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# DNA integrity of *Polyodon spathula* cryopreserved sperm

By P. Li[1,2], Q. Wei[1,2] and L. Liu[1]

[1]*Key Laboratory of Freshwater Fish Germplasm Resources and Biotechnology, Ministry of Agriculture, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Sciences, Jingzhou;* [2]*Fishery College, Huazhong Agricultural University, Wuhan, China*

## Summary

Comet assay was used to detect DNA integrity of paddlefish (*Polyodon spathula*) sperm following cryopreservation. At the same time, sperm velocities prior to freezing and post-thawing were also assessed by the computer-assisted sperm analysis (CASA) system. Significant differences ($P < 0.05$) were detected in the degree of DNA damage in cryopreserved sperm using different extenders. According to osmolality of the extenders, DNA damages of Sb (20 mM Tris, 75 mM sucrose, 0.5 mM KCl, pH 8.5) sperm was the least, which showed that the percentage of tail DNA of Sb (17.87–35.28%) was lower than those of Sa (20 mM Tris, 50 mM sucrose, 0.5 mM KCl, pH 8.5) and Sc (20 mM Tris, 100 mM sucrose, 0.5 mM KCl, pH 8.5). Moreover, A and B class sperm cells provided most of the Sb sperm (>50%). However, in light of the concentration of methanol, DNA damages of M8 (8% methanol concentration) sperm were the least, including a lower percentage of the tail DNA (21.56–30.86%), and C and D class sperm cells (<30%), regardless of the osmolality of the extenders. In conclusion, when the dilution was 20 mM Tris, 75 mM sucrose, 0.5 mM KCl, pH 8.5 and the concentration of methanol was 8%, the extenders were the best for cryopreservation of paddlefish sperm. In addition, the results indicated that the extent of damage to sperm motility caused by freeze-thawing (VCL, VSL) was correlated with DNA breakage ($|r| > 0.8$). This implied that cryopreservation could damage sperm DNA of paddlefish and affect the sperm velocities when the osmolality and the concentrations of the cryoprotectants of the extender were inappropriate.

## Introduction

Paddlefish (*Polyodon spathula*) are a highly endangered species that need stringent protection measures, including cultivation for release to support rebuilding populations. Cryopreservation plays an important role in assisting controlled reproduction because of the scarcity of spawning fish and the need to have sperm at least ready for use whenever a mature female is caught for use in controlled reproduction. Quality of cryopreserved sperm is certainly a factor affecting reproductive success, and quality testing for sperm was a key objective of the study. The comet assay possesses all of the characteristics for sensitivity and simplicity needed for extremely small sample sizes and is a common technique used for individual cells to measure DNA damages, especially single and double strand breaks (Singh et al., 1989; Tice et al., 1990; Collins et al., 1997). The method is widely applied in ecotoxicological studies with aquatic species and studies on DNA repair mechanisms, including fish (Mitchelmore and Chipman, 1998; Rocha-Olivares et al., 2000; Barbieri et al., 2002). However, the effect of cryopreservation on sperm DNA has been evaluated in only a few marine and freshwater fish species, such as trout (Billard, 1983; Labbé et al., 2001), carp (Kornilova et al., 1997) and sea bass (Zilli et al., 2003; Cabrita et al., 2005). At present, many effects of cryopreservation on paddlefish sperm are often assessed in terms of sperm motility, integrity of the acrosome, integrity of the membrane, ATP level and fertilization capacity (Cosson and Linhart, 1996; Ciereszko et al., 2000; Linhart et al., 2002, 2003). However, very rarely was the integrity of DNA in cryopreserved spermatozoa assessed. Sperm DNA integrity is an important issue to consider in terms of success of fertilization, including normal development of the resulting embryos or offspring (Lopes et al., 1998). Damage of DNA in the male genome, however, is a clearly potential reason for post-fertilization failure (Sakkas et al., 2002; Sergerie et al., 2005). Therefore, the study of DNA integrity of sperm after cryopreservation is imperative.

In this study, DNA damage in freeze-thawed paddlefish sperm was assessed using the comet assay. In addition, pre-frozen and post-thaw sperm characteristics were assessed by analysing sperm motility. The aim of the present study was to investigate the phenomenon of DNA fragmentation in cryopreserved paddlefish sperm, while testing the potential application value of the comet assay for this assessment.

## Materials and methods

### Semen collection

Sperm were collected in mid-April from two mature paddlefish maintained at a commercial aquaculture facility supplied with warm water from a plant in Hubei. Immediately after catch, the males were artificially induced for spermiation by injection with 10 mg LHRHa $kg^{-1}$ of body weight (Ningbo Hormone Product Co., China). When the males began to spermiate, their genital apertures were wiped dry and their abdomens pressed to collect sperm into contamination-free airtight plastic bags containing oxygen. Fresh semen were stored on ice and transferred to the laboratory where the motility of the fresh sperm was immediately assessed by computer-assisted sperm analysis (CASA) . Some of the sperm samples were cryopreserved directly after assessment; the remaining sperm were prepared for the comet assay as an unfrozen control.

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#### Cryopreservation

The cryopreservation process was amended according to Linhart et al. (2006). Three dilutions were tested: Sa (20 mM Tris, 50 mM sucrose, 0.5 mM KCl, pH 8.5), Sb (20 mM Tris, 75 mM sucrose, 0.5 mM KCl, pH 8.5) and Sc (20 mM Tris, 100 mM sucrose, 0.5 mM KCl, pH 8.5). Milt was diluted at a ratio of 1 : 3 at 4°C, then allowed to reach equilibration under refrigeration for 30 min. All dilutions were supplemented with cryoprotectant M6, M8, M10 (final methanol concentrations of 6%, 8% and 10%), respectively, leading to nine groups of extenders (Sa + M6, Sa + M8, Sa + M10, Sb + M6, Sb + M8, Sb + M10, Sc + M6, Sc + M8, Sc + M10).

Suspensions of extended sperm were drawn into 0.5 ml straws and immediately transferred to a pre-programmed device Kryo 550-16 (PLANER, UK). The freezing regime included several steps: the straws were cooled from 0 to −5°C at a freezing rate of 3°C $min^{-1}$, from −5 to −15°C at a freezing rate of 5°C $min^{-1}$, from −15 to −25°C at a freezing rate of 10°C $min^{-1}$, and from −25 to −80°C at a freezing rate of 20°C $min^{-1}$, then held for 5 min at −80°C before final transfer into liquid nitrogen (Linhart et al., 2006).

After about 1 month, the straws were thawed in a water bath at 40°C for 8 s. Following thawing, sperm motility was assessed immediately; the comet assay was prepared at the same time.

#### Comet assay

200 μl frozen sperm (50 μl fresh) were diluted in 5 ml of PBS (phosphate buffer solution, $Ca^{2+}$ and $Mg^{2+}$ free). Diluted samples (100 μl) were added to 800 μl of 0.8% NMPA at 42°C (normal melting point agarose in PBS, $Ca^{2+}$ and $Mg^{2+}$ free). A 200 μl aliquot of this mixture was dropped onto a rough slide, covered with a coverslip and refrigerated at 0–4°C to allow the agarose to solidify. After 10 min, the coverslip was gently removed and the slide immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% laurylsarcosine, 1% Triton X-100, 10% DMSO, pH 10) at 4°C for 1 h. Thereafter the slide was subjected to enzymatic treatment with proteinase K (2.5 M NaCl, 5 mM Tris, 0.05% laurylsarcosine, 0.5 mg $ml^{-1}$ proteinase K, pH 7.4) at 37°C. Twelve hours later, the slide was placed horizontally in an electrophoresis cube filled with freshly made electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 12), left for 20 min and then electrophoresized (13 V and 50 mA, 10 min). Following electrophoresis the slide was neutralized in 0.4 M Tris-HCl (pH 7.5) three times, each time for 10 min. Slides were prepared in ternary for each sample.

Immediately before scoring, the slide was dried and stained with DAPI (dihydrochloride, 5 μg $ml^{-1}$), examined at 400× on a LEICA DM 2500 epifluorescence microscope using fluorescence filters with an excitation wavelength at 510–560 nm and an emission wavelength at 590 nm. Each slide was analysed from front to back and from left to right and approx. 100 cells were photographed with a video monitor (JVC TK-U890EG) using the fish colour sperm image analysis system (FSQAS-2000, by Liu Ling cooperating with Wuhan Qianping Image Technology Co. Ltd, China).

The photographs were dealt with by high definition colour cell image analysis system (HCIAS-2000, Wuhan Qianping Image Technology Co. Ltd). This software allows the determination of several parameters, including comet area ($A_C$), mean comet intensity ($I_C$), head area ($A_H$) and mean head intensity ($I_H$). For each cell analysed, the pixels observed in the tail of the comet represent DNA fragments (damaged DNA) and the nucleus represent the head of the comet in which the undamaged DNA is located. The percentage of tail DNA is given by the formula: $\%DNA_T = 100 \times (DNA_C - DNA_H)/DNA_C$, in which $DNA_C = A_C \times I_C$, $DNA_H = A_H \times I_H$, respectively. According to the percentage of tail DNA, cells were visually allocated into four classes: A (no damage, $\%DNA_T < 15\%$), B (light damage, $15\% < \%DNA_T < 30\%$), C (medium damage, $30\% < \%DNA_T < 45\%$) and D (high damage, $\%DNA_T > 45\%$); the comet rate is the result of subtracting the percentage of A sperm cells.

#### Sperm motility assessment

Sperm velocity was examined at 100× magnification immediately after mixing 0.25 μl cryopreserved sperm (fresh sperm diluted at 1 : 3 before assessment) with 50 μl activating medium (20 mM Tris) supplemented with 1 mg $ml^{-1}$ BSA (bovine serum albumin) on a glass slide prepositioned on the microscope stage. The final dilution was 1 : 600. BSA was added to prevent sperm heads from sticking to the glass slide. Motility was evaluated within the first 150 s following activation using a microscope (LEICA DM 2500) connected to a video monitor (JVC TK-U890EG). The successive positions of the pixel spots of which sperm were in the image were analysed by means of FSQAS-2000 IMAGE software. The same process was repeated twice. Images which were 3 s and 72 frames were selected every 30 s after activation. System assessment parameters were set as 20 pixels (the smallest paced distance) and 180 (the present threshold). Two velocity parameters of significant value in predicting the quality of sperm assessed in the study were VCL-curvilinear velocity and VSL-straight line velocity (Kime et al., 2001).

#### Statistical analysis

The results were expressed as mean ± SD (n = 3) and analysed by one-way ANOVA ($P < 0.05$) using STATGRAPH Software (SPSS 13.0). A P-value $< 0.05$ was considered to be statistically significant.

## Results

#### Effect of dilution on integrity DNA of cryopreserved sperm

Figure 1 shows clearly that the extender Sb caused less DNA damage in sperm cells than the extenders Sa and Sc, where Sb + M8 and Sb + M10 did not cause significantly different DNA damage by tail %DNA compared to fresh sperm, but there were significant increases in per cent DNA fragmentation in the other samples compared to the control ($P < 0.05$). Figure 2 demonstrates DNA fragmentation at different degrees after cryopreservation, which can be observed by the extension pixels of the comets. The analysis of cells with different relative damages indicated that the comet rates of Sb were less among the cryopreserved samples, since their percentage of A class sperm was approaching the control value (40.13%), but were the highest (up to 34.48%). More DNA damage occurred in Sb, which was light (B class), in particular, Sb + M10 had no C and D class sperm. Whereas comet rates of Sa and Sc were more than 90%, the medium and highly damaged cells (C and D) were represented in a subpopulation with over 50% for the two samples (Table 1).

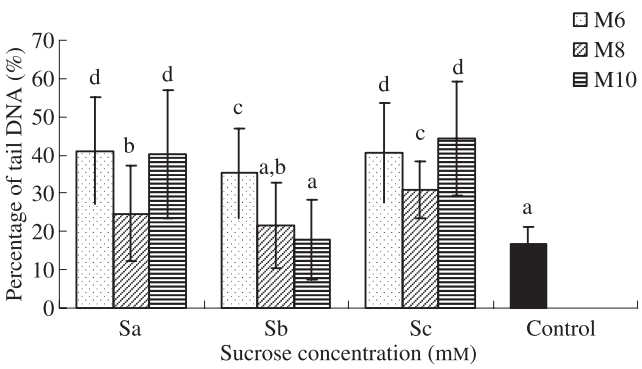


Fig. 1. DNA damage in cryopreserved sperm of paddlefish (*Poloydon spathua*) using three extenders: Sa, Sb, Sc, each supplemented at three levels of methanol (6%, 8% and 10%, labelled M6, M8 and M10, respectively). Sa = 20 mM Tris, 50 mM sucrose, 0.5 mM KCl, pH 8.5; Sb = 20 mM Tris, 75 mM sucrose, 0.5 mM KCl, pH 8.5 and Sc = 20 mM Tris, 100 mM sucrose, 0.5 mM KCl, pH 8.5. Values expressed as mean (columns) ± SD (bars). One-way ANOVA used for multiple comparisons of 100 determinations with three readings for each cell (total n = 300). Values with different letters (a, b, c, d) differed significantly ($P < 0.05$)

#### Effect of cryoprotectant on integrity DNA of cryopreserved sperm

Figure 1 shows that in samples of Sa and Sc, the percentage of tail DNA was less when the protectant was diluted with M8 compared to those with M6 and M10. In addition, Table 1 indicates that DNA damages in the samples with Sa + M8 and Sc + M8 cryoprotectant combinations were lower. For example, 25% of the frozen spermatozoa appeared undamaged (A class) in Sa + M8 treated sperm cells, while DNA damages in B class sperm were light. The percentages for D class sperm in samples treated in the Sc + M8 combination (5.56%) were much less than those in samples exposed to Sc + M6 and Sc + M10 treatments (over 40%). The results suggest that appropriate concentrations and compositions of cryoprotectant may serve as a compensation function.

#### Correlation analysis between sperm DNA damage and sperm motility

Extender type significantly affected ($P < 0.05$) the post-thaw sperm velocities such as VCL and VSL (Fig. 3). All results 150 s after activation were expressed as mean ± SD. When the motility velocities were analysed in detail, we observed that fresh samples showed velocities, whichever VCL (66.96 $\mu m\ s^{-1}$) or VSL (36.12 $\mu m\ s^{-1}$) was higher, than frozen sample velocities (14.03–40.44 and 7.08–30.53 $\mu m\ s^{-1}$ for VCL and VSL, respectively). Moreover, in cryopreserved samples, velocities of Sb + M8 and Sb + M10 (more than 30 $\mu m\ s^{-1}$) were the highest; next was Sa + M8; the others were much lower.

In paddlefish, there were highly negative linear correlations between tail % of DNA and sperm motility velocities ($|r| > 0.8$, $P < 0.01$; Fig. 4), i.e. DNA fragmentation increased along with declining sperm motility.

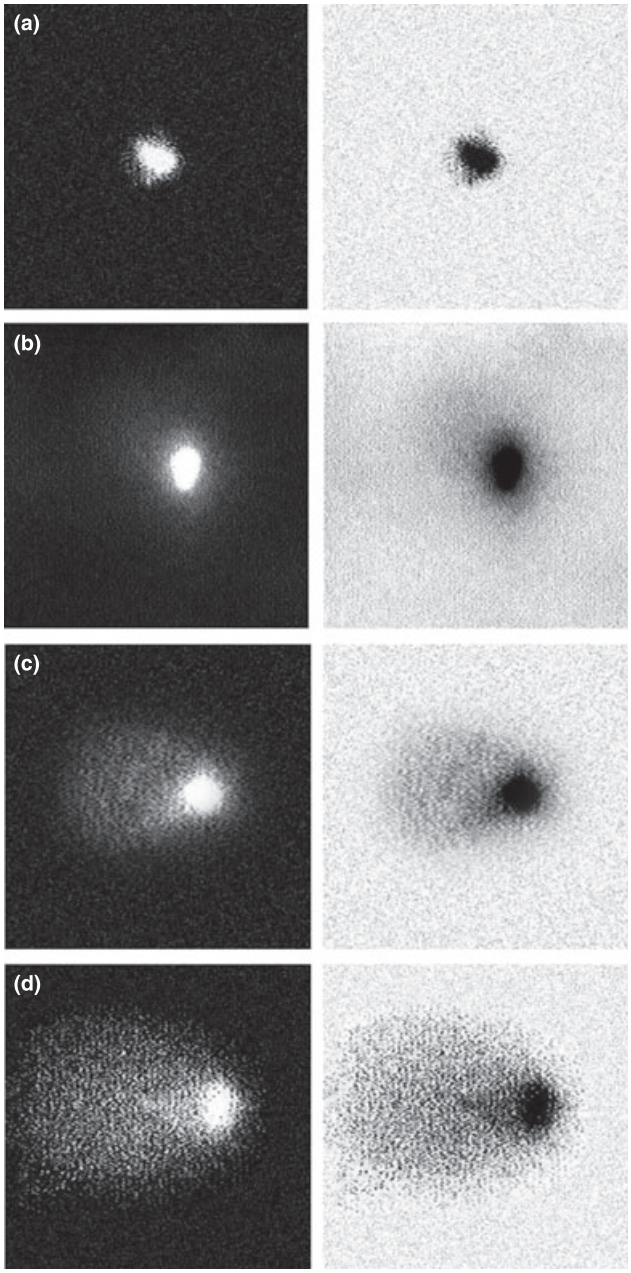


Fig. 2. Appearance of frozen-thawed paddlefish (*Poloydon spathua*) sperm with different degrees of DNA damage, arranged in four classes (a, no damage, $\%DNA_T < 15\%$; b, light damage, $15\% < \%DNA_T < 30\%$; c, medium damage, $30\% < \%DNA_T < 45\%$; d, high damage, $\%DNA_T > 45\%$) following preparation by comet assay. Right column = negative images of same preparation used to perform analyses

## Discussion

#### Application of the comet assay in cryopreservation of fish sperm

The comet assay, which is used as a common technique for assessing DNA damage, has been widely applied in radiation studies, assessment genotoxicity in human medication, etc. It is ideally suited as a non-specific biomarker of genotoxicity studies in aquatic species suffering from pollution (Mitchelmore and Chipman, 1998; Rocha-Olivares et al., 2000; Barbieri et al., 2002). Moreover, the comet assay was also successful in DNA damage assessment of cryopreserved sperm. Labbé et al. (2001), using the comet assay, demonstrated that cryopreserved rainbow trout sperm were slightly affected in DNA stability. Cabrita et al. (2005), using the same method, found a significant increase in fragmented DNA in cryopreserved rainbow trout sperm, while in gilthead sea bream the different effects on DNA integrity were caused by different dilutions following cryopreservation. Zilli et al. (2003) reported DNA fragmentation in cryopreserved sea bass

| Extenders | A | B | C | D | Comet rate |
|---|---|---|---|---|---|
| Sa + M6 | 1.72 | 31.03 | 29.31 | 39.66 | 98.28 |
| Sa + M8 | 25.00 | 53.57 | 17.86 | 3.57 | 75.00 |
| Sa + M10 | 4.23 | 30.99 | 29.58 | 35.21 | 95.77 |
| Sb + M6 | 5.26 | 45.61 | 26.32 | 22.81 | 94.74 |
| Sb + M8 | 26.25 | 61.25 | 8.75 | 3.75 | 73.75 |
| Sb + M10 | 34.48 | 65.52 | 0 | 0 | 65.52 |
| Sc + M6 | 11.11 | 23.81 | 20.63 | 44.44 | 88.89 |
| Sc + M8 | 6.94 | 59.72 | 27.78 | 5.56 | 93.06 |
| Sc + M10 | 3.23 | 25.81 | 21.31 | 50.00 | 96.77 |
| Fresh sperm | 86.74 | 13.26 | 0 | 0 | 13.26 |

Table 1
Cells with different degree of DNA damage (A, B, C, D), as identified in Fig. 1, by comet assay in paddlefish (*Poloydon spathua*) sperm after cryopreservation with different extenders and fresh sperm (control)

Data represent mean values (%) based on 100 cells measured, each redone three times.

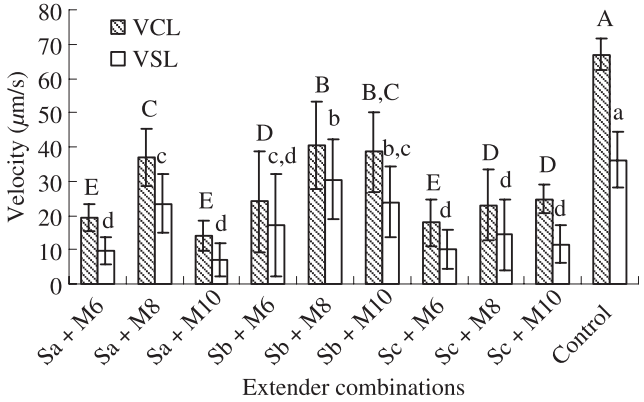


Fig. 3. Velocities (VCL, curvilinear velocity; VSL, straight line velocity) of frozen-thawed paddlefish (*Poloydon spathua*) sperm from different extenders and fresh sperm. Codes for extenders identical to those in Fig. 1. Values are expressed as mean (columns) ± SD (bars), n = 3. Values with different letters (VCL: A, B, C, D, E; VSL: a, b, c, d, e) differed significantly ($P < 0.05$)

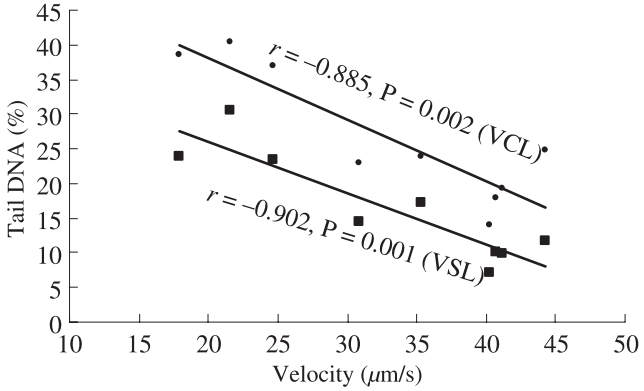


Fig. 4. Relationship of *Poloydon spathua* sperm DNA damage (% tail DNA as classified in Fig. 2) to sperm swimming velocities following freeze-thawing (n = 8). Upper and lower regressions show relationships of VCL and VSL to $\%DNA_T$, respectively

sperm, while certain cryoprotectants significantly reduced this effect. In this study we demonstrated different effects on DNA integrity after cryopreservation by different extenders. When sperm were cryopreserved in high osmolality (100 mM sucrose) or low osmolality (50 mM sucrose) media, a higher degree of DNA damage was observed. However, when the sucrose dilutent concentration was 75 mM, DNA damage in sperm was reduced, suggesting that an effective role of that medium was reflected in the results of the comet assay. Some authors affirmed that DNA-damaged spermatozoa would fertilize less efficiently and might result in embryo failure of embryonic development (Twigg et al., 1998; Morris et al., 2002; Tomsu et al., 2002). Unfortunately, fertility trials were not performed in the present study due to the absence of fertile eggs. Therefore, the degree to which damage may occur and affect fertility as well as normal embryonic development should be studied further.

Generally, the parameters that describe DNA damage include comet tail length, percentage of tail DNA, level of DNA, etc. (Billard, 1983; Kornilova et al., 1997; Labbé et al., 2001; Zilli et al., 2003). The nucleus of the paddlefish sperm assumes a cylindrical form and because of the long and short axis, it is difficult to confirm the diameter of the nucleus and measure the comet tail length and tail moment (tail length × percentage of tail DNA) because the sperm diffuse in agarose in all directions after electrophoresis. Moreover, in measuring the DNA damage categories, descriptions in the literature were not consistent. Xu et al. (2005) adopted a categorization in five damage classes according to the size of comet tail and sperm nucleus. Cabrita et al. (2005) selected some damage classes according to the percentage of the tail DNA, with 10% as a unit. In our study, according to percentage of tail DNA at 15% as a unit, four DNA damage classes were determined in paddlefish sperm cells. We considered that the measurement method of the comet assay had great changeability, for example, comet images varied with species, cell type, management methods, etc. thereby criteria of measurement on DNA damage cannot be standardized.

#### Osmolality of dilution and cryoprotectant

Some authors considered that osmolality played a key role in cryopreservation of fish sperm. Horváth et al. (2005) found that hyper- and hypo-osmolality of dilution were not favourable to cryopreserve *Acipenser brevirostrum* and *Scaphirhynchus albus* sperm. Cryoprotectants added to extenders can combine with the water molecule, leading to hydration, increase density and adhesion capacity of solution, and thereby weaken the process of water crystals in sperm (Yu et al., 2004). Linhart et al. (2003) confirmed that sperm motility and fertilization rates were enhanced using 8% methanol as cryoprotectant for the sperm of paddlefish. Our results demonstrated that 8% and 10% methanol were useful for paddlefish sperm, provided the osmolality of the extender was appropriate; however, where this is not the case, 8% methanol could at least mitigate the DNA damage.

#### DNA damage of sperm and sperm motility

According to Irvine et al. (2000) and Zini et al. (2001), sperm samples with low motility carried high loads of DNA damage.

In the current study, the motility of paddlefish sperm was reduced after cryopreservation; in addition, post-thaw sperm DNA integrity was simultaneously shown to be linked with sperm velocities (VSL, VCL). We did not anticipate seeing any cause–effect relationship between DNA damage and sperm velocity; however, the results suggest that different degrees of damage occur in different parts of the sperm during cryopreservation. In general, sperm motility relates to mitochondrial function (Fraser and Strzeżek, 2006). In the present study, the effects of cryopreservation on sperm velocities were more obvious than the ones on DNA integrity, although the impact of cryopreservation on mitochondria may be higher than on the DNA.

Our study demonstrates that DNA damage can be an additional cause of cryo-injury for paddlefish sperm. The data reported here also demonstrated the role of the quality of the extender (75 mM sucrose, 20 mM Tris, 0.5 mM KCl, 8% or 10% methanol) in reducing paddlefish sperm DNA fragmentation. Finally, from our results the effectiveness of the comet assay in revealing DNA fragmentation in paddlefish sperm during cryopreservation was also confirmed.

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**References**

Barbieri, E.; Oliveira, I. R.; Serralheiro, P. C. S., 2002: The use of metabolism to evaluate the toxicity of dodecil benzene sulfonate (LAS-C12) on the *Mugil platanus* (mullet) according to the temperature and salinity. J. Exp. Mar. Biol. Ecol. **277,** 109–127.

Billard, R., 1983: Ultrastructure of trout spermatozoa: changes after dilution and deep-freezing. Cell Tissue Res. **228,** 205–218.

Cabrita, E.; Robles, V.; Rebordinos, L.; Sarasquete, C.; Herráez, M. P., 2005: Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology **50,** 144–153.

Ciereszko, A.; Dabrowski, K.; Mims, S. D.; Glogowski, J., 2000: Characteristics of sperm acrosin-like activity of paddlefish (*Polyodon spathula* Walbaum). Comp. Biochem. Physiol. Part B **125,** 197–203.

Collins, A. R.; Dobson, V. L.; Dusinska, M.; Kennedy, G.; Stetina, R., 1997: The comet assay: what can it really tell us? Mutat. Res. **375,** 183–193.

Cosson, J.; Linhart, O., 1996: Paddlefish *Polyodon spathula* spermatozoa: effects of potassium and pH on motility. Folia Zool. **45,** 364–370.

Fraser, L.; Strzeżek, J., 2007: Effect of different procedures of ejaculate collection, extenders and packages on DNA integrity of boar spermatozoa following freezing-thawing. Anim. Reprod. Sci. **99,** 317–329.

Horváth, Á.; Wayman, W. R.; Urbányi, B.; Ware, K. M.; Dean, J. C.; Tiersch, T. R., 2005: The relationship of the cryoprotectants methanol and dimethyl sulfoxide and hyperosmotic extenders on sperm cryopreservation of two North-American sturgeon species. Aquaculture **247,** 243–251.

Irvine, D. S.; Twigg, J. P.; Gordon, E. L.; Fulton, N.; Milne, P. A.; Aitken, R. J., 2000: DNA integrity in human sperm: relationships with semen quality. J. Androl. **21,** 33–44.

Kime, D. E.; VanLooka, K. J. W.; McAllister, B. G.; Huyskensb, G.; Rurangwab, E.; Ollevier, F., 2001: Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp. Biochem. Physiol. Part C **130,** 425–433.

Kornilova, S. V.; Leont'ev, V. S.; Grigor'ev, D. N.; Blagoi, I. P., 1997: Effect of low temperatures on molecular parameters of DNA. Biofizika **42,** 99–104.

Labbé, C.; Martoriati, A.; Devaux, A.; Maisse, G., 2001: Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Mol. Reprod. Dev. **60,** 397–404.

Linhart, O.; Cosson, J.; Mims, S. D.; Shelton, W. L.; Rodina, M., 2002: Effects of ions on the motility of fresh and demembranated paddlefish spermatozoa. Reproduction **124,** 713–719.

Linhart, O.; Cosson, J.; Mims, S. D.; Rodina, M.; Gela, D.; Shelton, W. L., 2003: Effects of ions on the motility of fresh and demembranate spermatozoa of common carp (*Cyprinus carpio*) and paddlefish (*Polyodon spathula*). Fish Physiol. Biochem. **28,** 203–205.

Linhart, O.; Mims, S. D.; Gomelsky, B.; Cvetkova, L. I.; Cosson, J.; Rodina, M.; Horváth, Á.; Urbányi, B., 2006: Effect of cryoprotectant and male on motility parameters and fertilization rate in paddlefish (*Polyodon spathula*) frozen-thawed spermatozoa. J. Appl. Ichthyol. **22,** 389–394.

Lopes, S.; Sun, J. G.; Jurisicova, A.; Meriano, J.; Casper, R. F., 1998: Sperm deoxyribonucleic acid fragmentation is increased in poor quality semen samples and correlates with failed fertilization in a cytoplasmic sperm injection. Fertil. Steril. **69,** 528–532.

Mitchelmore, C. L.; Chipman, J. K., 1998: DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat. Res. **399,** 135–147.

Morris, I. D.; Ilotts, S.; Dixon, L.; Brison, D. R., 2002: The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (comet assay) and its relationship to fertilization and embryo development. Hum. Reprod. **17,** 990–998.

Rocha-Olivares, A.; Garber, N. M.; Stuck, K. C., 2000: High genetic diversity, large inter-oceanic divergence and historical demography of the striped mullet. J. Fish Biol. **57,** 1134–1149.

Sakkas, D.; Moffatt, O.; Manicardi, G. C.; Mariethoz, E.; Tarozzi, N.; Bizzaro, D., 2002: Nature of DNA damage in ejaculated human spermatozoa and possible involvement of apoptosis. Biol. Reprod. **66,** 1061–1070.

Sergerie, M.; Laforest, G.; Bujan, L.; Bissonnette, F.; Bleau, G., 2005: Sperm DNA fragmentation: threshold value in male fertility. Hum. Reprod. **20,** 3446–3451.

Singh, N. P.; Danner, D. B.; Tice, R. R.; McCoy, M. T.; Collins, G. D.; Schneider, E. L., 1989: Abundant alkali-sensitive sites in DNA of human and mouse sperm. Exp. Cell Res. **184,** 461–470.

Tice, R. R.; Andrews, P. W.; Singh, N. P., 1990: The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. Basic Life Sci. **53,** 291–301.

Tomsu, M.; Sharma, V.; Miller, D., 2002: Embryo quality and IVF treatment outcome may correlate with different sperm comet assay parameters. Hum. Reprod. **16,** 2160–2165.

Twigg, J.; Fulton, N.; Gomez, E.; Irvine, D. S.; Aitken, R. J., 1998: Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human sperm: lipid peroxidation DNA fragmentation and effectiveness of antioxidants. Hum. Reprod. **13,** 1429–1436.

Xu, X. C.; Ding, F. H.; Li, J., 2005: Cryopreservation caused sperm DNA damage in red sea bream *Pagrosomus major* and its detection. Trans. Oceanol. Limnol. **36,** 221–225 (in Chinese).

Yu, H. T.; Zhang, X. M.; Chen, C., 2004: Prospect of cryopreservation of fish spermatozoa. Trans. Oceanol. Limnol. **35,** 66–72 (in Chinese).

Zilli, L.; Schiavone, R.; Zonno, V.; Storelli, C.; Vilella, S., 2003: Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology **47,** 227–235.

Zini, A.; Bielecki, R.; Phang, D.; Zenzes, M. T., 2001: Correlations between two markers of sperm DNA integrity DNA denaturation and DNA fragmentation in fertile and infertile men. Fertil. Steril. **75,** 674–677.

**Author's address:** Prof. Ling Liu, Key Laboratory of Freshwater Fish Germplasm Resources and Biotechnology, Ministry of Agriculture, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Sciences, No. 41 Jianghan Road, Shashi District, Jingzhou City, Hubei 434000, China.
E-mail: liul@yfi.ac.cn