

Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success

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Abstract

Mammalian spermatozoa undergo a strong selection process along the female tract to guarantee fertilization by good quality cells, but risks of fertilization with DNA-damaged spermatozoa have been reported. In contrast, most external fertilizers such as fish seem to have weaker selection procedures. This fact, together with their high prolificacy and external embryo development, indicates that fish could be useful for the study of the effects of sperm DNA damage on embryo development. We cryopreserved sperm from rainbow trout using egg yolk and low-density lipoprotein as additives to promote different rates of DNA damage. DNA fragmentation and oxidization were analyzed using comet assay with and without digestion with restriction enzymes, and fertilization trials were performed. Some embryo batches were treated with 3-aminobenzamide (3AB) to inhibit DNA repair by the poly (ADP-ribose) polymerase, which is an enzyme of the base excision repair pathway. Results showed that all the spermatozoa cryopreserved with egg yolk carried more than 10% fragmented DNA, maintaining fertilization rates of 61.1 ± 2.3 but a high rate of abortions, especially during gastrulation, and only 14.5 ± 4.4 hatching success. Furthermore, after 3AB treatment, hatching dropped to 3.2 ± 2.2 , showing that at least 10% DNA fragmentation was repaired. We conclude that trout sperm maintains its ability to fertilize in spite of having DNA damage, but that embryo survival is affected. Damage is partially repaired by the oocyte during the first cleavage. Important advantages of using rainbow trout for the study of processes related to DNA damage and repair during development have been reported.

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Introduction

In recent years, sperm DNA damage assessment has gained special attention as an important sperm quality marker, linked with sperm fitness as well as with offspring quality. In this sense, some techniques to quantify sperm DNA integrity (sperm chromatin structure assay (SCSA), TUNEL, acridine orange test, Halomax, single cell gel electrophoresis (SCGE; comet assay), etc.) have been developed and applied to several species from different phyla (Singh *et al.* 1988, Evenson *et al.* 1991, Sun *et al.* 1997, Lee & Steinert 2003, Maluszynska & Juchimiuk 2005, Enciso *et al.* 2006, Andrabi 2007). Most of the reported data indicate that DNA damage is correlated with a reduction in fertilization ability, with development failure after fertilization and offspring problems (Kopeika *et al.* 2004, Evenson & Wixon 2006, Fernandez-Gonzalez *et al.* 2008, Barroso *et al.* 2009, Schulte *et al.* 2010). 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. (Tarin *et al.* 2000, Andrabi 2007, Pérez-Cerezales *et al.* 2009, Thomson *et al.* 2009). Some of these factors are also associated with other kinds of injuries which are directly

related to fertilization failure, such as membrane damage, immobility, and mitochondrial membrane depolarization (Ahmadi & Ng 1999, Irvine *et al.* 2000, Holt & Van Look 2004). In mammals, it has been suggested that the strong selective process undergone by sperm in the female tract (by elimination of defective spermatozoa, sperm competition, cryptic female choice, etc.) is probably unrelated to a DNA-scanning mechanism, and that some kind of link between sperm phenotype and genotype could be involved in this process (Holt & Van Look 2004, Khalil *et al.* 2006, Satake *et al.* 2006, Suarez & Pacey 2006, Rodriguez-Martinez 2007). Hourcade *et al.* (2010) showed that selection of DNA-undamaged spermatozoa occurs in mice during the transit through the oviduct as well as during the contact with the vitelline membrane, but this selective process is dependent on the source of sperm damage and probably related to the presence of damage in other cell structures. Sperm selection among mammals must be rigorous because a lot of time and energy is spent in obtaining a reduced number of embryos, which require an additional effort to develop properly. Taking into account that DNA damage can be promoted in different ways that do not necessarily correlate with changes in structures/molecules related

to survival in the female tract or with fertilization ability, the evaluation of the effects of fertilization with DNA-damaged spermatozoa on offspring development deserves more attention. The use of some assisted reproductive technologies (ARTs) for human reproduction and animal breeding facilitates fertilization success, avoiding the selection process that takes place in the female tract and during the interaction with the vitelline membrane in such a way that spermatozoa excluded during natural fertilization would be able to penetrate the oocyte and initiate development (Ahmadi & Ng 1999, Fernandez-Gonzalez *et al.* 2008, Zini & Sigman 2009). Studies performed with DNA-damaged human and mouse sperm demonstrated their fertilization ability in zona-free hamster oocytes (Ahmadi & Ng 1999) or via ICSI (Fernandez-Gonzalez *et al.* 2008), but a high degree of developmental failure, altered karyotypes, modified phenotypes, and even histopathological events were detected in embryos and newborn mice. Previous reports also suggest an excess of major and minor birth defects, low birth weight, and frequency of some imprinting-related syndromes in ART children, especially with the use of ICSI (Bowdin *et al.* 2007, Lim *et al.* 2009). These data reflect the importance of studying the consequences of fertilizing with DNA-modified sperm. DNA strand breaks and base oxidation are promoted during freezing–thawing, as has been described in several species (Baumber *et al.* 2003, Pérez-Cerezales *et al.* 2009, Thomson *et al.* 2009, Yamauchi *et al.* 2009). Thus, the use of cryopreserved sperm combined with other ARTs could increase the risk of undesirable modified genetic material being transmitted to the offspring. The analysis of the impact of fertilization with damaged pronuclei on the progeny is limited in mammals by the low number of embryos obtained per mating and the internal development of the embryo, which makes the progressive evaluation of development as well as the study of DNA repair mechanisms difficult. DNA damage such as simple or double strand breaks, base modifications, base mismatch, etc. could be repaired by the oocyte after fertilization, but there is not enough information about the extent of insults which can be repaired in the zygote or even about the eventual modifications in the DNA induced by the repairing mechanisms themselves. Deeper studies are required in this regard in order to assess the real impact of fertilizing with an injured genome in the future offspring. DNA-repairing pathways are highly conserved mechanisms, and the expression of different enzymes involved in the process has been detected during early development in different species from fish to mammals (Matsuda & Tobarí 1989, Hales *et al.* 2005, Marchetti *et al.* 2007, Fortier *et al.* 2009).

In external fertilizers, such as fish, sperm selection mechanisms before fertilization are different, and much simpler. Energy is not spent on offspring care, many embryos are produced and only a percentage of the

embryos survive, so selective pressure undergone by spermatozoa is lower than that undergone by spermatozoa in mammals. In fish, spermatozoa are delivered into water, an aggressive environment which activates the motility, and fertilization lasts no longer than some seconds or minutes after ejaculation when ATP stores are exhausted (Jalabert 2005, Cabrita *et al.* 2008, Cosson *et al.* 2008). So, natural selection is focused on fast fertilization with a simple spermatozoa structure and relatively simple strategies to ensure fertilization (increased ejaculate density, high spermatozoa velocity, chemotaxis, etc.; Holt & Van Look 2004, Cosson *et al.* 2008). In this case, spermatozoa selection and cryptic female choice could be less significant than in internal fertilizers, and fertilization may be a matter of certain probability (Holt & Van Look 2004). This suggests that natural fertilization with DNA-damaged spermatozoa could be more likely in fish than in mammals. Fish could be a very interesting model in this field if a weak selection process allowing natural fertilization with DNA-damaged spermatozoa were confirmed. The ease with which many fish species breed, external embryo development allowing embryo assessment and manipulation throughout development, and high prolificacy, allowing the generation of thousands of embryos per mating, are undoubtedly advantages.

The objective of this study was to check the suitability of rainbow trout as a model for the study of the effects of fertilization with spermatozoa containing damaged DNA promoted by cryopreservation, and evaluate the role of DNA damage in fertilization ability and embryo survival during development as well as analyze the activity of the base excision repair (BER) pathway through the inhibition of the poly (ADP-ribose) polymerase (PARP).

Results

Sperm evaluation

As shown in Table 1, fresh sperm viability (plasma membrane integrity) was $98.66\% \pm 0.39$, and a high percentage of damaged DNA ($31.99\% \pm 1.19$ tail DNA) was detected with a basal level of intracellular reactive oxygen species (ROS) of 2.35 ± 0.07 median fluorescent intensity (MFI). In the cryopreserved sperm, we detected a significant decrease in viability, with the use of egg yolk as cryoprotectant providing the lowest value ($13.69\% \pm 0.77$ viable cells). DNA fragmentation also increased after freezing–thawing, especially with the use of egg yolk ($56.91\% \pm 1.02$ tail DNA). The ROS content was higher in cryopreserved sperm than in fresh sperm (3.21 ± 0.14 in sperm frozen with egg yolk and 3.11 ± 0.13 with its low-density lipoprotein (LDL) fraction). When comet assay was combined with the use of restriction enzymes (endonuclease III (EndIII) and

Table 1 Sperm parameters in fresh and frozen sperm from rainbow trout (*Oncorhynchus mykiss*). Cell viability (%), percentage of tail DNA, and reactive oxygen species (ROS) level for fresh sperm, sperm cryopreserved with 10% egg yolk or 12% low-density lipoproteins (LDL), and sperm incubated with 500 mM H₂O₂ as positive control for oxidation. For viability and ROS, 10⁴ cells were acquired in duplicate per each male and treatment. One hundred cells per male and treatment were analyzed in comet assay. Values are expressed as mean ± S.E.M.

	Viability	Percentage of tail DNA	ROS
Fresh	98.66 ± 0.39 ^a	31.99 ± 1.19 ^a	2.35 ± 0.07 ^a
Egg yolk	13.69 ± 0.77 ^b	56.91 ± 1.02 ^b	3.21 ± 0.14 ^b
LDL	37.55 ± 1.35 ^c	49.21 ± 1.14 ^c	3.11 ± 0.13 ^b
H ₂ O ₂		77.36 ± 0.96 ^d	3.74 ± 0.07 ^c

Different letters in the same column indicate significant differences ($P < 0.05$).

formamidopyrimidine DNA glycosylase (Fpg)), DNA was cut into oxidized cytosines and guanines respectively, allowing the detection of oxidized bases (Fig. 1) through an increase in DNA fragmentation. Our results showed an increase in mean DNA fragmentation after digestion with Fpg in fresh sperm but not in cryopreserved samples, indicating a basal level of oxidized guanines in non-fragmented positions of the strands before cryopreservation.

The distribution of frequencies of DNA damage (Fig. 2) revealed that fragmentation in fresh sperm ranged from 0 to 80%, and over 10% of the cells displayed a level of damage lower than 10%. After cryopreservation, frequency distribution changed, reducing the number of cells in the lower ranges and increasing the cells in the higher ones. In samples frozen with egg yolk, DNA damage ranged from 10 to 90%, and no undamaged cells were detected

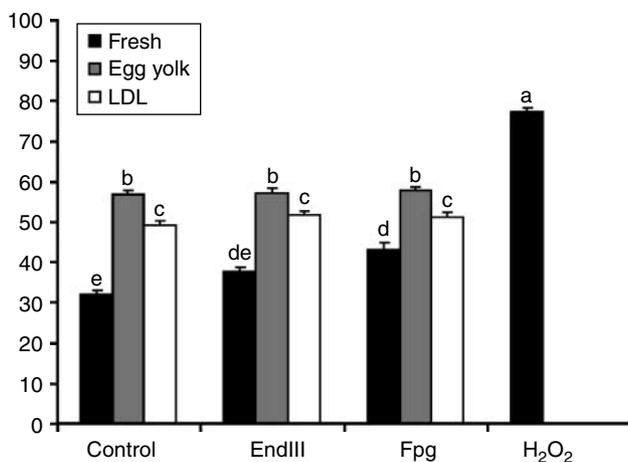


Figure 1 Percentage of tail DNA obtained by SCGE combined with the use of restriction enzymes (EndIII and Fpg from *E. coli*) for fresh sperm, sperm cryopreserved with egg yolk and 12% LDL, and samples treated with 500 mM H₂O₂ before the comet assay (800 cells per treatment). Bars indicate mean ± S.E.M. Different letters indicate significant differences ($P < 0.05$).

(range from 0 to 10%). Sperm cryopreserved with LDL showed a range of damage from 0 to 90%, with only 3% of cells showing damage in the 0–10% range.

By combining comet assay with enzymatic digestion (Fig. 2), we observed a reduction of cells with a low level of tail DNA (range from 0 to 20% and from 0 to 30 for EndIII and Fpg respectively) in fresh sperm, indicating the presence of both oxidized guanines and cytosines in non-fragmented positions of the strands, and also observed an increase in the number of cells with damage in the higher ranges (30–40, 60–70, and 60–80% for EndIII and Fpg respectively). Furthermore, cryopreservation with egg yolk also promoted an increase in the number of cells within the higher ranges (50–60%) of tail DNA, and a decrease in lower values (40–50%) when Fpg was used, but no effect was detected for EndIII, indicating the presence of oxidized guanines but not of cytosines. LDL promoted a significant increase in the number of cells with a high level of tail DNA when endonucleases were used (70–80% for EndIII and 40–50% for Fpg) and a decrease for lower ones (0–10 and 30–40% for Fpg), implying the presence of both oxidized bases.

Fertilization rates and embryo development

Fresh sperm showed higher fertilization (90.6 ± 2.46) and hatching rates (78.3 ± 5.4) than frozen sperm (Fig. 3). Furthermore, the use of egg yolk promoted lower fertilization (61.1 ± 2.3) and hatching rates (14.5 ± 4.4) than that of LDL (66.7 ± 2.1 and 38 ± 5.4 respectively). When eggs fertilized with fresh and LDL-cryopreserved sperm were incubated with 3-aminobenzamide (3AB), no differences were found in relation to non-treated ones in terms of fertilization and hatching rates. Nevertheless, the 3AB treatment promoted a decrease in hatching rates in batches of cryopreserved sperm fertilized with egg yolk (3.2 ± 2.2). As shown in Fig. 4, the percentage of abortions increased during development in embryos obtained from eggs fertilized with cryopreserved sperm, showing that most abortions were promoted in the transition from epibolia to somite stage (48.13 and 44.62% of the abortions with egg yolk and LDL respectively). This transition was also the most sensitive to the 3AB treatment in embryos obtained with egg yolk-cryopreserved sperm (58.21% of the abortions).

Discussion

It is suspected that, in mammals, DNA-damaged spermatozoa are impaired to fertilize under natural conditions by the high selective pressure occurring in the female tract and during sperm–egg recognition (Holt & Van Look 2004, Khalil *et al.* 2006, Satake *et al.* 2006, Suarez & Pacey 2006, Rodriguez-Martinez 2007, Yeates *et al.* 2009). These selection mechanisms are related to sperm structures that could also be modified in spermatozoa carrying damaged DNA

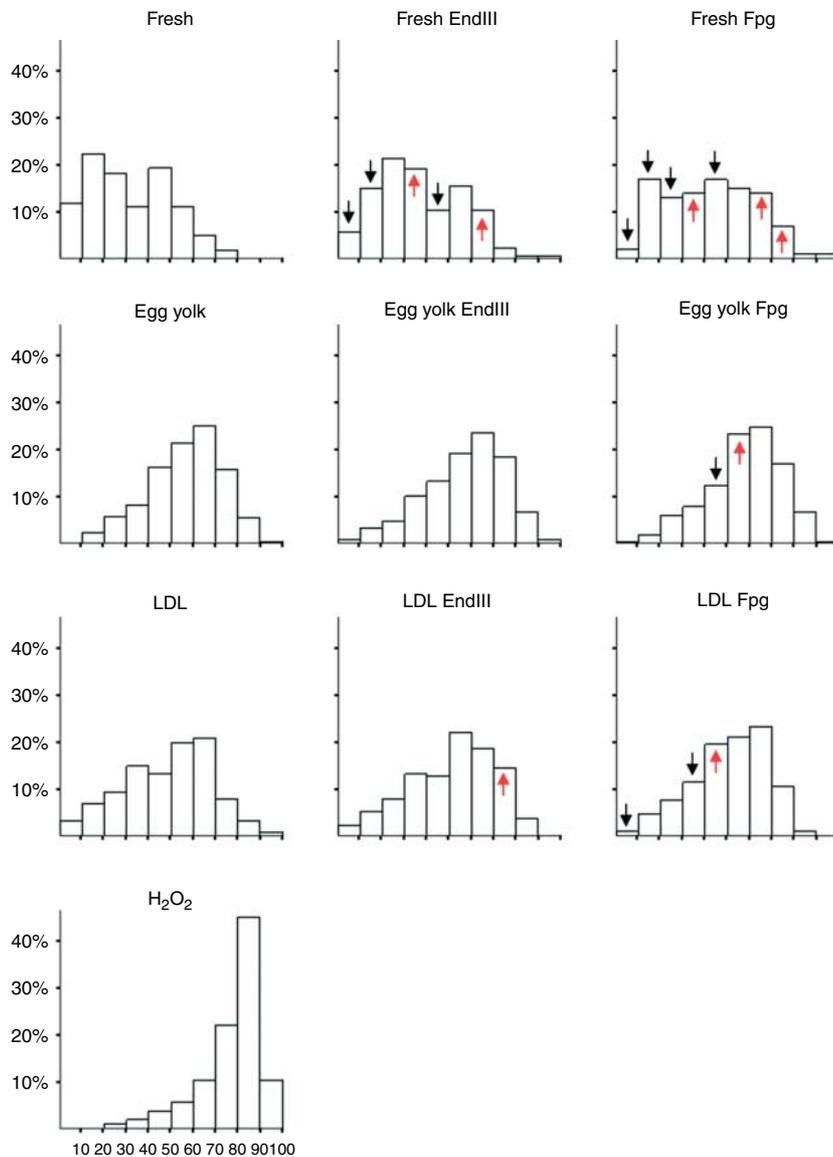


Figure 2 Cell frequencies in relation to the percentage of tail DNA ($n=300$) for fresh sperm, sperm cryopreserved with egg yolk and 12% LDL, and samples treated with 500 mM H_2O_2 before the comet assay (800 cells per treatment). Arrows pointing up or down indicate significant differences (increasing or decreasing respectively) for enzymes EndIII and Fpg compared with the respective treatment without enzymes for each range of tail DNA ($P<0.05$).

(Tarin *et al.* 2000, Andrabi 2007, Thomson *et al.* 2009). The use of ARTs could be bypassing the natural mechanisms selecting the spermatozoa competent to fertilize, allowing DNA-damaged sperm to reach and enter the egg with unknown consequences for the offspring (Zini & Sigman 2009). This effect could be magnified when ARTs are combined with sperm cryopreservation that promotes the production of DNA damage in spermatozoa. Previous works point to a lower selection pressure in external fertilizers, in which the encounter with eggs is suspected to be more a matter of probability, mainly depending on the presence of numerous spermatozoa close to the egg and on a superior ability to withstand the hyposmotic stress when released in water (Holt & Van Look 2004). This could facilitate the experimental application of fertilization processes with injured spermatozoa in order to

analyze the effects of sperm DNA damage after fertilization, its role during development, and the mechanisms to repair this damage by the oocyte. With this goal in mind, we decided to perform the study with rainbow trout, a species whose reproductive biology is well known, and which is easy to breed in captivity and very prolific.

In our study, we tested two cryopreservation procedures that promoted different degrees of sperm damage. When DNA fragmentation and membrane integrity were analyzed in fresh sperm, we found an apparently good status of plasma membrane and a high level of DNA fragmentation, $31.99\% \pm 1.19$, in comparison with other studies in the same species, reporting from 6 to 20% (Cabrita *et al.* 2005, Dietrich *et al.* 2005, Pérez-Cereales *et al.* 2009), with some of them using the same protocol. This could be related to semen

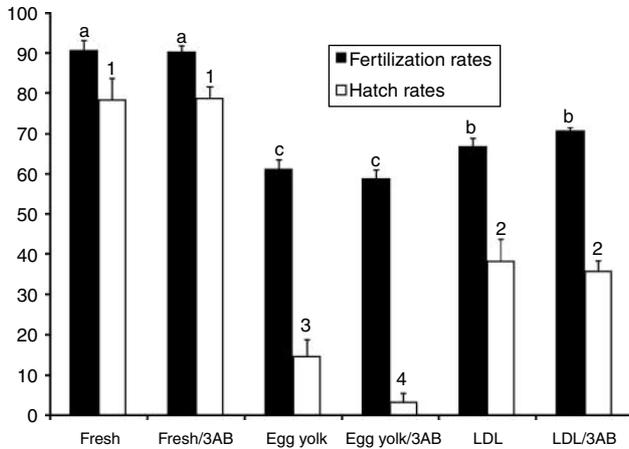


Figure 3 Fertilization and hatching rates after egg fertilization with fresh sperm and sperm cryopreserved with egg yolk and 12% LDL at hatching. 3AB indicates eggs treated with 3-aminobenzamide (3AB) after fertilization during the first cleavage (8 batches per treatment, 200 eggs per batch). Bars indicate mean \pm S.E.M. Different letters or numbers indicate significant differences ($P < 0.05$).

collection at the end of the optimal moment of the reproductive season when some sperm aging events appear, initially affecting DNA integrity but not membrane integrity, or fertilization capacity, as was described by Pérez-Cerezales *et al.* (2010) who compared the quality and freezability of the sperm collected during the reproductive season. Sampling was performed at this time, because a higher susceptibility to cryodamage was expected and this was considered beneficial for the objectives of the present work. Performance of the alkaline lyses at a pH of 12 allowed the identification of single and double strand breaks (Lee & Steinert 2003), with it being a more sensitive method than the application of TUNEL or comet assay at a neutral pH, which only reveals double strand breaks. This method is considered to detect the actual genotoxic effects of any treatment (Liao *et al.* 2009).

The analyzed parameters revealed that after freezing–thawing, considerable damage was produced. With the use of egg yolk as cryoprotectant, plasma membrane and DNA suffer a higher level of damage than with the use of LDL as was described previously in different species (Moussa *et al.* 2002, Hu *et al.* 2008, Yamauchi *et al.* 2009, Pérez-Cerezales *et al.* 2010). As was suggested by Pérez-Cerezales *et al.* (2010), protection of both additives is primarily directed to plasma membrane, which indirectly protects DNA. The LDL fraction of egg yolk concentrates the protective molecules, eliminating some others that could have an antagonist effect on pure egg yolk. The use of the two protectants provided samples with different levels of DNA fragmentation for further fertilization trials. The generation of ROS is considered as one of the main causes of cryodamage, especially in DNA injury, and antioxidant agents are commonly included in the

extenders (Baumber *et al.* 2003, Li *et al.* 2007, Thomson *et al.* 2009). Our results showed an increase in ROS production after cryopreservation, which was similar to the levels detected with both the tested cryoprotectants. Positive control incubated with H_2O_2 promoted significantly higher levels of radicals. To show the direct effect of oxidization on DNA molecules, we combined the use of EndIII and Fpg digestion with the comet assay showing the presence of oxidized bases in both fresh and cryopreserved samples located in the non-fragmented positions of the DNA strands. The presence of oxidized bases in fresh sperm could be related to the presence of senescent cells, and could affect DNA sensitivity to cryopreservation as it has been described that oxidized bases could transform directly into DNA breakages increasing fragmentation during freezing–thawing (Box *et al.* 2001, Thomson *et al.* 2009). A higher level of post-thaw DNA strand breaking was effectively detected. Our results also suggest that guanines are more prone than cytosines to be oxidized during freezing–thawing, as has been reported by other authors (Box *et al.* 2001). On the other hand, not all base oxidizations promote DNA strand breaks, because further digestion with specific endonucleases increased fragmentation rates. The presence of oxidized bases is not detected when DNA integrity is evaluated using the comet assay without digestion or other methods such as TUNEL and SCSA, and could have implications during embryo development.

It is important to highlight that sperm cryopreserved with egg yolk maintained 13% of cells with intact membrane integrity (and fertilizing spermatozoa should belong to this category), which produced levels of DNA damage higher than 10% of fragmented DNA in all the spermatozooids, plus additional oxidized guanines. This fact implies that every embryo produced from

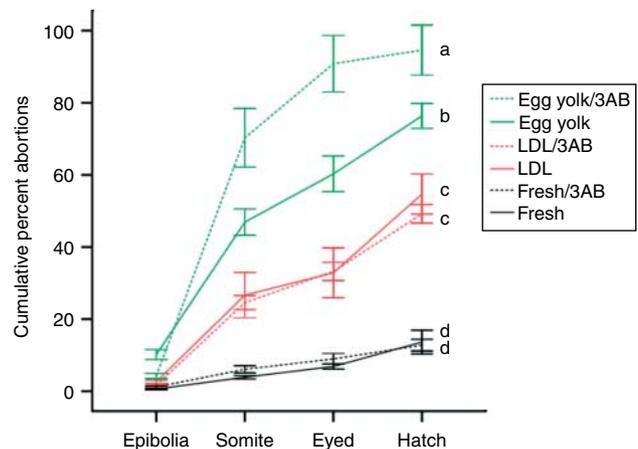


Figure 4 Cumulative percentage of abortions during development at different stages (epibolia, somite, eyed, and hatch) with or without 3AB incubation during the first cleavage (8 batches per treatment, 200 eggs per batch). Lines indicate mean \pm S.E.M. Different letters indicate significant differences between treatments ($P < 0.05$).

eggs fertilized with the damaged sperm was conceived with a male pronucleus with DNA damage promoted by cryopreservation, because unfragmented spermatozooids were not detected. The reduction in fertilization rates for both cryopreservation procedures in relation to fresh sperm was related to the damage promoted during freezing–thawing that could compromise different spermatozoa functions (Silva & Gadella 2006, Beirão *et al.* 2009). Nevertheless, fertilization rates were relatively high ($61.14\% \pm$ and $66.73\% \pm 2.10$ using egg yolk and LDL respectively) in relation to the apparently poor quality of the cryopreserved sperm. This fact could be associated with the high sperm:egg ratio applied in fertility trials (1.6×10^7 spermatozoa per egg). After fertilization, our results revealed a high number of abortions when both cryopreserved sperm were used. These abortions were related to the percentage of DNA damage detected after each cryopreservation procedure. Most of the abortions were produced in the transition from epibolia to somite stage, which entails a set of morphogenetic processes requiring bulk changes in gene expression with the transcription of a number of genes crucial for lineage specification (Solnica-Krezel 2006, Jaroudi & SenGupta 2007, Tam & Loebel 2007). This result reveals the capacity of DNA-damaged spermatozoa to fertilize, and points out their negative effect during early embryo development.

The ability of oocytes to repair DNA damage has been reported in different studies (Kopeika *et al.* 2004, Hales *et al.* 2005, Hakem 2008), but there are no references on the extent of damage that could be repaired during development. According to Ahmadi & Ng (1999), mouse oocytes are able to repair up to 8% of sperm DNA fragmentation, but no experimental data support this hypothesis. It is considered that many strand breaks from the male pronucleus could be repaired during the first cleavage. DNA repair is achieved by different mechanisms, the activity of which has been detected during early embryo development (Matsuda & Tobar 1989, Jaroudi & SenGupta 2007, Marchetti *et al.* 2007, Hakem 2008). In our study, we detected simple and double strand breaks as well as oxidized bases. Repairing of DNA simple strand breaks and oxidized base replacement is performed by the BER pathway, a highly conserved mechanism based on the PARP which has also been described in fish (Fortier *et al.* 2009), whereas double strand breaks are repaired by the homologous recombination (HR) pathway or the non-homologous end joining (NHEJ) pathway (Hakem 2008). When PARP was inhibited by 3AB during the first cleavage, 94.60% of the embryos fertilized with egg yolk-cryopreserved sperm died, showing that all fertilizing spermatozoa in these samples led to genetic damage requiring repair by the BER pathway of the oocyte. Without PARP inhibition, 76.34% of fertilized oocytes failed to hatch, indicating that a high percentage of fertilizing spermatozooids carry irreparable DNA damage

and that the successful 20% hatched embryos were conceived with DNA damage repairable by the BER pathway. These data suggest that trout oocytes are able to repair at least 10% of sperm DNA fragmentation, because no spermatozoa with a lower level of fragmentation were detected in these samples. This finding is in the range of that suggested by Ahmadi & Ng (1999) for mouse oocytes.

LDL-cryopreserved sperm promoted the abortion of 49.21% embryos showing the presence of unrepaired DNA damage. However, surprisingly, in contrast to embryos resulting from egg yolk-cryopreserved sperm, treatment with 3AB did not promote an increase in abortion. The basal percentage of abortions (in untreated 3AB embryos) could indicate fertilization with spermatozoa containing different genetic damage: simple or double strand breaks, base oxidization, packaging changes, or epigenetic modifications. The percentage of fertilizing spermatozoa carrying damage repairable by the BER pathway is not significant. This implies that the extent of double and simple strand breaks (non-distinguishable with this comet assay) could be different in fertile spermatozooids from samples frozen with egg yolk or with their LDL fraction. The use of different inhibitors of the repair system, during different stages of early development and fertilization with spermatozoa carrying different kinds of DNA damage, could help to characterize the repair process and to define the extent and type of damage that could be tolerated. Taking into account that repair DNA pathways such as BER, HR, or NHEJ are highly conserved mechanisms, fish could be very interesting models for this purpose. The high number of fertilized embryos that can be obtained and their external development – giving easy access to the embryo during development – are important advantages.

In conclusion, cryopreserved sperm from rainbow trout carrying damaged DNA are able to fertilize, affecting offspring survival. In addition, some degree of DNA fragmentation from the male pronucleus is repaired by the oocyte, and unrepaired damage promotes the abortion of the embryo. The presence of different kinds of damage such as simple or double strand breaks, base oxidization, or packaging modifications could imply developmental failures by different ways, and needs to be studied in depth. The numerous advantages of rainbow trout could help in understanding processes related to fertilization with DNA-damaged spermatozoa when processes of sperm selection are bypassed by ARTs in other species.

Materials and Methods

Reagents

All media components were purchased from Sigma–Aldrich or Merck unless another source is stated.

Animals

Fresh sperm and eggs were obtained from four males and two females rainbow trout from the Las Zayas fish-farm stock (León, Spain). The fish were maintained under natural photoperiod, and gametes were obtained during the natural reproductive season. Sperm were collected by cannulation and abdominal massage, and motility was checked according to the method described by Robles *et al.* (2003). Modified sperm were diluted 1:100 in SFMM (110 mM NaCl, 28.18 mM KCl, 1.22 mM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 1.77 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 10.05 mM bicine, and 9.99 mM HEPES), an immobilizing solution, and then, 10 μl of the sperm dilution were placed on a glass slide, and immediately, 100 μl of DIA532 (94.97 mM NaCl, 49.95 mM glycine, and 19.98 mM Tris) were added to activate motility. Only samples with more than 90% motile spermatozoa were used. Oocytes required for fertilization trials were extracted by stripping from females anesthetized with MS222. Eggs were pooled and divided into 200 egg batches for fertilization trials.

Sperm cryopreservation

Immediately after collection, sperm were frozen using the protocol described by Herráez *et al.* (2008). Sperm from each male were diluted 1:3 (sperm:extender) in #6 from Erdahl and Graham (0.7 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 1.08 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 1.49 mM Na_2HPO_4 , 34.30 mM KCl, 100 mM NaCl, 0.52 mM citric acid, 55.5 mM glucose, 226 mM KOH solution, 20 ml, 324 mM bicine solution, 20 ml, and 323 mOsm/kg, pH 7.4), containing 7% Me_2SO (v/v) as a permeable cryoprotectant and 10% (v/v) egg yolk or 12% LDL (w/v) as a membrane protector. LDL was obtained using the protocol described by Moussa *et al.* (2002). The sperm were then left to equilibrate for 10 min at 4 °C and loaded into French straws (0.5 ml). The loaded straws were kept for 10 min in a floating device 2 cm above liquid nitrogen in a styrofoam box. They were then immersed in liquid nitrogen and stored in a liquid nitrogen tank until analysis. Thawing was carried out in a water bath at 25 °C for 30 s, and the sperm analyses and fertilization trials were performed immediately.

Spermatozoa viability

Spermatozoa viability was evaluated using the fluorescent 'live/dead Kit' (Molecular Probes, Leiden, The Netherlands), containing propidium iodide (PI) and SYBR-14 and the flow cytometer (FACSort Plus Analyzer, Becton–Dickinson, Franklin Lakes, NJ, USA) method validated for freshwater fish by Flajshans *et al.* (2004). Each sample was diluted to 1.6×10^7 cells/ml in SFMM solution at a final volume of 1 ml. SYBR was added to a final concentration of 100 nM, and was incubated in the dark for 10 min. Then, PI was added to a final concentration of 12 μM , and was kept in the dark for 10 min. After this, flow cytometry analysis was performed for obtaining the percentages of red cells (cells with damaged membrane) and green cells (cells with intact membrane in this test).

Analysis of ROS in the sperm

For positive control of oxidization, one aliquot of each fresh sperm sample was 40-fold diluted in SFMM containing 500 mM H_2O_2 for 20 min at 4 °C. Fresh and frozen sperm aliquots were diluted to a final concentration of 2×10^6 in SFMM, and 5 μl of 0.5 μM H_2DCFDA were added to each sample for the detection of intracellular ROS. The aliquots were then incubated at 4 °C in the dark for 5 min, and analyzed by flow cytometry by the measurement of mean fluorescence emission for each sample. Results were reported in MFI as mean \pm S.E.M.

Flow cytometer configuration

The flow cytometer (FACSort Plus Analyzer, Becton–Dickinson) was equipped with standard optics and an argon ion laser (Innova 90, Coherent Inc., Santa Clara, CA, USA), with CellQuest software for Macintosh (version 3.0, Becton–Dickinson) for acquisition and analysis, and with FACSCComp software (version 1.0) for daily setup and quality control. The red fluorescence emitted by PI was detected using a 610-nm filter, and the green fluorescence emitted by SYBR-14 and H_2DCFDA was detected using a 516-nm filter. The evaluation was carried out in all samples and assays in triplicate, acquiring 10 000 cells each time.

Sperm DNA damage

DNA fragmentation was analyzed using the SCGE or comet assay. Prior to the assay, one aliquot of fresh sperm from each male was treated with H_2O_2 by 40-fold dilution in 500 mM H_2O_2 in SFMM at 4 °C for 20 min to be used as DNA-fragmented positive control. Fresh, cryopreserved, and H_2O_2 -treated sperm were diluted in SFMM to a final concentration of $8\text{--}10 \times 10^6/\text{ml}$. Frozen sperm were prepared immediately after thawing to prevent sperm degradation. Slides for sperm smears were prepared in advance as described by Cabrita *et al.* (2005). All buffers were prepared fresh. After spreading the sperm, the slides were immersed in a cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Tris, 1% Triton-X, 1% lauroyl sarcosine sodium salt, and 4 mM lithium diiodosalicylate, pH 8), and were incubated for 1 h at 4 °C. After lysis, the slides were washed in an enzymatic buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mg/ml BSA, pH 8.0) three times for 5 min, and were covered either with 40 μl of buffer alone or with endonuclease (1 $\mu\text{g}/\text{ml}$ of EndIII) or 22 $\mu\text{g}/\text{ml}$ of Fpg from *Escherichia coli*) and sealed with cover slips. Then, all the slides were incubated for 45 min at 37 °C to activate the enzymatic reaction. Then, the slides were placed in an electrophoresis cube (Bio-Rad) containing an electrophoresis buffer (0.3 M NaOH and 1 mM Na_2EDTA , pH 12) for 20 min at 4 °C. Electrophoresis was performed for 10 min at 25 V and 300 mA at 4 °C. The slides were then neutralized with 0.4 M Tris, pH 7.5, for 5 min at 4 °C. This operation was repeated three times to ensure the elimination of all alkali and detergents. The slides were fixed by immersion in a pure methanol solution for 3 min, and were left to drain in air and stored protected from light and dust.

For comet visualization, 40 µl of ethidium bromide, 0.5 µg/ml, were pipetted onto the slides, which were then covered with cover slips. Samples were observed using an epifluorescence microscope (Nikon Eclipse E800; Nikon, Lijnden, The Netherlands) fitted with a 510–560-nm excitation filter and a 590-nm barrier filter. Images were acquired from the microscope with a Nikon DXM1200F digital camera, acquiring ~50 cells from each slide using Nikon ACT-1 software (v. 2.62, Nikon). All the images were analyzed with the Komet imaging system software (version 5; Kinetic Imaging, Liverpool, UK). Two slides were analyzed per male and treatment. The percentage of tail DNA (fragmented DNA) was obtained for each treatment. Frequencies of cells for each degree of tail DNA were analyzed.

Fertilization and egg DNA repair inhibition

Eggs were fertilized with sperm from each male using fresh sperm diluted 1:3 (sperm:extender) in #6 from Erdahl and Graham and cryopreserved sperm (2400 eggs per male: four batches of 200 eggs/treatment). One milliliter of sperm was directly spread over 200 eggs, and motility was activated with 10 ml of DIA532 solution. Ten minutes later, the eggs were washed with water and incubated in the dark for 6 h at 4 °C (during the first cleavage) in 15 mM 3AB (two batches per treatment) to inhibit the PARP activity or in water (the two other batches). The eggs were washed with water and incubated until hatching.

Incubation and evaluation of development

The fertilized eggs were maintained in the dark under controlled conditions (10 °C) until hatching. Thirty embryos from each batch were taken on days 5, 9, 16, and 26 to evaluate embryo survival at four developmental stages: epibolia, somite, eyed, and hatch.

To visualize embryo development in epibolia, somite, and eyed stages, the eggs were treated (cleared) with a solution containing acetic acid, methanol, and H₂O (1:1:1).

Statistical analysis

Statistical analysis was carried out using the computerized package generated by SPSS 14.0 software for Windows (SPSS Inc., Chicago, IL, USA). The results were expressed as means ± S.E.M. Mean values of the percentage of tail DNA were obtained from 400 cells (100 from each male); mean values of viability and ROS were obtained from 10⁴ cells from each sample; and mean values of fertilization rates, hatching rates, and abortions were obtained from 800 eggs (200 from each male sperm sample and post-fertilization treatment). Means of spermatozoa viability, percentage of tail DNA, ROS, fertilization rates, and hatching rates were compared and analyzed by one-way ANOVA ($P < 0.05$). Frequencies of tail DNA were compared for enzyme treatments by χ^2 test ($P < 0.05$). Percentage of accumulative abortions was also analyzed using a linear mixed-effects model analysis ($P < 0.05$).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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