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Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm ☆

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Abstract

Cryopreservation causes several types of damage to spermatozoa, such as loss of plasma membrane integrity and functionality, loss of motility, and ATP content, resulting in decrease of fertility rates. This spermatozoal damage has been widely investigated for several marine and freshwater fish species. However, not much attention has been paid to the nuclear DNA. The objective of this study was to determine the degree to which cryopreservation induces spermatozoal DNA damage in two commercially cultured species, rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*), both of which could benefit from the development of cryopreservation strategies on a large scale. We have used the single-cell gel electrophoresis, commonly known as Comet assay to detect strand breaks in DNA. This technique was performed on fresh and cryopreserved sperm from both species. In rainbow trout there was a significant increase in the averages of fragmented DNA and Olive tail moment after cryopreservation (11.19–30.29% tail DNA and 13.4–53.48% Olive tail moment in fresh and cryopreserved sperm, respectively), as well as in the proportion of cells with a high percentage of DNA fragmentation. For gilthead sea bream there were no significant differences in the percentage of tail DNA between the control samples and sperm diluted 1:6 and cryopreserved (28.23 and 31.3% DNA_t, respectively). However, an increase in the sperm dilution rate produced an increase in the percentage of DNA fragmentation (41.4%). Our study demonstrates that cryopreservation can induce DNA damage in these species, and that this fact should be taken into account in the evaluation of freezing/thawing protocols, especially when sperm cryopreservation will be used for gene bank purposes.

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The effect of the freezing/thawing process on sperm is often assessed in terms of sperm motility, cell viability and fertilization capacity. Several studies have demonstrated the existence of spermatozoal damage associated with loss of motility and fertility, low ATP content, loss of plasma membrane, mitochondrial integrity, and function in cryopreserved fish sperm [5,6,29,32,42]. However, these parameters provide no information about the integrity of DNA and chromatin. Damage to spermatozoa DNA has been observed in several mammalian species, such as human, mouse, horse, and pig [1,3,19,23,37,40], using different techniques. One common technique is the single-cell gel electrophoresis assay, commonly known as comet assay, which determines DNA fragmentation in individual cells. The method was described by Ostling and Johanson [33] and consists, basically, of electrophoresis of cells embedded and lysed in agarose, on a microscope slide. After lyses, nuclear DNA is placed in an alkaline solution and begins to unwind from sites of strand breakage, resulting in structures resembling comets [10,17,36]. The comet visualization and analysis can be performed using a fluorescent dye and image analysis software. There are some important parameters that describe the damage occurring in the cell DNA. The percentage of tail DNA, that directly indicates DNA fragmentation, and the Olive tail moment, that includes both length and intensity of DNA in the tail, appear to be the preferred parameters for characterization of the comets [2,10,24,34]. This technique has been applied not only to sperm cells, to detect damage associated with cryopreservation, but also for the detection of DNA damage, induced by genotoxicants in marine and freshwater vertebrates and invertebrates [30,31]. It has also been used to assess DNA repair and apoptosis in aquatic organisms. Modifications of the comet assay have allowed the detection of specific DNA lesions, such as strand breaks, modified bases, DNA–DNA and DNA–protein crosslinks [30]. This method is considered a simple and reliable method for measuring DNA strand breaks in eukaryotic cells, and is recommended for DNA damage and repair studies [10].

There is little information on the effects of cryopreservation on the DNA of fish spermatozoa. The only studies performed demonstrated the existence of DNA damage in trout sperm [4,28] and in sea bass [43]. There is also indirect evidence, provided by Kopeika et al. [25,26], suggesting DNA damage in loach sperm during cryopreservation. The evaluation of DNA status in the assessment of cryopreserved sperm can be particularly important for commercial scale application of cryopreservation, or for the assessment of samples stored in gamete banks, since a loss of genetic information, or the appearance of larval malformations associated with this damage could not be accepted. The aim of the present study was to investigate the phenomenon of DNA fragmentation in rainbow trout and gilthead sea bream cryopreserved spermatozoa, using a standard cryopreservation method, intended for application on a commercial scale.

Materials and methods

Broodstock and gamete collection

The present work was carried out during the reproductive period of rainbow trout (November to March in León, North of Spain) and gilthead sea bream (December to March in Cádiz, South of Spain). Ripe male rainbow trout were maintained in the Lillogen fishfarm (León, Spain) and sperm was collected in these facilities, while gilthead sea bream broodstock was supplied by CUPIMAR, SA fish farm (San Fernando, Cadiz, Spain) before the beginning of the reproductive season and was maintained in the Institute of Marine Science of Andalucia, Spain (ICMAN-CSIC) facilities during the experiments. Both species were kept under natural photoperiod conditions. Gilthead sea bream males (2-years old, approximately 1.2 kg) and females (3-years old approximately 3.5 kg) were maintained in a 20,000 L tank in a recirculating seawater system with compressed air supply. The individuals were fed ad libitum every day with squid.

The water temperature in the tank was 19 °C ± 1. Before sperm collection, rainbow trout males were anaesthetised with 100 mg/L MS-222 (Sigma) and sea bream with 125 mg/L of the same anaesthetic. For semen collection, rainbow trout males were catheterized, introducing a catheter into the urogenital pore, to avoid urine contamination during the process. For sea bream, a 1 mL syringe, without needle, was used to aspirate milt released by abdominal massage. A previous gentle pressure was applied to eliminate urine in the ducts in both species. Some samples were discarded due to urine contamination, detected by changes in sperm viscosity, pH, and osmolarity. After sperm collection, samples were kept in centrifuge tubes (15 and 50 mL) on ice until analysis. Gilthead sea bream sperm was maintained in a polystyrene support to avoid ice contact (approximately 7 °C). A preliminary sperm standard analysis of osmolarity (Osmomat 030 Gonotec), pH (micro pH 2000 Crison) and cell motility (sperm activation under light microscopy) was performed to discard bad quality and contaminated samples. Only samples whose quality parameters ranged between the following values were used: for rainbow trout, osmolarity 310–319 mOsm/kg, pH 8.08–8.42, and motility 75–100%, and for sea bream, osmolarity 363–408 mOsm/kg and motility 75–100%. Sperm was pooled in equal amounts using semen from three individuals. For rainbow trout, three pools were used in the experiments and for sea bream four pools were analyzed. For both species, 3/4 of each sample was used for cryopreservation procedures and the remainder was used to perform the fresh analysis.

Sperm cryopreservation

Rainbow trout sperm was cryopreserved using the method described by our group [7]. Briefly, samples were diluted 1:3 in the extender # 6 from Erdhal and Graham [13] (0.103 g/L $CaCl_2.2H_2O$, 0.22 g/L $MgCl_2.6H_2O$, 0.263 g/L Na_2HPO_4, 2.557 g/mL KCl, 0.1 g/L citric acid, 10 g/L glucose, 10 mL KOH solution—1.27 g/100 mL, 20 mL bicine solution—5.3 g/100 mL, and 323 mOsm/kg, pH 7.4) using 7% Me_2SO as permeable cryoprotectant and 10 mg/mL BSA as non-permeable cryoprotectant. Sperm was loaded into 5 mL macrotubes and placed in a horizontal rack 1 cm above liquid nitrogen in a styrofoam box. Sperm freezing was performed in nitrogen vapour during 10 min. After that time, the macrotubes were immersed in liquid nitrogen and stored in a nitrogen container until analysis. For thawing, macrotubes were immersed in a water bath at 50 °C for 10 s and sperm was kept in 15 mL centrifuge tubes on ice until analysis. Gilthead sea bream sperm was cryopreserved using the extender proposed by Fabbrocini et al. [15] (1% NaCl, 300 mOsm/kg plus 5% Me_2SO) modified by adding 10 mg/mL BSA to protect plasma membrane and avoid sperm aggregation. Two sperm dilutions in the extender, 1:6 and 1:20 (sperm:extender), were used. Sperm was loaded into 5 mL macrotubes and frozen using the procedure previously described. Sperm samples were thawed in a water bath at 60 °C for 30 s.

Single-cell gel electrophoresis procedure

Sample and slide preparation

Fresh and frozen/thawed sperm were diluted in seminal fluid mineral medium—SFMM—(6.42 g/L NaCl, 2.1 g/L KCl, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.26 g/L $CaCl_2 \cdot 2H_2O$, 1.63 g/L bicine, and 2.38 g/L Hepes), a non-activating motility medium for rainbow trout [4], or in the non-activating medium from Fauvel et al. [16] for marine fish spermatozoa (3.5 g/L NaCl, 0.11 g/L KCl, 1.23 g/L $MgCl_2$, 1.68 g/L NaH_2CO_3, 10 mg/mL BSA, and 310 mOsm/kg, pH 7.7) until a final concentration of $8–10 \times 10^6$ spermatozoa per mL. Frozen sperm was prepared immediately after thawing to prevent sperm degradation. Slides were prepared in advance (1 day before the experiments) applying a thin layer of normal melting point agarose (100 μL) and spreading it across the length of the slide, eliminating agarose in excess. This agarose solution was prepared by heat dissolving 0.5% agarose powder in milli-Q water. The slides were stored at room temperature and protected from dust and light. Low melting point agarose at the same concentration described before, was prepared next day for the second layer, using the same procedure. Two hundred and twenty-five microliters of this agarose were added to 30 μL of each sperm suspension in an Eppendorf tube. The second slide layer was made by adding

85 μL of this suspension and covering the slide with a coverslip. This layer was left to solidify at 4 °C for 15 min. After this period of time, the coverslip was removed and the peripheries of the second agarose layer were coated with low melting point agarose, avoiding the loss of sample during the next procedures. Slides were left to solidify at 4 °C for 10 min. For each type of sperm and pool, three slides were prepared.

Spermatozoa lysis, DNA denaturation, and electrophoresis

Lysis buffer, DNA denaturation solution, and electrophoresis buffer were freshly prepared with milli-Q water before the beginning of the experiments. The slides were placed into a coplin jar containing lysis solution (2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100, and 1% lauryl sarcosine) for 1 h at 4 °C. To decondense the DNA, dithiothreitol was added to the lysis buffer, to a final concentration of 10 mM, and slides were immersed for 30 min at 4 °C. After this period of time, lithium diiodosalicylate was added to the previous solution to a final concentration of 4 mM and slides were left to incubate for 90 min at room temperature. The slides were removed from the solution, excess solution eliminated, and they were placed horizontally in a electrophoresis cube (Bio-Rad), filled with freshly made electrophoresis solution (0.3 M NaOH, 1 mM Na_2–EDTA, pH 12) for 20 min at 4 °C to allow the DNA to unwind. Electrophoresis was then conducted for 10 min at 25 V and 300 mA at 4 °C. Amperage was controlled by adjusting the volume of the electrophoresis buffer. After electrophoresis, slides were drained and placed into a coplin jar with neutralizing solution (0.4 M Tris, pH 7.5) for 5 min at 4 °C. This operation was repeated twice with freshly prepared solution to assure the elimination of all alkali and detergents. Slides were left to drain and sample fixation was performed by immersing the slides in a pure methanol solution for 3 min. The slides were then left to drain in the air and were stored protected from light and dust.

Slide analysis

For comet visualization 40 μL ethidium bromide were pipetted into the sample and covered with a coverslip. The ethidium bromide at final concentration 0.5 μg/mL was prepared from a stock solution 5 μg/mL. Samples were observed in an epifluorescence microscope (Nikon Eclipse E800) fitted with an excitation filter of 510–560 nm and a barrier filter of 590 nm. Each slide was analyzed from front to back and from left to right randomly selecting several fields for image recording. Approximately 80 cells from each slide were photographed with a digital camera (Nikon DXM1200F) using the software Nikon ACT-1 (v. 2.62, Nikon). Comet analysis was performed with the imaging system Komet software (version 5, Kinetic Imaging, UK). This software allows the determination of several parameters related with length and intensity of the comet tail and head. For each cell analyzed, the pixels observed in the tail of the comet represent DNA fragments (damaged DNA) and the nucleus represents the head of the comet in which is located the undamaged DNA. From the several parameters analyzed by the Komet software, the percentage of tail DNA (% DNA_t) and Olive tail moment (M_t) were used to characterize fresh and cryopreserved rainbow trout and sea bream sperm. The percentage of tail DNA is given by the formula: % $DNA_t = 100 \times (DNA_c - DNA_h)/DNA_c$, in which DNA_c and DNA_h represent the sum of the intensities of the pixels in the comet area and in the head area, respectively. The Olive tail moment (M_t) is given by the formula: $M_t = (DNA_c - DNA_h) \times lt$, in which lt represents the comet tail length.

Data were expressed as averages and the percentages of cells with different degree of damage were also established.

Statistical analysis

The percentile data obtained from comet analysis were normalized, though arcsine transformation and the results were expressed as means ± SD and analyzed by one-way ANOVA. Significant differences between the percentage of DNA in tail (% DNA_t) and Olive tail moment (M_t) obtained for fresh and cryopreserved sperm were detected by a multiple range test, SNK test (Student–Newman–Keuls) ($p < 0.05$) [39].

Results

The results shown in Fig. 1 demonstrate that there was an increase in DNA fragmentation after cryopreservation that can be observed by the pixels and the length of the tail of the comets. This information is seen after comet analyses as an increase in the percentage of tail DNA (DNA fragmentation) and Olive tail moment in cryopreserved samples, especially in rainbow trout spermatozoa (Table 1). The analysis of the percentages of cells with different degrees of damage showed that more than 85% of fresh spermatozoa had less than 20% of DNA in tail, whereas only 22% of frozen spermatozoa showed the same degree of damage, and more than 73% had between 20 and 50% of DNA_t (Fig. 2). Undamaged cells ($DNA_t < 10\%$) represented a subpopulation of 60% for fresh sperm and 10% for cryopreserved spermatozoa.

In sea bream, differences between fresh and frozen sperm were less pronounced, and the fresh samples showed a more heterogeneous sperm population than in rainbow trout. No differences were observed in the average of tail DNA or in Olive tail

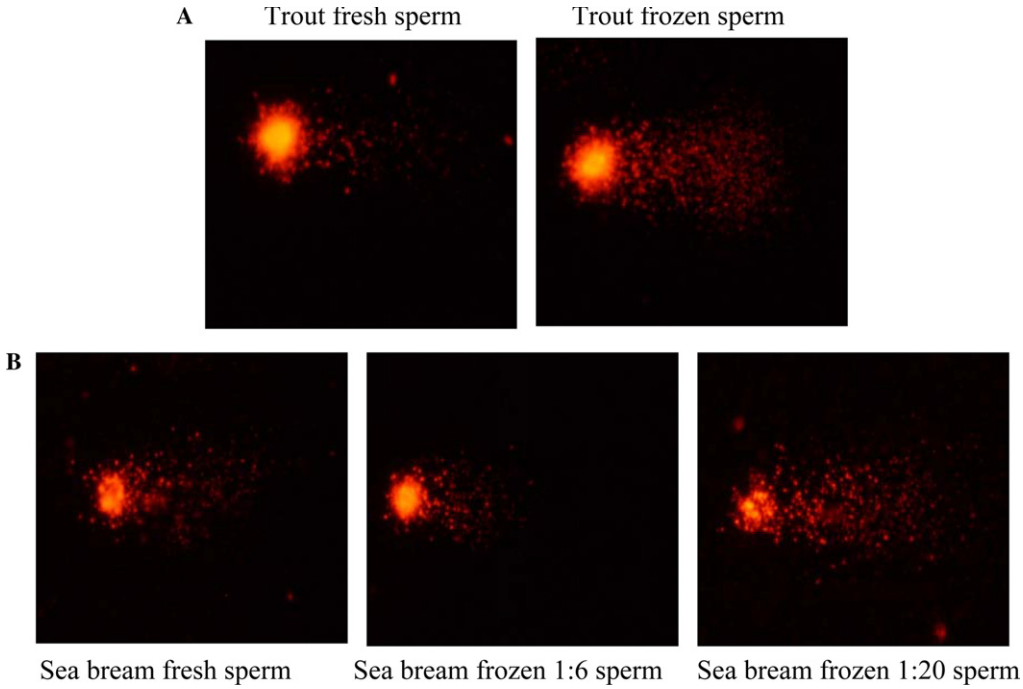

Fig. 1. Single-cell gel electrophoresis of fresh and frozen spermatozoa from (A) rainbow trout and (B) gilthead sea bream. Each comet represents the type of damage most commonly analyzed.

Table 1
Effect of cryopreservation on spermatozoa DNA from rainbow trout and gilthead sea bream: percentage of tail DNA and Olive tail moment in fresh and cryopreserved sperm

	% Tail DNA	Olive tail moment
Rainbow trout fresh sperm	11.19 ± 9.2^{b}	13.40 ± 9.8^{b}
Rainbow trout frozen sperm	30.29 ± 13.9^{a}	53.48 ± 31.7^{a}
Seabream fresh sperm	28.23 ± 15.0^{b}	37.36 ± 25.6^{a}
Seabream frozen sperm 1:6	31.35 ± 15.8^{b}	33.16 ± 23.8^{b}
Seabream frozen sperm 1:20	41.44 ± 15.0^{b}	40.19 ± 21.9^{ab}

Significant differences between fresh and cryopreserved sperm for each species are indicated by different letters ($p \leqslant 0.05$). Data are expressed as mean values ± SD ($n = 3$, $n = 4$).

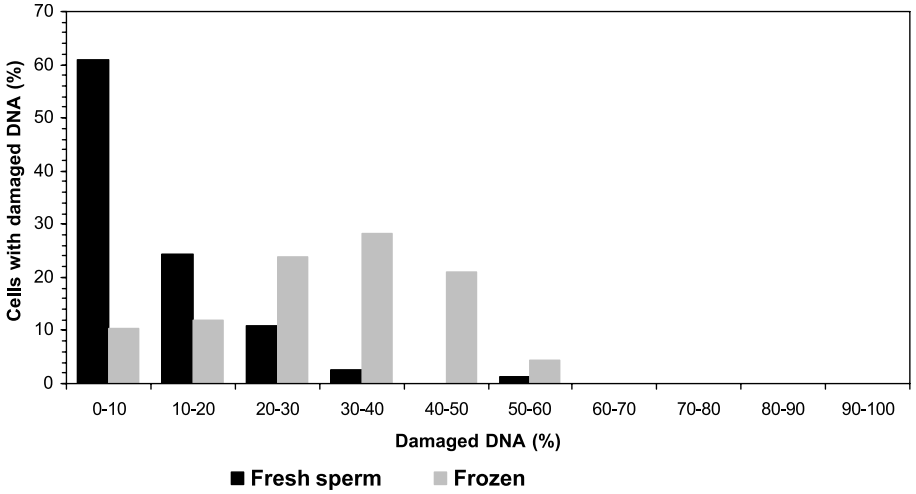

Fig. 2. Distribution of DNA damaged cells in fresh and cryopreserved rainbow trout spermatozoa. Cells were grouped in classes (from 0–10% DNA_t to 90–100% DNA_t) according to the percentage of damaged DNA determined for each cell. A total of 240 cells were analyzed.

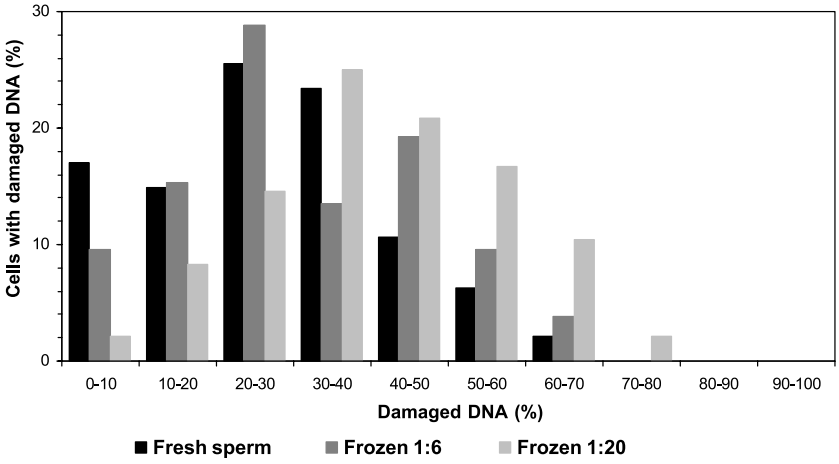

Fig. 3. Distribution of DNA damaged cells in fresh and cryopreserved sea bream spermatozoa. Cells were grouped in classes (from 0–10% DNA_t to 90–100% DNA_t) according to the percentage of damaged DNA determined for each cell. A total of 240 cells were analyzed.

moment between fresh sperm and sperm frozen at 1:6 dilution (Table 1). High dilutions in the extender increased the effects on DNA, since there was a significant increase in the percentage of DNA fragmentation in samples diluted 1:20 (41.35%) when compared with samples diluted 1:6 (31.35%) (Table 1). When the degree of damage was analyzed in detail (Fig. 3), we observed that fresh samples showed a percentage of undamaged cells (cells with less than 10% DNA_t) (17%) higher than frozen samples (9% and 2% for sperm dilution rate 1:6 and 1:20, respectively). The percentage of cells showing more than 40% of DNA fragmented was higher in frozen spermatozoa (32 and 49%) than in fresh sperm (19%). Moreover, in cryopreserved sperm, the percentage of cells with more than 30% of DNA damaged was higher at 1:20 dilution rate than for cells at 1:6 dilution, with 75 and 46% damaged cells, respectively. Fresh samples also showed a high percentage of damaged

cells (42%) with more than 30% of DNA in comet tails.

Cells with more than 75% of fragmented DNA, which could be considered apoptotic cells, were not detected in either species.

Discussion

The effect of cryopreservation on sperm DNA has been evaluated in few marine and freshwater fish species. Billard [4] observed chromatin ultrastructural alterations in thawed sperm of rainbow trout and brown trout, and Kornilova et al. [27] detected the same damage in carp sperm. Recently, Labbé et al. [28], using the comet assay, demonstrated that cryopreservation of rainbow trout sperm slightly affected sperm DNA stability. Zilli et al. [43] applying the same method, reported that cryopreservation induced DNA fragmentation in sea bass sperm and that cryoprotectants significantly reduced this effect. Sperm chromatin is considered less susceptible to damage than that in somatic cells, due to the high level of chromatin packaging [37]. Nevertheless, the evaluation of the extent of damage is very important in order to prevent possible loss of fertilization ability or genetic variability during sperm storage and to understand possible developmental failures during embryo development.

Several authors have reported DNA fragmentation in cryopreserved sperm and associated this finding with a decrease in fertility and abnormal embryo cleavages in human [41], oyster [18], and mouse [1]. Moreover, there is some evidence that changes in genetic material from cryopreserved spermatozoa are inherited by offspring, and the functional and physiological status of which is affected. Dulioust et al. [11] reported significant differences in morphology and behavior of mice originating from fresh and cryopreserved embryos. Higher immunological reactivity was also observed in young carp grown from eggs fertilized by cryopreserved sperm [35]. Despite the potential significance of the effect of cryopreservation on the genome of reproductive cells, reports in the literature on this subject have been limited.

In the present study, we have analyzed the effect of cryopreservation on DNA in two farmed species with high potential for the application of sperm cryopreservation as routine or standard procedure. This analysis is very important, especially when cryopreservation is going to be applied for the creation of a GenBank. We have detected an increase in the percentage of DNA fragmentation and an increase in the percentage of cells with high level of DNA damage in cryopreserved sperm when compared with fresh sperm, especially for rainbow trout.

There have been some disagreements among authors regarding the importance of DNA damage in reproductive cells. The main questions are: does the damage occur in all cryopreserved spermatozoa? and, if damage has occurred, does it affect fertility and normal embryonic development? or, is there a degree of damage that can be overcome by repair mechanisms after fertilization? According to Kopeika et al. [26], the use of 3-aminobenzamide to block the DNA repair system in loach embryos, enhances the developmental failures attributed to sperm DNA damage after freezing. These authors suggested that cryopreservation could induce DNA instability that could be repaired by the oocyte after fertilization. Nevertheless, the use of caffeine with the same purpose provided opposite results [25]. Most of these controversies are due to the lack of data reported (hatching rate provided but not the fertility rate, no DNA analysis performed, or insufficient sperm quality analysis) and sometimes to the species-specific response to cryopreservation. It has been showed that different levels of chromatin packaging and of histone/protamine rate in spermatozoa from different species are responsible for the DNA vulnerability to denaturation. This could explain the fact that some authors detected DNA damage in some species (human) that were not reported in others (boar) [14,19,38].

Suquet et al. [42] and Chereguini et al. [9], demonstrated that fertility, hatching rate, and different parameters related to larval development (survival rate, weight, and length) were similar whether fresh or cryopreserved turbot sperm had been used to produce the zygotes. However, a DNA analysis of those cells was not performed. Other studies developed in sea bass did not obtain significant differences in hatching rate when fresh or cryopreserved

sperm was used for fertilization, although DNA damage in cryopreserved spermatozoa had been noticed [43]. It is possible that, depending on the nature or the level of the damage, they can be repaired by the oocyte repair mechanism during early embryogenesis and therefore do not affect embryo development, but large damage will induce changes in embryo development, reducing hatchability. This finding was also observed by Ahmadi and Soon-Chye [1] working with mouse sperm.

In the present study, fertility trials were not performed. When we compare our data with previous studies [6–8], our results are consistent with the previous findings, regarding hatching rates for both species. In rainbow trout, previous reports [7] pointed out that fertilization with sperm cryopreserved using the same protocol as applied in the present study, provoked a slight reduction in the hatching rate (73.2% respect to control—78.4%) and that this reduction was more pronounced if the cells were not well protected by the freezing medium [5,6]. The comet assay revealed in this species a 30.3% DNA damage (% DNA_t) for cryopreserved sperm, against 11.1% damaged DNA for fresh sperm, and the percentage of undamaged nuclei was significantly reduced after freezing. This chromatin damage could also be responsible for the decrease in hatchability, which was always attributed to fertilization failures caused by sperm plasma membrane damage. Therefore, rainbow trout sperm suffers damage at different levels during cryopreservation and the sum of the different types of damage could be responsible for the decrease in the hatching rates obtained. This work proved that DNA fragmentation is one more of the sperm cryoinjuries in this species.

For sea bream, our previous studies [8], resulted in similar hatching rates with fresh and frozen sperm using the same cryopreservation procedure and a 1:6 sperm dilution in the extender (77.1 and 75.6% with fresh and frozen sperm, respectively). In this species, DNA analysis showed that cryopreservation, using an appropriate freezing medium and dilution rates, slightly affected DNA structure, since there were no significant differences between the percentages of tail DNA detected for fresh and cryopreserved sperm with 1:6 dilution. The percentage of cells having more than 40% of DNA fragmented increased after freezing/thawing. However, taking into account fertility data, it seems that this extent of damage (until 40%) could be repaired by oocyte DNA repair mechanism during fertilization, and may be irrelevant in terms of hatching success.

The nature of DNA damage is not clear, but evidence indicates that freezing/thawing process could induces damage more than the toxicity of cryoprotectants. It has been proposed that an increase in intracellular calcium concentration, caused by cryopreservation, could raise the frequency of nuclear DNA break through endonuclease activation [20]. In addition, the production of reactive oxygen species (ROS) induced by cryopreservation can be responsible for an increase in peroxidation, leading to DNA fragmentation [1]. Strand breaks can also be introduced directly by genotoxic compounds [12], but the hypothesis that cryoprotectants could have some responsibility for that damage should be discarded with respect to Me_2SO. In fact, their addition to cell suspensions is recommended before performing the comet assay, to prevent oxidant-induced DNA damage [21]. Zilli et al. [43] demonstrated that when Me_2SO and BSA were added to the extender medium, the DNA damage in sea bass sperm decreased significantly when compared to spermatozoa frozen only in the extender without cryoprotectants. Studies carried out by Labbé et al. [28] demonstrated that the simple exposure of rainbow trout sperm to freezing extender containing 10% Me_2SO did not cause significant DNA fragmentation. All these data point to the conclusion that the freezing/thawing process is responsible for the DNA fragmentation. In the present study, there was a significant increase in DNA damage when sea bream sperm was diluted 1:20 in the extender, demonstrating that in this species high sperm dilutions reduce the protection of cells and promotes more damage. The high levels of DNA fragmentation observed in fresh sea bream sperm and the detection of a heterogeneous sperm population, with a wide range of fragmented DNA, could be attributed to the fact that sperm was obtained at the end of the reproductive season and different sperm subpopulations (some of them over-ripened) could be present in the milt. Nevertheless, another possible

explanation for our results with fresh sperm could be related to the comet assay method. Zilli et al. [43] concluded that this analysis is appropriate for sea bass sperm. Nevertheless, not much information exists on the chromatin structure of marine fishes. The possible presence of many alkali sensitive sites in sea bream sperm chromatin could induce strand breaks during cell lysis. Moreover, according to Hasimoto et al. [22] hypertonic stress can induce DNA fragmentation and apoptosis in fish cells, and the cell lysis procedure in the comet assay was carried out in a very hyperosmotic solution for a long period of time (1 h). This fact has to be confirmed, because, none of the analyzed spermatozoa had more than 75% of DNA fragmentation, a percentage which is usually used as apoptosis index.

In conclusion, DNA damage can be one more cause of cryoinjury for rainbow trout sperm. The higher cryoresistance of marine spermatozoa when compared with freshwater sperm cells is also evident when DNA damage is evaluated after cryopreservation. More studies on the correlation of DNA damage with fertility and hatching rates and, especially with genetic variability of the offspring, should be performed to determine the degree of damage that can be accepted for the use of cryopreserved sperm for commercial and GenBank purposes.

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