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Evaluation of the damage in fish spermatozoa cryopreservation*

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Abstract Cryodamages occur during sperm cryopreservation. Cryopreservation of fish sperm usually results in marked decrease in sperm quality, such as swelling or disruption of the plasma membrane, mitochondrial dysfunction, diminished sperm motility, impaired velocity, shorter motility period, denaturation, and release of some enzymes from spermatozoa. In this paper, damages in morphology, physiology, biochemistry and metabolism, and genetic integrity of fish semen after cryopreservation are discussed. New approaches in assessment of fish thawed sperm quality such as computer assisted sperm analysis, flow cytometic analysis combined with fluorescent probes and single cell gel electrophoresis are also briefly reviewed.

Key words: cryodamage; cryopreservation; fish sperm; freezing and thawing

1 INTRODUCTION

Over years of sperm cryopreservation work, empirically-derived protocols have been established, and considerable improvement has been achieved in sperm cryopreservation (Dreanno et al., 1997; Lahnsteiner et al., 2000; Fabbrocini et al., 2000; Zhang et al., 2003; Chen et al., 2004) since the first successful cryopreservation of herring sperm 50 years ago (Blaxter, 1953). However, the cryopreserved semen of fish species is not yet widely used in large scales because post-thaw viability and fertility of the cryopreserved sperm are reduced dramatically as a consequence of accumulated cellular damage that arise throughout the freezing-thawing process.

Recently, most researches in this field are focused on optimizing protocols for cryopreservation of fish sperm, while few studies have been carried out on the damage to spermatozoa during freeze-thaw cycle. The cryopreservation process involves the steps of temperature reduction, cellular dehydration, freezing and thawing. Sperms are not adapted to tolerate low temperature; therefore, during the process of freezing and thawing, spermatozoa are subjected to a series of markedly changes. Cellular damage in different degrees is induced by distinct mechanisms at each of the cryopreservation phases, and the functional state of the frozen-thawed spermatozoa is the result of the injuries accumulated throughout the freezing and thawing process.

The reasons of cryopreservation damage are commonly explained by a two-factor hypothesis (Mazur, 1970). If sperm is frozen too rapidly, adequate dehydration does not occur and intracellular ice formation will induce mechanical damage to cell organelles and membranes, even lead to lethal injuries to the cells. Otherwise, if the freezing rate is too slow, ice forms in the extracellular solution first and leads to an increase in the electrolyte concentration of the unfrozen fraction. The increased concentration induces consequent cellular dehydration. The cells dry up as freezing proceeds extracellularly, with the rate and extent of this process depending upon the rate of freezing and permeability of the cells. If this dehydration is too severe, formation of toxicity or injury can be induced due to high concentration of electrolytes. This condition is collectively called "solution effect" that damages to the sperm (Mazur, 1970; Mazur and Rigopoulos, 1983). High concentrations of electrolytes affect ionic in-

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teractions, including those that help stabilize the native state of enzymes. Because both solute concentration and intracellular ice formation contribute to cell damage, an ideal freezing rate should be neither too fast nor too slow.

Further sperm damage may be caused during thawing process as the ice melts or recrystallizes. Slow thawing is most likely to induce injury, as it allows time for consolidation of microscopic ice-crystal into large forms. Sperm survival is also dependent upon extender composition, osmotic stress and other factors during freezing and thawing. In the present paper, cryodamage to the spermatozoa of fish after freezing and thawing are discussed, and recently published methods of laboratory assessment on sperm quality are also briefly reviewed.

2 CRYODAMAGE FOLLOWING CRYOPRESERVATION

Cryodamage to the sperm has been reported in many fish species. The cryopreservation of spermatozoa is known to result in considerable damage to cellular structures such as plasma membrane, nucleus, mitochondria, and flagellum (Lahnsteiner et al., 1992; 1996a; Drokin et al., 1998; Conget et al., 1996; Zhang et al., 2003). These damages may lead to further consequence including leakage and denaturation of proteins, structural deformation of the cell organelles, abnormalities of sperm chromatin structure, and genome alterations (Billard, 1983). The magnitude of cryoinjuries during freezing and thawing may result in diminished motility in association with impaired velocity and reduced fertility.

2.1 Morphological damage

It is a common knowledge that the cryopreservation process has the most significant influence on morphological and functional integrity of spermatozoa. Plasma membrane integrity and mitochondria function are two most important factors for keeping viability of the sperm. The evaluation of sperm membranes can help indicating the success of cryopreservation since the membranes are extremely susceptible to cryoinjury. Ultrastructure cryodamages to fish sperm cells were assessed by electron microscopy and permeability to different fluorescent dyes such as SYBR-14, propidium iodide (PI) and rhodamine 123 (Rh 123) used fluorescent staining method.

The morphological damages observed in frozen-thawed sperm were mainly swelling and plasmalemma rupture in the head, mid-piece, and tail, as well as in the mitochondria (Gwo et al., 1992; Billard, 1983; Lahnsteiner et al., 1992; 1996a; Gwo et al., 1999; Yao et al., 2000; Zhang et al., 2003) (Figs.1, 2). After cryopreservation of grayling sperm, a marked decrease in sperm quality was observed, 40%–50% of the spermatozoa had been completely damaged, 30%–40% altered and only 10%–20% were intact in morphology (Lahnsteiner et al., 1992). Similar morphological changes have also been reported in post-thawed sperm of ocean pout (Yao et al., 2000), rainbow trout (Lahnsteiner et al., 1996a), and Atlantic croaker (Gwo et al., 1992). These results are in accordance with the reports in cryopreservation of rainbow trout spermatozoa by use of flow cytometic analysis (FCM) in combination with dual fluorescent staining method. The percentage of spermatozoa with an intact membrane and a functional mitochondrion varied below 18% only (Ogier de Baulny, 1997). Some authors suggest that the swelling of the spermatozoa and the rupture of the plasmalemma after thawing may have been caused by damage of the cells through intracellular ice-crystal growth or instability of the membrane, and their losses for osmoregulation (Grout and Morris, 1987; Lahnsteiner et al., 1996a). However, detailed analysis in future is needed.

The flow cytometic analysis in double- or triple-staining procedure has been widely used for the assessment of sperm quality in mammalian species for its advantages of rapid and precise method for measuring multiple sperm attributes on thousands of sperm cells in a short period of time (Garner et al., 1986; Graham et al., 1990; Garner and Johnson 1995; Papaioannou et al., 1997; Gravance et al., 2001; Love et al., 2003; Kavak et al., 2003). In recent years, FCM has been introduced to study of fresh and cryopreserved sperm from fish species. Double-staining method has been performed on fresh and cryopreserved sperm after fluorescent staining with Rh 123 and PI to quantify mitochondrial function and to assess plasma membrane integrity in rainbow trout (Ogier de Baulny et al. 1997), Nile tilapia (Segovia et al., 2000), European catfish (Ogier de Baulny et al., 1999). The fluorescent probe Rh 123 is accumulated in the mitochondrial turning functional mitochondria into fluorescent green. Sperm having a damaged plasma membrane would permit PI to enter the cell and bind the DNA in a fluorescent red, whereas the plasma membrane

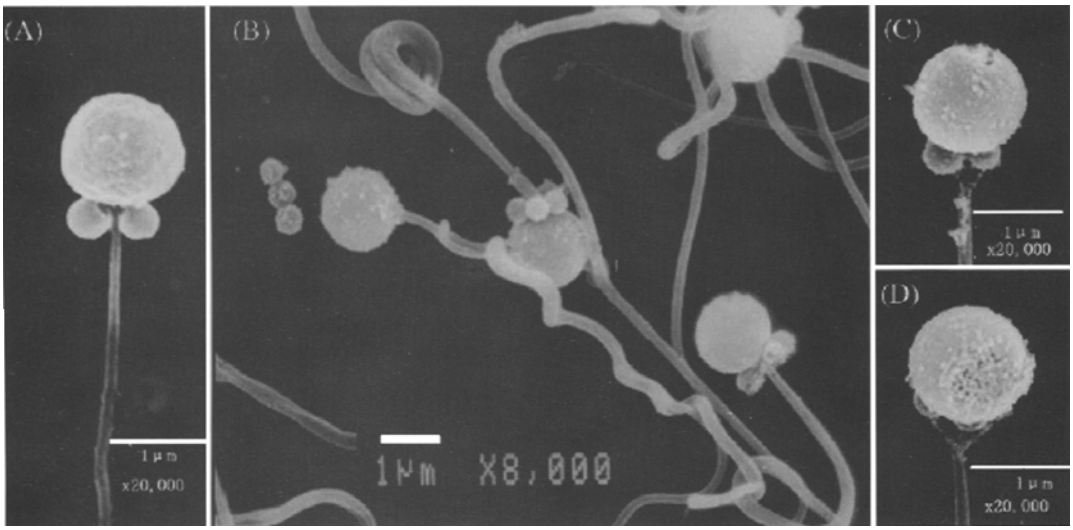

Fig.1 Scanning electron microscopic photographs of fresh sperm (A) and frozen–thawed sperm cryopreserved in the extender with Gly. Note the loss of the mitochondria (B), membrane damage in the head (C, D) and mitochondria swelling at the neck (C) (Zhang et al., 2003)

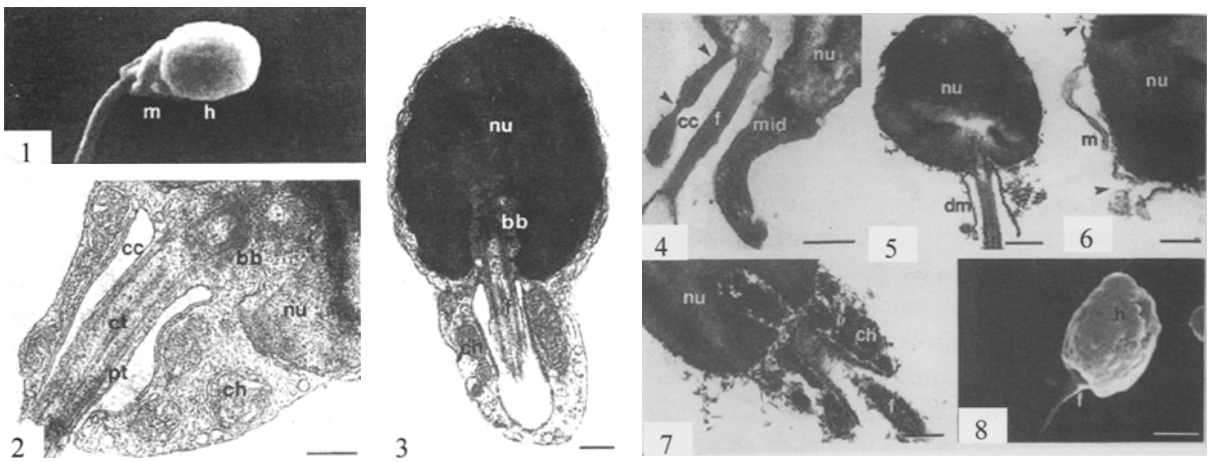

Fig.2 Untreated spermatozoa of *T. thymallus*

1–3.Spermatozoa after cryopreservation; 4. Deformed mid-piece region (mid). Note the reduction of cytoplasm (arrowheads). Scale bar=0.2 μm; 5. Damaged mid-piece region (dm), Scale bar=0.1 μm; 6. Destabilization of nuclear envelop and plasmalemma (arrowheads). Scale bar=0.5 μm; 7. Mid-piece region after freezing and thawing. Scale bar=0.2 μm; 8. Residues of the head region of a damaged spermatozoon after freezing and thawing. Scale bar=1 μm (Lahnsteiner et al., 1992)

Abbreviations: h: head; m: mid-piece; bb: base body; f: flagellum; ch: chondriosome; nu: nucleus; cc: cytoplasmic channel; ct: central tubules of the flagellum; pt: peripheral tubules

of viable spermatozoa is impermeable to PI. This staining method quantifies three different populations: a) live spermatozoa with nuclei emitting high green fluorescence; b) dead spermatozoa with nuclei emitting red fluorescence; c) moribund spermatozoa with nuclei emitting green and red fluorescence. Therefore, the double-staining protocol provides rapid identification between viable and nonviable spermatozoa.

2.2 Physiological impairment

Post-thaw sperm quality has been mostly evaluated through motility analysis since motility is

important characteristic for estimating the quality of fresh, untreated sperm as well as cryopreserved sperm (Lahnsetiner et al., 1996c). An objective evaluation of sperm motility by computer assisted sperm analysis (CASA) has been reported for fish sperm (Kime et al., 1996; Lahnsteiner et al., 1996a, 1997; 1998). This method is rapid, objective and accurate, and enables quantification of physical components of sperm movement. It is a powerful for analyzing fish sperm quality, therefore, CASA has been successfully used to study the fish sperm quality before and after cryopreservation (Dreanno et al., 1997; Lahnsteiner et al., 2000; Wamecke and Pluta et al., 2003).

The cryopreservation often results in reduced viability compared with fresh sperm. Usually cryopreserved sperm have a lower percentage of motility, shorter motility period and slower circular movement (Dreanno et al., 1997; Lahnsteiner et al., 1996a, 2000; Wamecke and Pluta, 2003). The motility of post-thaw common carp sperm were remarkably reduced and could not reach half the values of the corresponding fresh ones. The initial velocity decreased to 50–60 μm/s in average. The lateral head displacement (ALH around 1.0 μm) and the linearity (70%–80%) were less influenced by freezing/thawing processes (Wamecke and Pluta, 2003). Although the exact cryodamage that reduced the swimming velocity is still unknown, but the decreased low velocity may reduce the probability of spermatozoa to reach micropyle (Lahnsteiner et al., 2000). In rainbow trout, cryopreservation also induced overall motility and changed from circular to linear for post-thaw sperm, which was caused by the decrease in intracellular Ca^{2+} depots due to membrane leakage or the alternation in phosphorylation cascade. However, the swimming velocity of spermatozoa was not influenced by the cryopreservation process (Lahnsteiner et al., 1996a, c). Dreanno et al. (1997) reported that in cryopreservation of turbot sperm, the percentage of motile frozen-thawed spermatozoa was also significantly lower than those of fresh ones while the velocity and the duration of motion were not significantly modified.

Although high post-thaw sperm motility and fertility have been achieved in some fish species due to considerable progress made recently in development of technique and applications of optimal settings in cryoprotectant, dilution ratio, freezing rate and thaw temperature for the cryopreservation, the motility and fertility levels were still very lower than fresh ones. The cryodamage to the sperm in morphology could partially explain the low viability and fertility rates achieved with croyopreserved sperm in our case.

2.3 Biochemical and metabolic change

Recent studies on biochemical characteristics for evaluatig changes during cryopreservation were based on an assumption that damage to sperm cells during freezing-thawing process can be monitored by measuring the levels of substances, mostly enzymes and some metabolites in seminal fluid before and after cryopreservation. Deficiency or low activity of enzyme could possibly interrupt metabolic pathways and limit sperm viability, or hinder sperm from entering the egg micropyle. Localization of these substances in spermatozoa should pinpoint which structure has been damaged, and allow better understanding of the roles of these substances playing in metabolic changes in sperm under cryopreservation. Previous assays of activities of L-lactate dehydrogenase (LDH) and aspartate aminotransferase (AspAT) are promising tests for evaluation of quality of fresh sperm in fish (Mcniven et al., 1992; Ciereszko and Dabrowski, 1994).

Lahnsteiner et al. (1996a) evaluated the activities of enzymes in fresh and cryopreserved sperm of rainbow trout. The activities of isocitrate dehydrogenase, malate dehydrogenase (MDH), LDH, ATPase were significantly lower in thawed sperm than controls. The influence of cryodamage on sperm metabolism can be monitored by the activities of some enzymes released from spermatozoa during freezing and thawing process and used to predict cryopreservation success. Lahnsteiner et al. (1996c) reported statistically reliable correlation among enzyme activities, organic compounds of sperm and the fertilizing rate of cryopreserved sperm. Seminal fluid LDH is associated with a low sperm quality for cryopreservation. A significant negative correlation of post-thaw sperm fertilizing ability to acid phosphatase (AcP) activity and a positive linear regression for activity of adenylate kinase were found. Negative significant correlations were also reported between the percentage of hatched larva and AspAT or LDH activity from injured spermatozoa in rainbow trout. Positive significant correlations were observed among AspAT, AcP, and LDH activities (Babiak et al., 2001). Activities of AspAT and AcP were assayed both in supernatants and in spermatozoa of brown, rainbow and brook trout (Głogowski

et al., 1996). It was found the AspAT activity in cryopreserved spermatozoa was correlated positively with fertilization success in all three species, and the enzyme activity in supernatants was usually higher than that in spermatozoa because of protein leakage from injured cells.

As mentioned above, it seems that seminal plasma biochemical assays may be applicable to fish sperm for evaluation of the cryopreservation success. On the other hand, measurements of the spermatozoa-associated enzymes could be useful to further investigate the cryodamage to the sperm for better understanding of the mechanism of the cryodamage. Studies on changes in sperm biochemical parameters provide new insights into the nature of cryopreservation.

2.4 DNA damage

Although the effect of freeze-thaw process on sperm vitality is commonly assessed in terms of motility, fertility, and ultrastructural alterations, but the information on the genetic integrity is rarely reported, which is crucial for accurate transmission of genetic information to future generation. Labbe et al., (2001) reported that rainbow trout sperm cryopreservation only affected sperm DNA stability slightly and that the use of cryopreserved spermatozoa did not impair offspring survival and quality. Sea bass sperm comet assay (Fig.3) clearly revealed that a significant level of DNA fragmentation occurred in frozen-thawed sperm samples with respect to that of fresh sperm, whereas the cryopreservation protocol did not affect either the percentage of motile sperm or the percent of fertilization (Zilli et al., 2003). Similarly, no effect of the freeze-thaw process on the nucleus of Atlantic croaker spermatozoa was reported (Gwo et al., 1992). It appears that cryopreservation of spermatozoa from fish does not have any deleterious effect of sperm DNA integrity and offspring survival.

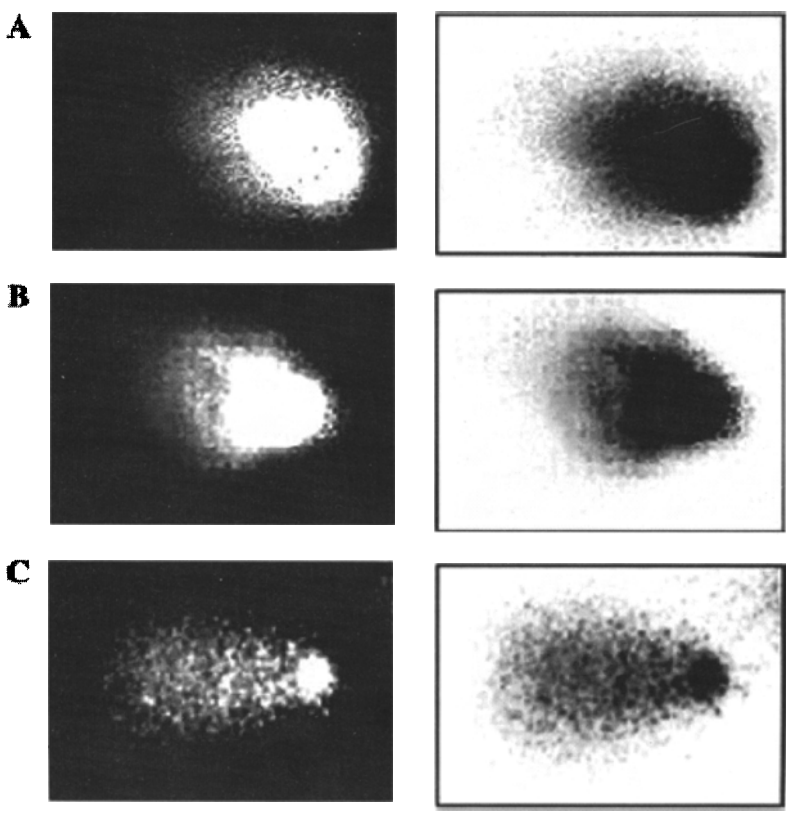

Fig.3 Patterns of SCEG assay for fresh (A), frozen-thawed (B) and unprotected frozen-thawed (C) sea bass sperm

The left panel is positive whereas negative counterparts are in the right panel (Zilli et al., 2003)

A number of techniques have been used to examine sperm genetic integrity. The sperm chromatin structure assay (SCSA) measures the susceptibility of the DNA to acid denaturation (Evenson et al., 1999). DNA laddering has also been used to obtain a qualitative analysis of DNA fragmentation (Duke and Cohen, 1986; Sun et al., 1999; Homma-Takeda et al., 2001). The terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay was another technique for identifying DNA strand breaks in sperm (Sun, 1997; Sakkas et al., 1999). Single cell gel electrophoresis (SCGE) assay has been introduced and recognized as a fast and sensitive method for detecting DNA damage in spermatozoa in recent years (Hughes et al., 1999; Labbe et al., 2001; Gwo et al., 2003; Zilli et al., 2003). The SCGE assay is more commonly called "Comet assay" as a damaged DNA looks like a comet. The method is based on the electrophoresis of cells embedded and lysed in agarose on a microscope slide. During electrophoresis, broken strands of DNA are drawn out of the sperm nucleus, with smaller fragments usually traveling further than the larger fragments to form a comet tail leaving a head of intact DNA. Undamaged DNA appears as intact comet heads while damaged DNA can be seen streaming as a comet tail (Fig.3).

3 CONCLUSION

Cryopreserved fish sperm is generally acknowledged to have an impaired quality by comparison with fresh sperm. The loss of motility and fertility arise from a lower physiological activity, structure deformation, biochemical and metabolic changes, and a series of alterations in additional undetected sublethal cryodamage. Studies on cryodamage to sperm not only can pinpoint which structure has been damaged and allow better understanding of changes in sperm by cryopreservation but can provide new strategies to improve the outcome using very recent technical innovations in the sperm cryopreservation protocols. Cryopreservation of fish sperm will play a crucial role in the improvement of bloodstock management, conservation of genetic diversity and continuous and stable supply of gametes for hatchery seed production or laboratory experimentation.

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