopreservation, which subsequently harms the function and fertility of sperm cells after thawing (Graham and Mocé, 2005; Long, 2006). During cryopreservation, several factors have been reported to contribute to the deleterious effects of freezing and thawing on sperm biological function; these factors include the constituents of freezing media, cryoprotectant agents (types and concentrations) and the cooling and warming rates (Watson, 2000). Cryoprotectant agents (CPAs) are the compounds included in semen extenders to protect the spermatozoa from freezing-inflicted damage (Gao *et al.*, 1997), categorised into permeating and non-permeating cryoprotectants according to their ability to penetrate the cell membranes. Permeating CPAs are able to pass through the cell membranes and are of low molecular weight, such as glycerol, dimethyl sulphoxide (DMSO), dimethyl formamide (DMF) and acetamide. In contrast, non-permeating CPAs cannot pass through the cell membranes and have a high molecular weight, such as sucrose, glucose, trehalose and lactose (Ashwood-Smith, 1987). It is well documented that rabbit spermatozoa

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<https://doi.org/10.4995/wrs.2019.10892>

are relatively sensitive to cryoprotectants, particularly those containing hydroxyl groups such as glycerol (Hanada and Nagase, 1980). This sensitivity of rabbit spermatozoa has been related to the characteristic features of their plasma membranes, as they have high activation energy and low water permeability coefficient (Curry *et al.*, 1995). Therefore, amides and other methyl group cryoprotectants which are known for their lower molecular weights and higher membrane permeability could be effectively used in the preservation of rabbit semen (Darin-Bennett and White, 1977). Dimethyl formamide (DMF) is an amide that has been successfully applied to preserve spermatozoa in different species including rabbits (Domingo *et al.*, 2018). Cryopreservation of stallion spermatozoa in the presence of DMF has been shown to improve sperm quality after thawing better than those preserved in glycerol-based extenders (Olaciregui *et al.*, 2014; Pukazhenthil *et al.*, 2014). On the other hand, previous studies showed that cryopreservation of spermatozoa from dogs (Mota Filho *et al.*, 2011), goats (Bezerra *et al.*, 2011) and fowl (Chalah, 1999) in DMF based media reduced sperm quality more than those cryopreserved in glycerol-based extenders. These studies confirm the notion of species differences in the response of the spermatozoa to cryoprotectants. In addition to DMF, DMSO also has been used successfully for cryopreservation of rabbit spermatozoa (Viudes de Castro and Vicente, 1996). However, the combined effect of DMF and DMSO in protecting rabbit spermatozoa against freezing-inflicted damage has not been addressed before.

This study evaluated the effect of supplementation of INRA-82 semen extender with DMSO versus DMF on the quality of frozen/thawed rabbit spermatozoa. We also investigated the combined effect of both cryoprotectants on motility, viability and DNA integrity of rabbit spermatozoa after cryopreservation.

MATERIALS AND METHODS

Animals

Animal care procedures followed the ethical protocols approved by the Ethics Committee for Animal Use at Cairo University (approval number: CU II S 718). The study was carried out on eight (8-10 mo old; 4-6 kg body weight) healthy rabbit bucks. The animals were housed individually in separate cages and exposed to a 16 h light / 8 dark photoperiod, with temperature ranging from 19 to 25°C. The animals were supplied with a pelleted balanced diet composed of crude protein 18%, crude fat 3%, crude fibre 19%, calcium 1.00%, phosphorus 0.5% and vitamins. Green fodder and fresh water were also offered *ad libitum*.

Semen collection and processing

Semen was collected from each buck early in the morning using a lubricated and pre-warmed (42-45°C) artificial vagina (All Vet. Supply Inc., France) on a regular basis (2 ejaculates/buck week). The experiments were performed during the period from April 2018 to June 2018. Immediately after semen collection, the gel plug was removed and the ejaculates were placed in an incubator at 37°C. Collected ejaculates from each buck were pooled to rule out the individual variations among animals (ten replicates were performed). Pooled semen samples with a final volume of about 8.00 mL were evaluated for basic semen characteristics including motility, percentage of live spermatozoa (mentioned in *Examination of processed semen* section) and sperm cell concentration. Semen volume was estimated using a graduated Eppendorf tube and sperm concentration was assessed with a haemocytometer. Samples showing more than 70% motility, 75% live sperm and 250 million sperm cell/mL were processed for freezing. Pooled ejaculates were diluted to ratio 1:1 in INRA-82 extender (glucose 25 gm/L, lactose 1.5 gm/L, raffinose 1.5 gm/L, Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 4.76 gm/L, potassium citrate 0.41 gm/L, sodium citrate 0.25 gm/L, 0.15% skim milk and supplemented with 20% egg yolk; Vidament *et al.*, 2000) supplemented with 8% DMSO or 8% DMF or a combination of 4% DMSO plus 4% DMF. The diluted semen samples were then gradually cooled to +5°C within 90 min. The samples were kept in the refrigerator at this degree for 15 min. For cryopreservation, 0.25 mL plastic straws were filled with diluted semen sample and the straws were sealed with a sealing powder and placed at the level of 4 cm above the liquid nitrogen (LN₂) vapour in a foam box for 10 min before being plunged into LN₂ (Cristanelli *et al.*, 1985).

Examination of processed semen

Two straws per treatment were thawed in a water bath at 50°C for 7 s (Di Iorio *et al.*, 2012) and samples were then examined to assess progressive motility, sperm viability sperm abnormalities, osmotic resistance, acrosome integrity, viability index, and DNA integrity.

Evaluation of sperm motility

For motility evaluation, a small drop of frozen-thawed semen was placed onto a pre-warmed glass slide and covered with a coverslip. The slides were then examined under the microscope, and 2 hundred sperm cells (motile and non-motile) were counted. The percentage of motile sperm cells was then calculated. Viability index (VI) was recorded for the first 3 h after thawing and calculated based on the following equation (Milovanov, 1962): $VI = \frac{M(T-R)}{2}$, where VI is the viability index, M is the percentage of sperm motility, T is the time of next determination of motility, and R is the time for previous determination of motility. This index is used to evaluate how many sperm cells were viable for more than 3 h after thawing to be used for artificial insemination.

Determination of sperm viability and sperm abnormalities

Percentages of live spermatozoa and sperm abnormalities were evaluated using eosin-nigrosin staining according to the method described by Evans and Maxwell (1987). Two hundred spermatozoa were counted under a microscope (1000×), and the percentages of live spermatozoa (spermatozoa that remained unstained indicating intact plasma membrane), and the percentage of the sperm showing abnormalities were recorded.

Osmotic resistance

To evaluate the functional integrity of the spermatozoan plasma membrane, the osmotic resistance test was performed. Briefly, a 10 µL aliquot of frozen/thawed semen sample was mixed with 40 µL of distilled water and incubated for 5 min at 37°C. After that, a drop of the mixture (10 µL) was placed on a microscope slide and covered with a coverslip. Slides were then examined under a phase contrast microscope using ×400 power. One hundred spermatozoa were counted and evaluated for signs of viability (swelling and curling in the sperm tail) as indicated by (Partyka *et al.*, 2012).

Acrosome integrity

Acrosome integrity was assessed using patent specific stain (Spermac Stain™) as previously described (Ghallab *et al.*, 2017). After staining, the slides were left in the air to dry; 200 sperm cells were then examined under the microscope (at ×1000 power) and the percentage of intact acrosome was calculated according to the method described by (Chan *et al.*, 1999).

DNA integrity

DNA integrity was assessed using the alkaline COMET assay. Diluted semen samples processed through centrifugation, encapsulation, lysis and denaturation of double-stranded DNA using electrophoresis. Then, the slides were stained with ethidium bromide. Using fluorescent microscope (Olympus, Japan) at a magnification of 400×, fragmented DNA with a tail which migrated from the sperm head, producing a 'comet' pattern was evaluated as mentioned by Sariozkan *et al.* (2009).

Statistical analysis

Data were presented as mean±standard error of mean and normalised using arcsine transformation. Data were analysed with a simple one-way ANOVA followed by Duncan's comparison test. Significant differences were set at $P < 0.05$. All statistical analyses were performed with the SPSS package Version 22 for Windows (SPSS Inc., Chicago, IL) to determine the effects of the treatment and the time of analysis.

Table 1: Effect of supplementation of INRA-82 extender with different cryoprotectants on the quality of frozen/thawed New Zealand White rabbit spermatozoa (mean±standard error of mean).

Semen parameters	DMSO 8%	DMF 8%	DMSO 4%+DMF 4%
Progressive motility (%)	33.25±0.65 ^b	24.25±0.91 ^c	42.00±0.60 ^a
Live sperm (%)	35.40±0.68 ^b	27.05±0.93 ^c	45.30±0.65 ^a
Sperm abnormalities (%)	33.30±0.77 ^b	35.55±0.40 ^b	29.10±0.81 ^a
Functional membrane (%)	26.80±0.80 ^b	23.30±0.67 ^c	37.80±0.35 ^a
Intact acrosome (%)	54.80±0.72 ^b	47.50±0.63 ^c	59.75±0.75 ^a

Means with different superscripts (^{a, b, c}) within the same row are significantly different at $P<0.05$.

DMSO: Dimethyl sulphoxide. DMF: Dimethyl formamide. n=10.

RESULTS

Effect of supplementation of INRA-82 extender with different cryoprotectants on the quality of frozen/thawed rabbit semen

Data presented in Table 1 showed that dilution of rabbit buck semen with INRA-82 extender supplemented with a combination of DMSO 4% and DMF 4% before freezing significantly ($P<0.05$) improved post-thaw progressive motility, percentage of live spermatozoa, percentage of spermatozoa with intact membranes and acrosome integrities in comparison with those diluted in INRA-82 supplemented only with either DMF 8% or with DMSO 8%. Moreover, the percentages of abnormal spermatozoa were significantly reduced ($P<0.05$) in the DMSO and DMF (4% each) supplemented group compared to the other 2 groups. The results also showed that supplementation of INRA-82 extender with 8% DMF alone adversely affected the quality of frozen/thawed rabbit semen (Table 1).

Effect of supplementation of INRA-82 extender with different cryoprotectants on the viability index of frozen/thawed rabbit semen

The effect of supplementation of INRA-82 extender with different cryoprotectants on viability indices was evaluated and the results are presented in Table 2. Viability index was significantly higher ($P<0.05$) in the DMSO and DMF (4% each) supplemented group (105.00±2.27) than those diluted in DMSO 8% or DMF 8% alone (66.87±2.50 and 40.87±2.81, respectively).

Effect of supplementation of INRA-82 extender with different cryoprotectants on the DNA integrities of frozen/thawed rabbit semen

As shown in Table 3, the percentages of spermatozoa with non-fragmented DNA were significantly higher ($P<0.05$) in the 4% DMSO plus 4% DMF supplemented group than those supplemented with either DMSO or DMF alone (86.04±0.92 vs. 79.05±0.45 and 77.21±0.23). However, the tail length, tail moment and the percentage of DNA in the tail were significantly lower in the 4% DMSO plus 4% DMF combined group than in the other 2 groups.

Table 2: Effect of supplementation of INRA-82 extender with different cryoprotectants on viability indices of frozen/thawed New Zealand White rabbit spermatozoa (Mean±SEM).

Viability (% progressive motility)	DMSO 8%	DMF 8%	DMSO 4%+DMF 4%
At 0 h	33.25±0.65 ^b	24.25±0.91 ^c	42.00±0.60 ^a
At 1 h	25.02±0.25 ^b	15.27±0.67 ^c	35.32±1.20 ^a
At 2 h	14.42±0.95 ^b	10.35±1.20 ^c	30.15±0.33 ^a
At 3 h	10.81±0.62 ^b	3.00±0.03 ^c	18.53±0.14 ^a
Viability index (for the first 3 h post-thawing)	66.87±2.50 ^b	40.87 ^c ±2.81 ^c	105.00±2.27 ^a

Means with different superscripts (^{a, b, c}) within the same row are significantly different at $P<0.05$.

DMSO: Dimethyl sulphoxide. DMF: Dimethyl formamide. n=10.

Table 3: Effect of supplementation of INRA-82 extender with different cryoprotectants on DNA integrity of frozen/thawed New Zealand White rabbit spermatozoa (mean±standard error of mean).

Criteria	DMSO 8%	DMF 8%	DMSO 4%+DMF 4%
Non-fragmented DNA (%)	79.05±0.45 ^b	77.21±0.23 ^b	86.04±0.92 ^a
Tail length (Px)	13.00±0.47 ^b	14.66±0.53 ^b	9.40±0.22 ^a
DNA in tail (%)	4.02±0.42 ^b	4.19±0.62 ^b	2.03±0.04 ^a
Tail moment	0.44±0.05 ^b	0.53±0.04 ^c	0.30±0.02 ^a

Means with different superscripts (^{a, b, c}) within the same raw are significantly different at $P < 0.05$. DMSO: Dimethyl sulphoxide. DMF: Dimethyl formamide. n= 10.

DISCUSSION

The results of the current study demonstrate that dilution of rabbit semen in INRA-82 extender supplemented with a combination of DMSO and DMF (4% each) improved sperm quality after freezing and thawing. We also speculate that supplementation of INRA-82 with DMSO or with DMF alone at higher concentrations (8%) negatively impacts the quality and function of rabbit spermatozoa. These results indicate that both DMSO and DMF could have a synergistic effect on enhancing the characteristics of rabbit semen after freezing and thawing. The positive effects of DMSO and DMF on protecting spermatozoa against freezing-inflicted damage have been reported in different species (Mocé and Vicente, 2009). Previous studies showed that DMSO could interfere with the production of intracellular free oxygen radicals, which subsequently improves the permeability of cell membranes (Kashiwazaki, 2006). Moreover, DMF can protect the sperm cells by lowering the osmotic stress due to its low molecular weight compared to other cryoprotectants such as glycerol (Hanada and Nagase, 198), which could be supported by using skim milk extender (INRA-82). The lower sperm motility and viability in semen samples diluted in media supplemented with 8% of DMSO or 8% of DMF agrees with the notion that cryoprotectant toxicity is exacerbated as the concentration and the exposure time are increased (Holt, 2000). DMSO at high concentrations such as 8% or more has been shown to be detrimental to frozen/thawed sperm and this could be due to the ability of DMSO to interact with egg yolk that is present in freezing extender (Courtens, 1995; Viudes de Castro and Vicente, 1996; Gogol, 1999). Therefore, using a combination of DMSO and DMF at 4% would minimise the possibilities of harmful interaction between DMSO and the egg yolk. Contrary to our results, previous studies in stallions showed that supplementation of INRA-82 extender with DMF alone improved the quality of cryopreserved semen (Medeiros *et al.*, 2002; Squires *et al.*, 2004; Alvarenga *et al.*, 2005; Mesa and Henao, 2012; Fadl, 2016). The discrepancies between the results may be due to the species differences. The adverse effects of DMF at high concentration (8%) on the quality of frozen/thawed rabbit semen that were clear in our studies could be due to the harmful interaction between DMF and the skim milk that is present in INRA-82. Our results also indicate that DMSO alone is less harmful to the frozen/thawed rabbit semen when compared with DMF, as it improved the sperm quality to some extent. These findings are in agreement with those of previous studies on cryopreservation of rabbit semen (Hanada and Nagase, 1980; Di Iorio *et al.*, 2012 and Hall *et al.*, 2017).

In conclusion, the results of the present study indicate that supplementation of INRA-82 extender with a combination of DMSO and DMF at 4% each improved the quality and biological function of New Zealand White rabbit spermatozoa. Our results also suggest that both DMSO and DMF at lower concentrations can act synergistically to protect the rabbit spermatozoa against freezing-inflicted damages. Further studies are needed to evaluate the effects of different cryoprotectants on the *in vivo* fertility of frozen/thawed rabbit semen.

Acknowledgements: The authors would like to thank Dr Adel R. Moawad, for his careful revision and critical inputs on the manuscript.

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