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Effect of DNA damage caused by cryopreservation of spermatozoa using a modified Single cell gell electrophoresis in the freshwater catfish *Pangasianodon hypophthalmus* (Fowler, 1936)

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PEER REVIEW

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Comments
The present study deals with the effect of DNA damage of *P. hypophthalmus* caused by cryopreservation of spermatozoa using the SCGE method. The experiments used are appropriate and the findings are interesting.
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ABSTRACT

Objective: To detect the integrity of sperm DNA in the catfish *Pangasianodon hypophthalmus* cryopreserved with Hanks balanced salt solution, 5%–30% dimethyl acetamide (DMA) using the single-cell gel electrophoresis in order to test how sperm cryopreservation affected nuclear DNA stability.
Methods: Electrophoresis was conducted for 60 min at 130 mA and 15 V. The comet images were analyzed using software CometScore 1.5, and parameters such as comet length, tail length and percentage DNA in the tail were obtained. Then the comet rate and damage coefficient were calculated.
Results: There were no significant differences in motility, comet rate and damage coefficient between fresh sperm and cryopreserved sperm stored in 5%, 10%, 15% and 20% DMA, while the sperm cryopreserved with 25% and 30% DMA had lower motility, higher comet length and damage coefficients than those of fresh sperm.
Conclusions: There was a positive correlation between comet rate of cryopreserved sperm and the concentration of DMA. The toxicity of cryoprotectant is the main factor for DNA damage in cryopreserved sperm.

KEYWORDS
Pangasianodon hypophthalmus, Sperm, Cryopreservation, Genetic damage, Single-cell gel electrophoresis

1. Introduction

Pangasianodon hypophthalmus (*P. hypophthalmus*) is a freshwater fish species of considerable commercial importance in the aquaculture industry in Southeast Asia. Overfishing and pollution have substantially reduced the wild populations[1]. Reliable methods for sperm cryopreservation could particularly be beneficial and improve the breeding of this species. Another index of sperm quality is DNA stability[2]. However, there is little information available on the effects of cryopreservation on the DNA of fish spermatozoa. Hagedorn and Carter used *in vitro* fertilization to increase sperm fertility after cryopreservation in zebrafish[3]. Beirão *et al.* introduced antifreeze proteins to improve sperm cryopreservation in seabream[4]. Lin and You used glycerol as cryoprotectants for the successful storage of large yellow croaker sperm[5]. Lin *et al.* discovered effects of the freeze-thaw process on sperm ultrastructure of large yellow croaker[6]. DNA damage could be caused in spermatozoa by many different factors, from sperm aging to the effect of toxic agents, freezing process, X-ray irradiation, *etc*[7-9]. DNA-repairing pathways are highly conserved mechanisms, and the expression of different enzymes involved in the

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process has been detected during early development in different species from fish to mammals[10–12]. Damage of *P. hypophthalmus* sperm DNA by cryopreservation, however, has not been assessed as yet.

The comet assay or single–cell gel electrophoresis (SCGE) is a classical method for the detection of DNA damage. The alkaline comet assay was firstly described by Singh *et al*[13]. This assay follows the process of cell lysis and DNA unwinding, the DNA fragments flow from the nucleus and move to the positive pole, generating a comet–like band. After fluorescent staining, DNA–damage of the cells can be observed under the microscope. The fluorescence intensity of the comet tail depends on the degree of DNA damage. The degree of DNA damage is one of the important indicators of sperm quality. It is of high sensibility, fast, and straightforward. SCGE is widely used for detection of DNA damage in plant cells[14], human sperm[5], and fish sperm[1,15].

The DNA damage of *P. hypophthalmus* sperm after cryopreservation in different cryoprotectant [dimethyl acetamide (DMA)] concentrations of 5%; 10%; 15%; 20%; 25% and 30%, and the effect of DMA on sperm nucleus DNA stability were analyzed, in order to optimize the cryoprotectant concentrations for cryopreservation of *P. hypophthalmus* sperm and to provide a reliable method for cryopreserving sperm.

2. Materials and methods

Mature fishes were obtained from integrated fish farm Vandalur, Tamil Nadu. Sodium sarcosinate, ethidium bromide staining solution, proteinase K, Tris, Triton X–100, dimethylsulfoxide, ethylene diamine tetraacetic acid, and low melting–point agarose were purchased from Sigma–Aldrich (USA). All reagents were pure grade. Nikon ECLIPSE fluorescence microscope was bought from Nikon Corporation, Japan; CometScore 1.5 image analysis software was from TriTek Corporation, USA.

2.1. Sperm cryopreservation

Fresh sperm was collected from five anesthetized male fish of a weight from 7 to 8 kg. Then 1 volume of sperm was mixed with 3 volumes of Hanks balanced salt solution at different concentrations in 5%–30% DMA; each treatment was repeated for four times. The suspension was balanced on ice for 10–15 min and then it was injected into 500 µL straws that were horizontally placed 3–5 cm above the surface of liquid nitrogen. After a pre–freezing period of 5–8 min the straws were immersed in liquid nitrogen for storage.

2.2. Sperm motility assessment

The motility of fresh sperm and cryopreserved sperm was monitored for 30 d following the procedure of Tiersch *et al*. and Lin and You[5,15]. In brief, motility was determined with a dark–field microscope. Thawed samples were diluted in a cryopreservation extender. Then 2 µL of sperm were placed on a microscopic slide and diluted with filtered sea water. Sperm became motile and initiated rapid swimming when diluted. The percentage of sperm swimming actively in a forward direction was estimated at 200× magnification. Motility observations were divided into three periods: 1) time required to reach maximum motility after addition of water; 2) duration of maximum motility, and 3) time until complete cessation of motility.

2.3. SCGE procedure

SCGE was performed in 9 steps as follows: (1) Dilution: Straws were thawed in a water bath at 40 °C, after centrifugation (2 000 r/min) and washing with phosphate buffer solution (pH=7.4) at 4 °C twice. Sperm suspension (50 µL, at concentrations of about 8×10^6 sperm/mL) was diluted with 350 µL of 1% low melting–point agarose mixture in a 5 mL centrifuge tube to obtain a concentration of 10^6 sperm/mL. (2) Lysis: Sperm suspension was placed in a lysing solution (2.5 mol/L NaCl, 100 mmol/L ethylene diamine tetraacetic acid, 10 g/L sodium sarcosinate, 10% dimethylsulfoxide, 1% Triton X–100, pH 10) for 1 h at 100 °C. The lysis and the steps 3–7 were performed in the dark. (3) Digestion: Sperm suspension was placed in a digestive solution (2.5 mol/L NaCl, 5 mmol/L Tris, 0.5 g/L sodium sarcosinate, 0.5 g/L proteinase K, pH 7.4) in a water bath at 55 °C for 3 h. (4) After washing with phosphate buffer solution twice, the mixture was melted at 700 °C in a water bath for 3 min, then a 100 µL drop of the mixture was pipetted on a slide, covered with a coverslip and allowed to solidify at 10 °C for 10 min. (5) Unwinding: The slides were placed in a horizontal electrophoresis bath with a fresh alkaline electrophoresis solution (300 mmol/L sodium acetate, 100 mmol/L Tris, pH 10.0) for 30 min to allow the DNA to unwind. (6) Electrophoresis was carried out using a current of 130 mA and 15 V for 1 h. (7) Neutralization: The slides were neutralized in a freshly prepared Tris–HCl buffer (0.4 mmol/L, pH 7.0) for 15 min. (8) Staining: The slides were stained with ethidium bromide solution (50 µg/mL) for 10 min. (9) Observation: The slides were observed and photographed on an epifluorescent microscope at an excitation wavelength of 580 nm. Each sample was measured 4 times, and 100 individual sperm were observed from each sample.

2.4. Image analysis

Comet images were analyzed with CometScore 1.5 software to obtain parameters such as comet length, tail length, and % DNA in the tail.

2.5. Comet rate and damage coefficient calculation

Comet rate and damage coefficient were calculated as follows: comet rate=(comet cell number/total cell number)× 100%. Damage coefficient=[(grade 0 cell number×0)+(grade I cell number×1)+(grade II cell number×2)+(grade III cell number×3)+(grade IV cell number×4)][16].

2.6. Statistical analysis

Analysis of variance was performed using SPSS for Windows 17.0, with a significance level of $P<0.05$.

3. Results

There was no significant difference among 5%–30% DMA treated groups (D1, D2, D3, D4) and control (fresh sperm) concerning activation rate, movement time and life time, whereas the values of D5 and D6 were significantly lower than the fresh sperm (Table 1).

Table 1
Motility of fresh sperm and cryopreserved sperm.

Group	Activation rate (%)	Movement time (min)	Life time (min)
Control	91.21±2.59	10.32±1.25	15.61±2.06
D1	89.72±3.04	8.63±0.55	12.63±0.95
D2	90.91±1.33	9.90±0.70	15.82±0.40
D3	89.61±2.04	9.62±0.84	14.53±0.61
D4	89.49±0.70	8.85±0.40	15.51±1.17
D5	67.44±2.64*	7.16±0.66*	11.46±1.00*
D6	62.15±1.96*	4.20±0.36*	8.55±1.04*

Control: Fresh sperm; D1: 5% DMA; D2: 10% DMA; D3: 15% DMA; D4: 20% DMA; D5: 25% DMA; D6: 30% DMA.
*: Significant effect of the treatment, $P<0.05$.

Results of SCGE detection on fresh sperm and cryopreserved sperm demonstrated that comet length, tail length, and concentration correlated positively with the cryoprotectant DMA (Table 2). Comet length and tail length

of D1–D4 treatments were similar to fresh sperm, but D5 and D6 showed significant differences to fresh sperm.

Comet images were analyzed with CometScore 1.5; the data of sperm nucleus DNA damage are displayed in Table 3. DNA damage was divided into 5 grades according to the method of Goerin[17]. The sperm of D1–D4 treatments showed DNA damage of different degrees, comet rate and damage coefficient were not significantly different from those of fresh sperm. In grade I and IV damages, only D6 sperm was significantly different from the control; nucleus DNA damage of D5 and D6 sperm were more severe, because there were significant differences from the fresh sperm in grades 0, II, III damage, comet rate and damage coefficient.

Table 2
Comet length and tail length of sperm (n=400, mean±SD).

Group	Comet length (μm)	Tail length (μm)
Control	17.72±2.33	0.91±0.43
D1	22.70±4.08	1.06±0.44
D2	24.45±2.57	4.60±3.09
D3	25.22±4.23	4.93±2.86
D4	26.89±1.24	5.09±1.83
D5	34.77±2.37*	8.29±2.70*
D6	40.00±10.96*	10.30±2.08*

Control: Fresh sperm; D1: 5% DMA; D2: 10% DMA; D3: 15% DMA; D4: 20% DMA; D5: 25% DMA; D6: 30% DMA.
*: Significant effect of the treatment, $P<0.05$.

The comet rate and damage coefficient of cryopreserved sperm were similar to those of fresh sperm (Figure 1).
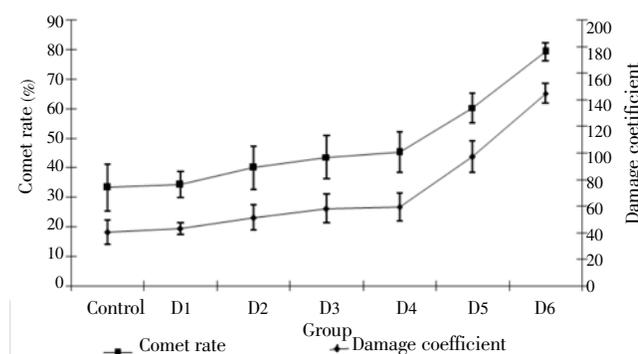
Figure 1. Comet rate and damage coefficient of sperm in different DMSO concentrations.
Control: Fresh sperm; D1: 5% DMA; D2: 10% DMA; D3: 15% DMA; D4: 20% DMA; D5: 25% DMA and D6: 30% DMA.

Table 3
Classification of DNA damage detection with comet rate.

Group	Grade 0 (%)	Grade I (%)	Grade II (%)	Grade III (%)	Grade IV (%)	Comet rate (%)	Damage coefficient
Control	66.73±7.80	26.52±6.66	6.53±1.29	0.25±0.50	0.00±0.00	33.22±7.80	40.21±9.36
D1	70.26±5.10	23.75±3.77	5.27±1.71	1.22±0.82	0.00±0.00	34.32±4.35	43.21±4.55
D2	60.48±7.26	30.77±6.60	7.26±2.06	2.23±0.82	0.00±0.00	40.89±7.26	51.25±9.54
D3	56.51±7.33	31.25±4.92	9.11±2.83	2.71±1.26	0.00±0.00	43.53±7.33	58.21±10.80
D4	54.72±6.80	34.24±4.86	8.33±2.94	3.66±2.58	0.00±0.00	45.28±6.80	59.26±10.66
D5	39.72±4.99*	35.37±2.06	14.96±0.58*	9.06±3.59*	1.02±0.68	60.66±4.99*	97.21±11.97*
D6	20.97±2.99*	37.53±3.11*	21.11±2.75*	16.43±2.63*	3.59±2.21*	79.61±2.99*	144.42±7.27*

Control: Fresh sperm; D1: 5% DMA; D2: 10% DMA; D3: 15% DMA; D4: 20% DMA; D5: 25% DMA; D6: 30% DMA.
*: Significant effect of the treatment, $P<0.05$.

There was a positive correlation between comet rate and cryoprotectant concentration for cryopreserved sperm (Figure 2 and 3).
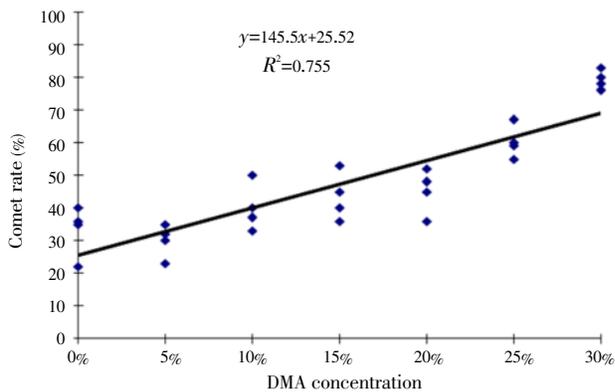
Figure 2. Comet rate and DMA concentration of fresh sperm and cryopreserved sperm.
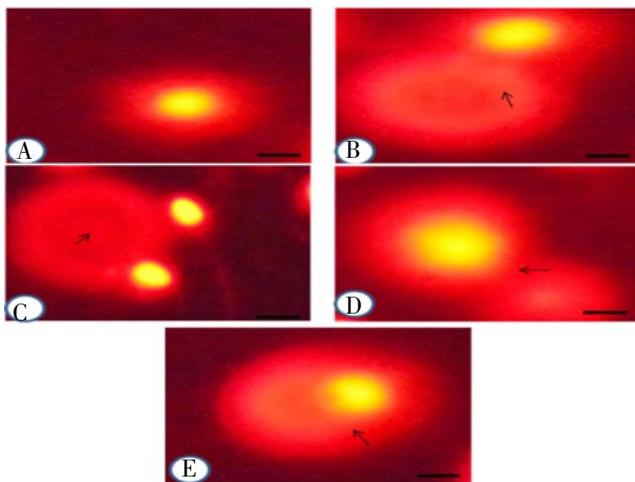
Figure 3. Photomicrographs of single–cell electrophoresis of fresh and stored spermatozoa in *P. hypophthalmus*.

A. comet image of control (unstained) spermatozoa, without any DNA damage. B. comet image of cryopreserved spermatozoa with DMA (10%) showing minimum tail damage. C. comet image of spermatozoa cryopreserved with DMA (15%) showing minimum tail damage. D. comet image of spermatozoa cryopreserved with DMA (20%) showing head undamaged and tail damaged. E. comet image of spermatozoa cryopreserved with DMA (25%) showing maximum tail damage. The arrow indicates the damage.

4. Discussion

Continuous improvement was made to detect not only double–strand breaks but also single–strand breaks at alkaline fragile sites quickly and with high sensitivity[18,19]. Based on the characteristics of the *P. hypophthalmus* sperm, we made some modifications to the classic SCGE method in the study of this species that were similar to *Sparus macrocephalus*[1].

Ice crystal formation and recrystallisation in the freeze–thaw procedures are the main factors that induce mechanical injury to frozen cells, and the degree of injury depended on intracellular fluid content. Although sperm consists of haploid cells with super spiral tetramers combined with nucleoprotamine and DNA, chromatin is highly aggregated with little intracellular fluid, and mechanical injury of sperm is much less than

that of body cells during cryopreservation. There was negligable mechanical injury on sperm DNA stability, as demonstrated by Song *et al*[20]. The study of Suquet *et al*. suggested that the freeze–thaw process did not cause genome alterations in turbot *Psetta maxima* sperm since the fertilization rate, the hatching rate, the larval survival rate (up to 10 d) and the larval weight were similar between fresh and frozen–thawed sperm[11]. Similarly, no effect of the freeze–thaw process on the nucleus of Atlantic croaker *Micropogonias undulatus* spermatozoa was reported[10]. But Cabrita *et al*. indicate that the freeze–thaw process could induce DNA damage to sperm of rainbow trout and gilthead sea bream *Sparus aurata*[21].

DNA damage detection for cryopreserved sperm of red sea bream *Pagrosomus major* carried out by Xu *et al*. indicated that comet rate and DNA damage had a positive correlation with DMA concentration[22]. In our study, the degree of DNA damage in *P. hypophthalmus* sperm increased with increasing DMA concentration. When DMA concentration ranged between 5% and 30%, the DNA damage was similar to the fresh sperm and there was no significant difference between frozen sperm and fresh sperm in sperm motility; sperm DNA damage increased and sperm motility decreased when DMA concentration was higher than 30%.

The sperm for the different experimental treatments were from the same mature male fish individuals. The sperm aliquots were treated in identical ways except different DMA concentrations. Since mechanical injury effects had no significant influence on DNA stability, we explain the differences of comet rate and damage coefficient of cryopreserved sperm as caused by elevated DMA concentrations.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Comments

Background
Overfishing and pollution have substantially reduced the wild populations of *P. hypophthalmus*. Reliable methods for sperm cryopreservation could particularly be beneficial and improve the breeding of this species. Another index of sperm quality is DNA stability. However, there is little information available on the effects of cryopreservation on the DNA of fish spermatozoa.

Research frontiers

The present study aimed to optimize the cryoprotectant concentrations for cryopreservation of *P. hypophthalmus* sperm and to provide a reliable method for cryopreserving sperm.

Related reports

The study of Suquet *et al.* suggested that the freeze–thaw process did not cause genome alterations in turbot *Psetta maxima* sperm since the fertilization rate, the hatching rate, the larval survival rate (up to 10 d) and the larval weight were similar between fresh and frozen–thawed sperm. But Cabrita *et al.* indicate that the freeze–thaw process could induce DNA damage to sperm of rainbow trout and gilthead sea bream *Sparus aurata*.

Innovations and breakthroughs

Authors in this research work used the classic SCGE method with some modifications to investigate the effect of DNA damage of *P. hypophthalmus* caused by cryopreservation of spermatozoa.

Applications

This study provide information to optimize the cryoprotectant concentrations for cryopreservation of *P. hypophthalmus* sperm, which is useful for the further research work.

Peer review

The present study deals with the effect of DNA damage of *P. hypophthalmus* caused by cryopreservation of spermatozoa using the SCGE method. The experiments used are appropriate and the findings are interesting.

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