ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 10 (6): 514-520, 2011
ISSN 1682-296X / DOI: 10.3923/biotech.2011.514.520

DNA Fragmentation, Linear Velocity and Fertilising Ability of Reactivated Cryopreserved Goldfish Sperm using Different Cryoprotectants

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Abstract: The motility, fertilising ability and DNA integrity of freeze-thawed goldfish sperm was evaluated using computer-aided sperm motility analysis and the comet assay. The results indicate that sublethal effects during the freeze-thawing procedure compromised sperm fertilising ability, DNA integrity, velocity linearity and the duration of swimming. Compared to 10% Ethylene glycol (EG), 5% and 10% Egg yolk (EY) and DMSO treated sperm exhibited higher percentage of motile sperm and higher path velocity (VAP); straight-line velocity (VSL) linearity (LIN), DNA integrity and fertilising ability. There was a low but significant ($r = 0.67$, $p<0.05$) correlation between DNA integrity and the maximum Duration of Forward Progressive Swimming (DFPS) ability of cryopreserved goldfish sperm. DNA integrity and to a lesser degree the simple assessment of the DFPM of thawed sperm may be reliable indicators of sperm integrity for the development and the rapid assessment of fish sperm cryopreservation protocols.

Key words: Spermfreezing, DNA integrity, sperm motility

INTRODUCTION

Cryopreserved fish sperm is routinely used in fish hatcheries. Several biological, chemical and technical parameters can influence the quality of fresh and cryopreserved sperm. These factors include freezing and thawing rates, the chemical composition of cryoprotectants and extenders and initial quality of the fresh sperm (Acosta-Salmon *et al*., 2007; Basavaraja *et al*., 2006; Billard and Zhang, 2005).

The evaluation of cryopreserved fish sperm is important for optimising freeze-thawing procedures for commercially important fish species. The comet assay is a technique which can evaluate the DNA integrity of cells. Comet assay allows comparisons using a simple procedure and requires small sample size. DNA integrity of sperm estimated on the basis of single gel electrophoresis and DNA damage, especially single and double strand breaks (Warnecke and Pluta, 2003) are reflected in a comet like dispersion of the DNA fragments. The method is widely accepted in experimental medicinal research (Barbouti *et al*., 2002). In fish however, the effect of cryopreservation on DNA integrity of the sperm varies between different species (Cabrita *et al*., 2005) and has been evaluated in only a few marine and freshwater fish including loach (Kopeika *et al*., 2004), trout (Cabrita *et al*., 2005; Labbe *et al*., 2001), sturgeon (Li *et al*., 2008) sea bass (Zilli *et al*., 2003) and sea bream (Cabrita *et al*., 2005).

Goldfish are important ornamental fish with a significant global commercial presence. Selection of commercially important phenotypes requires several generations. The application of simple cryopreservation protocols revolutionised the aquaculture industry including ornamental fish production. Sperm cryopreservation can facilitate artificial reproduction and rapid output of ornamental fish with desirable traits in a commercial fish hatchery unit. Furthermore, biotechnological procedures for fish hatcheries (Horvath and Urbanyi, 2000) would benefit from the long term storage of selected phenotypes and the cost of maintaining a large number of broodstock would be significantly reduced.

Various cryopreservation protocols have been developed for several cyprinids species (Babiak *et al*., 1997; Magyary *et al*., 2000; Morisawa *et al*., 1983) with reported 95% fertilisation and hatching rate.

Several factors can influence the cryopreservation success. A successful cryopreservation procedure involves the use of a solution that maintains sperm inactivity without compromising the cell membrane integrity and in combination with cryoprotectants, protecting sperm from injury during the freeze-thawing cycle. Evaluation of the percentage of motile cells is an indicator of sperm quality, but sub-lethal damages to cryopreserved sperm may result in reduced sperm motility and fertilising ability or in compromised sperm DNA

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integrity (Lewis *et al*., 2008; Kopeika *et al*., 2004; Li *et al*., 2008). Any changes in the motility patterns (for example swimming speed and forward or progressive movement) after a freeze-thaw cycle would indicate a cryopreservation induced alteration in the swimming ability of sperm. The evaluation of sperm motility with computer assisted motility analysis (CASA) is an objective method which uses a computerised sperm motility tracking system to analyse sperm motility parameters. Image processing software detects and tabulates the motility data of sperm cells. Sperm velocity and other motion characteristics are useful indicators for the objective evaluation of sperm quality (Pavlov, 2006).

Published works on the cryopreservation of fish sperm indicate that success varies with species, cryoprotecting agent and ionic composition of the immobilising solution (Billard *et al*., 1995; Warnecke and Pluta, 2003; Woolsey *et al*., 2006). Reports on the sperm cryopreservation of cyprinids, indicate good motility and fertilisation rates when using Dimethyl Sulfoxide (DMSO) as a cryoprotecting agent (Warnecke and Pluta, 2003). However, very limited information is available on the swimming parameters of reactivated goldfish sperm (Babiak *et al*., 1997) and in and the possible relationship between DNA integrity and kinetics of cryopreserved fish sperm. The evaluation of DNA integrity and motility is crucial for integrating sperm freezing protocols in commercial fish hatcheries and for evaluating the quality of fish sperm. Nevertheless, the cryopreservation induced changes in DNA integrity and motility may vary, for example between different sperm dilution and cryoprotectant (Acosta-Salmon *et al*., 2007) treatments and it is not clear which sperm quality parameter is the most suitable for assessing different freezing protocols.

Many laboratories need to evaluate and optimise different sperm freezing protocols. In Ornamental fish hatcheries, the sperm of male fish with a rare phenotype is valuable and sperm banking may be an appropriate option. The evaluation of sperm integrity is crucial for optimising cryopreservation protocols and there are several sperm quality parameters which can be used, however, the use of some of these are technically difficult. For example, the motility of freeze-thawed sperm is important parameter for ensuring that sperm will reach the egg in time but dedicated laboratory equipment is required to analyse the sperm kinetic parameters. Similarly, post-thaw DNA integrity is crucial for the fertilization success and the normal embryonic development of the fertilised eggs but protocols for assessing sperm DNA integrity, are technically challenging and time consuming (Cabrita *et al*., 2005). Moreover, different cryoprotecting solutions may offer different level of protection on post-thaw sperm DNA and motility. The purpose of this study was to directly compare the DNA integrity and sperm kinetics as indicators of sperm quality of cryopreserved goldfish sperm using different cryoprotecting solutions.

MATERIALS AND METHODS

Fish rearing conditions: Selected male goldfish (n = 15, size range: 3.2-6.9 cm total length) were held in a 0.5 m^3 circular tank. Temperature (22-23°C), oxygen concentration (>8.0 mg L^{-1}) and pH (7.2-7.4) was monitored daily. Photoperiod was maintained at 14 h light/10 h darkness. Fish were fed using commercially available dry feed. Feeding was maintained at 5% body wet weight, calculated weekly.

Sperm and egg collection: The day before stripping, hormone was injected into the dorsal musculature (dosage: Males: 1 mg kg^{-1} of carp pituitary extract (CPE) in 0.65% NaCl solution, females: 3.5 mg kg^{-1} CPE administrated over two dosages, 10% at 24 h and 90% at 10 h before stripping). Milt was collected into 1 mL pipettes and eggs were collected in glass dishes. Eggs and sperm from a pool of two males and two females were used for the fertilisation experiments with two replicates. For sperm motility analysis, n = 3 male fish with two replicates per treatment were used. All values presented are mean values±SD.

Exposure of fresh goldfish sperm in cryoprotectants: In order to assess the influence of different cryoprotecting agents on the motility particularly on the maximum duration of motile sperm, samples of freshly collected sperm were diluted at a ratio of 1:3 with an extender and cryoprotecting media. The mixture of extender-cryoprotecting solution contained 80 mM NaCl, 60 mM KCl, 25 mM Tris, pH 8.0 (Billard *et al*., 1995) and one of the following cryoprotecting agents: 5% egg yolk+5% Dimethyl Sulfoxide (DMSO), 10% egg yolk+10% DMSO, 10% methanol and 10% ethylene glycol. The milt was incubated in the extender-cryoprotecting solution for 10 min at 4°C. Undiluted fresh sperm was used as control. Sperm motility was analysed after the 10 min exposure. The sperm was activated after dilution (1:2000, sperm:activating solution) in an activating solution containing 5 mM KCl, pH 8.0 or in fresh water and examined rapidly by placing 20 μL of the activated sperm on a Leja counting glass slide (Leja Netherlands, 100 μm chamber depth). The initial percentage of motile cells and the total duration of sperm motility of each preparation was monitored by visual observation of movement until full cessation of forward swimming activity.

Sperm cryopreservation: Semen was diluted at a ratio of 1:3 with an extender containing 80 mM NaC1, 60 mM KCl, 25 mM Tris, pH 8.0 and different concentrations of cryoprotectants (5% egg yolk+5% DMSO, 10% egg yolk+10% DMSO and 10% ethylene glycol). Sperm were exposed to cryoprotectants for 10 minutes and subsequently placed into 0.5 mL plastic straws (IMV France) and frozen in liquid nitrogen vapour at a height of 2.5 cm above the liquid nitrogen surface. After 15 min, straws were plunged into liquid nitrogen and stored for 24 h. After storage in liquid nitrogen, samples were thawed in a warm water bath (30°C) for 30 sec. The thawed sperm was used immediately for sperm motility analysis or for the fertilisation experiments.

Sperm motility analysis: All measurements were performed with activating solutions at 20°C. Sperm motility parameters were measured using a computer-aided sperm motility analysis (CASA, Test Sperm 2.1, Videotest, St. Petersburg, Russia) software. Sperm motility was recorded with "Pinnacle studio" software (Pinnacle System, GmbH, Braunschweig, Germany). The system included a microscope with a phase-contrast device, a standard microcellular camera for sperm, a colour analogous system to transfer images to the computer, and special software (Videotest-sperm 2.1). Videotaping of sperm motility was conducted at a rate of 25 frames/s and the files were saved in AVI format. The duration of each single videotaping was 1s, three videotapings for each sperm sample. The equipment used for the analysis of sperm motility included a phase contrast microscope (Olympus BX41) and a video camera (Sonny Exwave HHD) connected to a computer. Videotaping was conducted at a total magnification of 400X. The settings and the procedures used in the present work were based on a previously published work (Pavlov, 2006). Briefly, in the present study, a Makler chamber (Sefi Medical Instruments, Israel) was used (10 depth, 20 μm sample volume). Within 20-30 sec after mixing with activating solution, sperm were placed in the Makler chamber and the preparation was focused. This time is considered as the initial swimming time for the present study. Recording of motility was initiated within 10 sec of focusing. As a result the exact time of the registration of motility was within 30-40 sec after mixing the thawed sperm with an activating solution. Randomly selected microscopic fields were used (n = 6.7) and at least 150 motile spermatozoa (n>50 in each replica) from each male fish (n = 3 fish, total >150 spermatozoa per cryoprotectant) were observed.

To avoid possible overestimation errors of the percentage of motile spermatozoa, the proportion of immotile cells (%) was not determined using the automated CASA method but it was determined subjectively based on visual determinations. The CASA parameters evaluated in this study were:

- **Averaged path velocity (VAP; in micrometers per second):** A smoothing of the path of the centre of brightness of the spermatozoon, which reduces the effect of the lateral head displacement
- **Straight-line velocity (VSL; in micrometers per second):** The distance between the first and last tracked point of the spermatozoon trajectory divided by the time elapsed
- **Curvilinear velocity (VCL; in micrometers per second):** The sum of the distances between each centre of brightness, during each frame, divided by the time elapsed
- **Lateral head displacement ALH:** The maximum value of the distance of any point on the track from the corresponding average path, multiplied by two
- **Strait direction of sperm motion (STR):** Measures the departure of the cell path from a straight line. STR is derived from the ratio of VSL/VAP multiplied by 100
- **Linearity (LIN):** Measures the straightness of the path. LIN is derived from the ratio of straight-line velocity (VSL) divided by curvilinear velocity (VCL) as a percentage

Linearity indicates the similarity of the actual path (of a moving sperm) to a linear sperm path (Warnecke and Pluta, 2003). Linearity is an indicator of how effective the swimming motion is in dispersing the sperm. Spermatozoa were classified according to their curvilinear swimming velocity (VCL) into the following categories: Immotile and low motility spermatozoa (VCL ≤10 μm sec^{-1}) and fully motile spermatozoa (VCL >10 μm sec^{-1}).

COMET assay analysis of frozen sperm DNA integrity: Frozen samples were thawed and 5 μL were diluted in phosphate buffered saline (PBS), pH 7.4. Samples were centrifuged, supernatants were discarded and sperm cells were resuspended in PBS. Subsequently, cell number in each sample was measured and 1.5×10^5 sperm cells were suspended in 1% low melting point agarose in PBS, pH 7.4 and pipetted on to super frosted glass microscope slides precoated with a layer of 1% normal melting point agarose (warmed to 37°C prior to use). The agarose was allowed to set at 4°C for 10 min and then the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris at pH 10, 1% Triton X-100 v/v) at 4°C for 1 h in order to dissolve cellular proteins and lipids. Slides were then placed in single rows in a 30 cm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA, pH 13, at 4°C for 40 min in order to allow for separation of the two DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 30 V, 300 mAmps for 30 min. The slides were then washed three times for 5 min each with 0.4 M Tris, pH 7.5, at 4°C before staining with Hoechst 33342 (35 μM).

Hoechst-stained nuclei were examined under a UV microscope and a magnification of 400. The damage was not homogeneous and visual scoring of the cellular DNA on each slide was based on characterisation of 100 randomly selected nucleoids. The comet-like DNA formations were categorised into 5 classes (0, 1, 2, 3, and 4) representing an increasing extent of DNA damage seen as a "tail." Each comet was assigned a value according to its class. The overall score for one hundred comets ranged from 0 (100% of comets in class 0) to 400 (100% of comets in class 4). In this way, the overall DNA damage of the cell population can be expressed in arbitrary units. Visual observations and analyses of the results were carried out by the same experienced person, using a specific pattern when moving along the slide. In previous studies we compared the scoring obtained by visual observation of damaged DNA, with results obtained when other parameters as the percentage of DNA in the tail estimated after computer image analysis using specific software package (Barbouti *et al*., 2002). Visual scoring correlated linearly with the percentage of DNA in the tail. The same linear correlation between visual scoring and computer image analysis has also been reported by other laboratories.

The fertilising ability of cryopreserved sperm: Cryopreserved sperm were mixed with eggs (0.5 mL of thawed sperm with 0.5 g of eggs). This resulted in a sperm/egg ratio of about 3.5×0^{6} spermatozoaria per egg, which is within the optimal range for sperm fertilisation in cyprinids (Lahnsteiner *et al*., 2003). The sperm were activated by addition of 10 mL of activating solution. After 2 min of incubation, the activating solution was gradually replaced with a solution of 4 NaCl and 3 g L^{-1} urea to prevent stickiness of the eggs. After several washes, eggs were placed in zug-jars and incubated at 20°C. The percentage of fertilised eggs was evaluated 10 h later using a microscope and counting the number of fertilised eggs in two samples of approximately 50 eggs siphoned from each zug-jar.

Statistical analysis: All values are expressed as mean values±SD. Data were analysed using ANOVA followed by Student-Neuman-Keuls multiple range test ($p<0.05$). For the Least-squares regression analyses, percentage data and motility parameters were subjected to logarithmic transformation. For assessing the significance of differences between different cryoprotecting solutions, data were arc-sin transformed prior to statistical analysis. Data are presented as means of duplicates from three selected male fish ±SD. Prior to statistical comparison data were tested for normal distribution (Shapiro-Wilk test). The level of significance was set at 0.05. The DNA integrity and other sperm quality parameters were analysed by Spearman correlation test.

RESULTS

Exposure of freshly collected sperm of goldfish to 5% egg yolk +5% DMSO and 10% egg yolk did not influence sperm motility duration. The use of methanol or ethylene glycol as cryoprotecting agents resulted in reduced duration of motility (Fig. 1). In the same manner, cryopreservation of goldfish sperm with 5% egg yolk +5% DMSO and 10% egg yolk +10% DMSO resulted in improved sperm kinetics with higher VAP, VSL sperm motion linearity and the duration of sperm motility compared to the 10% ethylene glycol (Fig. 2a, b).

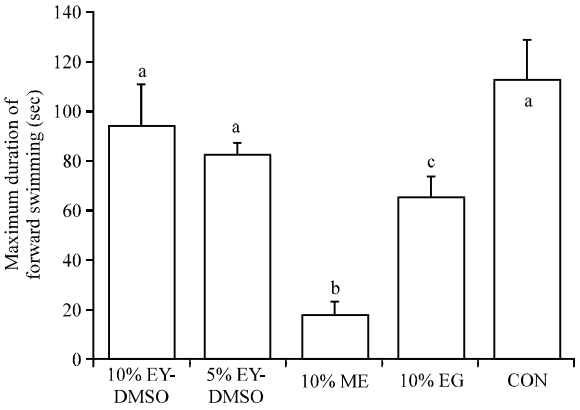

Fig. 1: The influence of different cryoprotectants on the maximum sperm motility duration observed in fresh goldfish sperm stored at 4°C for 10 min and activated with an activating solution containing 5 mM KCl, pH 8.0 (Billard and Cosson, 1992). Fresh goldfish sperm was exposed to different cryoprotecting solutions for 10 min: (10% EY-DMSO) 10% egg yolk+10% DMSO; (5% EY-DMSO): 5% egg yolk+5% DMSO; (10% ME): 10% methanol; (10% EG): 10% ethylene glycol; (Diluted CON): fresh sperm was diluted in extender (80 mMNaC1, 60 mM KCl, 25 mM Tris, pH 8.0) and activated with activating solution; (Undiluted CON): Fresh sperm activated immediately after collection in fresh water. Different letters indicate significant differences ($p<0.05$). Values derived from three male fish (in duplicates, in total six measurements for each cryoprotectant). Error bars indicate the SD

Table 1: Correlation of the sperm DNA fragmentation with other sperm quality parameters which varied significantly after the freezing and thawing process of all treatments

	% Motility	%Fertilisation	Max duration	VAP	VSL	LIN
DNA damage	-0.78*	-0.97**	-0.66*	-0.96**	-0.93**	-0.96**

The asterisks indicates significant correlation (*p<0.05; **p<0.001) as analysed by Spearman correlation test

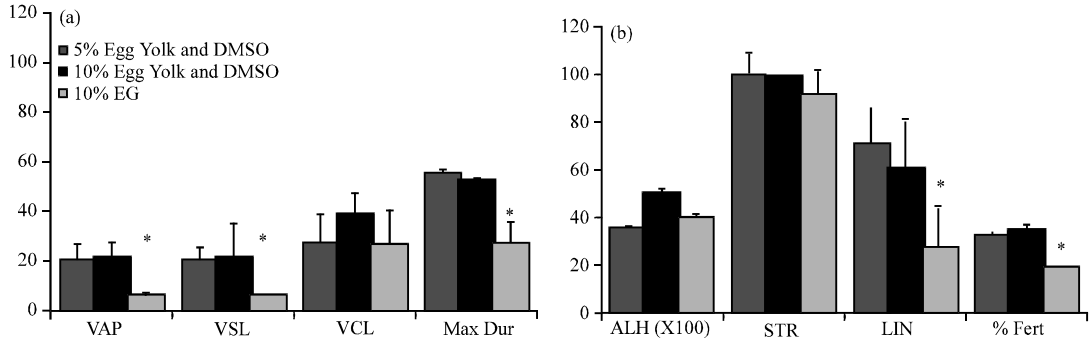

Fig. 2(a-b): The swimming parameters and fertilisation ability of freeze-thawed goldfish sperm using different cryoprotecting solutions: 5% egg yolk+5% DMSO (grey bars); 10% egg yolk+10% DMSO (black bars); 10% Ethylene glycol (dotted bars) (a): VAP: Average path velocity (μm/s), VSL: Straight velocity (μm/s), VCL: Curvilinear velocity (μm/s) and maximum duration of forward movement (b) ALH: Lateral head displacement amplitude (μm/s), STR%: Straightness, LIN%: Linearity and Fertilising ability of freeze-thawed sperm. Activating solution used was 5 mM KCl, pH 8.0. An asterisk indicates significant difference (p<0.05) Error bars indicate the SD

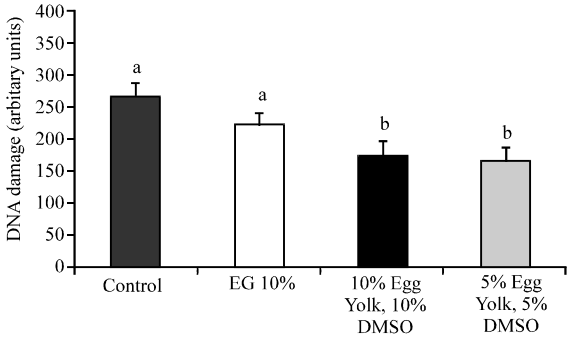

Fig. 3: DNA damage in freeze-thawed goldfish sperm exposed to different cryoprotectants. Control: fresh sperm frozen without cryoprotecting agents. EG: ethylene glycol. Error bars indicate the standard deviation. Different letters indicate significant differences (p<0.05)

The fertilising ability of sperm cryopreserved in both 10 and 5% Egg Yolk+DMSO were significantly higher than those obtained in 10% EG (Fig. 2b, ANOVA, p = 0.033). There was no correlation between the pre and post-thawed duration of motility of all the experimental treatments (results not shown). Nevertheless, there was a low (Spearman correlation, r = 0.67, p<0.05) but significant correlation between DNA integrity and the maximum duration of forward progressive swimming ability of cryopreserved goldfish sperm post-thawed sperm motility. The DNA integrity of thawed sperm correlated with some of the CASA measured motility parameters including VAP; VSL;LIN, duration of motility and fertilising ability (Table 1). The DNA integrity of cryopreserved sperm was compromised when EG was used as cryoprotectant. On the contrary, 10 or 5% EY and DMSO provided sufficient protection and DNA integrity which varied significantly from the EG group (Fig. 3).

In general, DNA damage explained most of the differences in the sperm kinetics and the fertilisation ability of cryopreserved sperm.

DISCUSSION

Forward swimming and linear motion are important for increasing the chances of sperm to reach the ova successfully (Lahnsteiner *et al*., 2003). The initial sperm velocity is important for ensuring that sperm reach the ova in good time, whereas a low linearity indicates an erratic swimming pattern which in turn indicates a deterioration of the sperm energy stores (Perez-Cerezales *et al*., 2010). The experiments with freeze-thawed goldfish sperm indicated a significant deterioration in the VAP, VSL and linearity of the sperm cryopreserved in 10% ethylene glycol, whereas cryopreservation of goldfish sperm with 5% egg yolk +5% DMSO and 10% egg yolk +10% gave superior results. The superior fertilisation ability of sperm

cryopreserved with egg yolk and DMSO was also reflected in the very satisfactory kinetics, whereas ethylene glycol (EG) at a concentration of 10% resulted in significant deterioration in the motility characteristics and the fertilising ability of the sperm.

In general, fish sperm motility correlates well with the fertilisation ability of the sperm. In the present work, different cryoprotectants resulted in different motility and fertilisation ability of cryopreserved fish sperm. A significant relationship between some of the sperm motility parameters (percentage of motile cells, VAP, VSL and LIN) and the fertilisation ability of cryopreserved goldfish sperm was observed. Sperm motility patterns may be altered by the cryopreservation procedure with consequences on the fertilising ability of freeze-thawed sperm. These phenomenon is associated with sublethal effects of cryopreservation procedures in relation to membrane integrity of the sperm which can result from lipid peroxidation and osmotically drive changes in the cellular volume during the freezing-thawing cycle (Billard and Zhang, 2005). For example the decreased duration of forward spermatozoa movement, observed in the EG treated sperm, may be a result of sub lethal damage to the membrane integrity with consequences for the intracellular ionic conditions and ATP stores which both can influence the velocity of cryopreserved sperm after activation (Cosson *et al*., 2008). This partially reduced sperm motility is commonly observed in fish hatcheries where an increased sperm/eggs ratio is recommended to compensate the reduced motility when using frozen-thawed sperm (Lahnsteiner *et al*., 2003).

Sperm cells can escape both "solution effects" injury and the dangers of intracellular freezing when appropriate cryoprotectants are present. Nevertheless cryoprotectants may themselves be damaging to cells or may not provide sufficient protection. This may be a result of damages in sperm membrane function or DNA integrity (Kopeika *et al*., 2004). For example, in the present work, both the DNA integrity and the percentage of motile cells was reduced when EG was used in the cryoprotecting solution. This reduction influenced the fertilising ability of the sperm. As a result, both comet assay and sperm motility parameters indicate that EG exhibited poor cryoprotecting ability for goldfish sperm. These results are in agreement with the results of other fish species in which sperm motility and fertilisation ability of the sperm varies according to the cryoprotecting agent (Horvath and Urbanyi, 2000) and the DNA damage of cryopreserved sperm (Cabrita *et al*., 2005). In addition to structural damage during the freeze-thawing cycle, the cryopreservation induced damages to sperm DNA could also be attributed to the release of reactive oxygen species released after thawing, resulting in oxidative stress (Perez-Cerezales *et al*., 2010). Sperm motility is important for fertilisation success, motile sperm with compromised DNA may retain the ability to fertilise eggs. Early embryonic development may progress and DNA repair mechanism could correct some of the DNA damage (Kopeika *et al*., 2004). In the same manner, compromised membrane integrity may result in the leakage of functional enzymes and ATP molecules but still sperm may exhibit a capacity for fertilisation. Nevertheles, it can be assumed that if both membrane and DNA integrity are compromised the fertilisation success will be significantly reduced.

In conclusion, the results indicate that DMSO and Egg yolk can provide sufficient protections to sperm during the freezing thawing cycle. Sublethal effects during the freezing-thawing procedure were observed with the cryoprotecting solutions used in the present work. These sublethal damages can result in significantly compromised post-thaw sperm DNA integrity, velocity and linearity with consequences for the fertilising ability of the cryopreserved goldfish sperm.

ACKNOWLEDGMENTS

The authors are grateful to Prof D. Pavlov, Department of Ichthyology, Moscow State University, for comments and suggestions on an earlier version of this manuscript. Thanks are also due to Prof. D. Galaris, Laboratory of Biological Chemistry, University of Ioannina Medical School who kindly advised us on the comet assay procedure.

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