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Ultrastructure of fresh and post thawed sperm of pejerrey *Odontesthes bonariensis* (Atheriniformes)

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In the present study it was showed for the first time the ultrastructural morphology of *O. bonariensis* sperm using electron microscopy techniques. Different kinds of abnormalities were described in fresh and post thawed sperm caused by crogenic protocols. Pejerrey spermatozoon is uniflagellated and is differentiated into three parts: a small roundish head (~1.80µm in length and 1.67µm in width), a midpiece or transitional region (~1.11µm in length and 1.56µm in width), and a long tail or flagellum (~29.08µm). Samples of fresh and post thawed sperm showed evidence of morphological anomalies affecting various intracellular compartments. Spermatozoa with swollen, ruptured, or absent membranes in the head showing excess of cytoplasm, and with alteration of the spatial orientation of the mitochondria were observed. A swollen flagellum was observed containing cytoplasmic vesicles, distributed along the whole length or concentrated in a restricted part of the tail. It was also found a high level of abnormalities (60%) in frozen sperm when compared with normal sperm (18%) reflecting the damage provoked by cryopreservation procedures.

No presente estudo mostrou-se pela primeira vez a morfologia estrutural dos espermatozoides de *O. bonariensis* utilizando técnicas de microscopia eletrônica. Diferentes tipos de anormalidades foram descritas para sêmen fresco e descongelado. O espermatozoide de Pejerrey é uniflagelado e dividido em três partes: uma cabeça pequena e arredondada (~1.80µm de comprimento e 1.67µm de largura), uma parte intermediária ou região de transição (~1.11µm de comprimento e 1.56µm de largura) e uma cauda longa ou flagelo (~29.08µm). Amostras de sêmen fresco e descongelado mostraram evidências de anormalidades morfológicas afetando vários compartimentos intracelulares. Na cabeça haviam espermatozoides com membranas dilatadas, rompidas ou ausentes, mostrando excesso de citoplasma e alteração na orientação espacial das mitocôndrias. Um flagelo dilatado foi observado contendo vesículas citoplasmáticas, as quais estavam distribuídas ao longo de todo o seu comprimento ou concentradas em uma parte restrita da cauda. Também foi encontrado um alto nível de anormalidades (60%) em sêmen congelado em comparação com o sêmen normal (18%), refletindo os danos provocados pelos procedimentos de criopreservação.

Key words: Cryopreservation, Morphology.

Introduction

Atheriniformes are widespread, abundant and ecologically important forage fishes in near-shore marine, estuarine, and freshwater habitats (Bloom *et al*., 2012). Pejerrey (*Odontesthes bonariensis)* is an inland water fish native from Buenos Aires Province (Argentina) and the South of Brazil (Dyer, 2006). It is a brackish species (Tsuzuki *et al*., 2000) considered the largest atheriniform in the world (Dyer & Chernoff, 1996). This species is highly appreciated because of the quality of its flesh and also as a game fish, and probably is the most popular and known fish in Argentina (López *et al*., 2001). Although, pejerrey aquaculture is considered regionally important, it has not been fully developed (Somoza *et al*., 2008). One technical problem is the obtaining of enough number of embryos for the operation of a hatchery in an efficient way. For this purpose, it was necessary to solve some reproductive aspects like the marked asynchrony between females during spawning season, the relatively low fecundity, and the low volume of expressible milt (Strüssmann, 1989; Miranda *et al*., 2001). For these reasons, some experiments were performed using environmental and hormonal treatments to increase pejerrey sperm volume and synchronize spawning in females (Miranda *et al*., 2001, 2005, 2006; Miranda & Somoza, 2009). Recently, pejerrey sperm have been successfully cryopreserved using simple and reliable techniques which produced thawed

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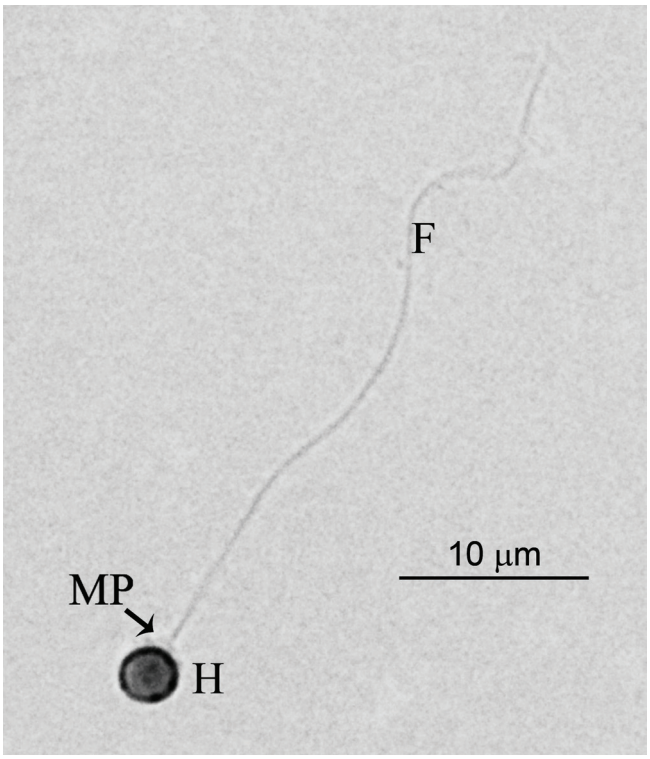

Fig. 1. Fresh pejerrey spermatozoa stained with eosin. H: Head; MP: Midpiece; F: Flagellum.

spermatozoa capable of fertilizing oocytes with values between 44-80% depending on the method used. In that study, the best results were obtained using a saline based solution as extender, dimethyl sulfoxide (DMSO) or ethylene glycol (EG) as cryoprotectants, and liquid N_2 as freezing method (Lichtenstein *et al*., 2010). These values, demonstrated that cryopreservation affected viability and fertilizing capacity of frozen thawed spermatozoa.

In the last years, great emphasis has been placed on the study of the effects of cryosolutions and cooling procedures on the structure and physiology of spermatozoa (Lahnsteiner *et al*., 1996; Cabrita *et al.*, 1998; Rodriguez-Martinez *et al.*, 1998, Yao *et al*., 2000). It has been observed that different types of alterations and damages could be provoked or increased by cryopreservation procedures. For example, ruptured plasmatic membranes in the spermatozoa head, midpiece, and flagellum, as well as swollen mitochondria and broken axoneme were frequently found after thawing (Gwo *et al*., 1992; Lahnsteiner *et al*., 1996; Yao *et al.*, 2000; Taddei *et al*., 2001). Besides, the examination of sperm morphology at ultrastructural level provides useful information to develop cryopreservation protocols (He & Woods, 2004), and also for doing phylogenetic analysis; Mattei, 1991; Gwo *et al*., 2004a, b; França *et al*., 2007; Jamieson, 2009). Between the methods that are used to investigate cellular damages, electron microscopy techniques provide detailed information on the subcellular ultrastructure representing a useful tool for the evaluation of morphological changes that can affect sperm functionality (Taddei *et al*., 2001).

In order to analyze the damage produced during freezing procedures, and due to the lack of information about pejerrey spermatozoa morphology, in this study we investigated the anomalies caused by cryogenic protocols at ultrastructural level.

Material and Methods

Milt collection and cryopreservation procedure

Pejerrey male spawners reared at IIB-INTECH aquaculture facilities were used during spawning season (spring). They were kept in 3000 L indoors tanks with an open flow system keeping water temperature and salinity around 18°C and 1.5% respectively. Five males were anesthetized with benzocaine (100 ppm) and the milt was collected by gentle abdominal pressure into a 200µl tips adapted for 1ml syringes positioned at the genital pore. Once filled, each tip was placed inside 1.5 ml tube and kept on ice. All the samples that showed maximum motility (index of 5, Strüssman *et al*., 1994) were collected in two pools of l00µl. Sample 1 (S1) was directly processed for transmission electron microscopy (TEM)., sample 2 (S2) was diluted (1:5) with a Mounib modified solution (127mM $NaHCO_3$, 159mM Sucrose, 0.025g/ml Reduced Glutathione; pH 8; osmolality 400mOsm/Kg), and 10% of DMSO. Then, it was freeze in liquid N_2 and after 24 hs was thawing following the methodology described in Lichtenstein *et al.* (2010), and then processed for TEM as it was described above.

Also, 1µl of fresh sperm was diluted in 500 µl of 2% glutaraldehyide and dyed with 5% alcoholic eosin for 10 min. Spermatozoa were observed and photographed using a light microscope (Nikon Eclipse E 600) attached to a digital camera (Nikon Digital Sight DS-U2).

Transmission electron microscopy

Sperm samples (S1 and S2) were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4 (LADD Research Industries, Williston USA) for 2 hours at 4ºC. Then they were post fixed

Table 1. Measurements of the different parts of pejerrey spermatozoa (length, width and diameter) n=20.

Name of the structure or microstructure	Mean ± SE (µm)
Length of the head	1.808 ± 0.060
Width of the head	1.673 ± 0.066
Length of the midpiece	1.115 ± 0.032
Width of the midpiece	1.565 ± 0.050
Length of the flagellum	29.084 ± 1.242
Diameter of the flagellum	0.373 ± 0.053
Diameter of the flagellum with lateral extensions	1.418 ± 0.068

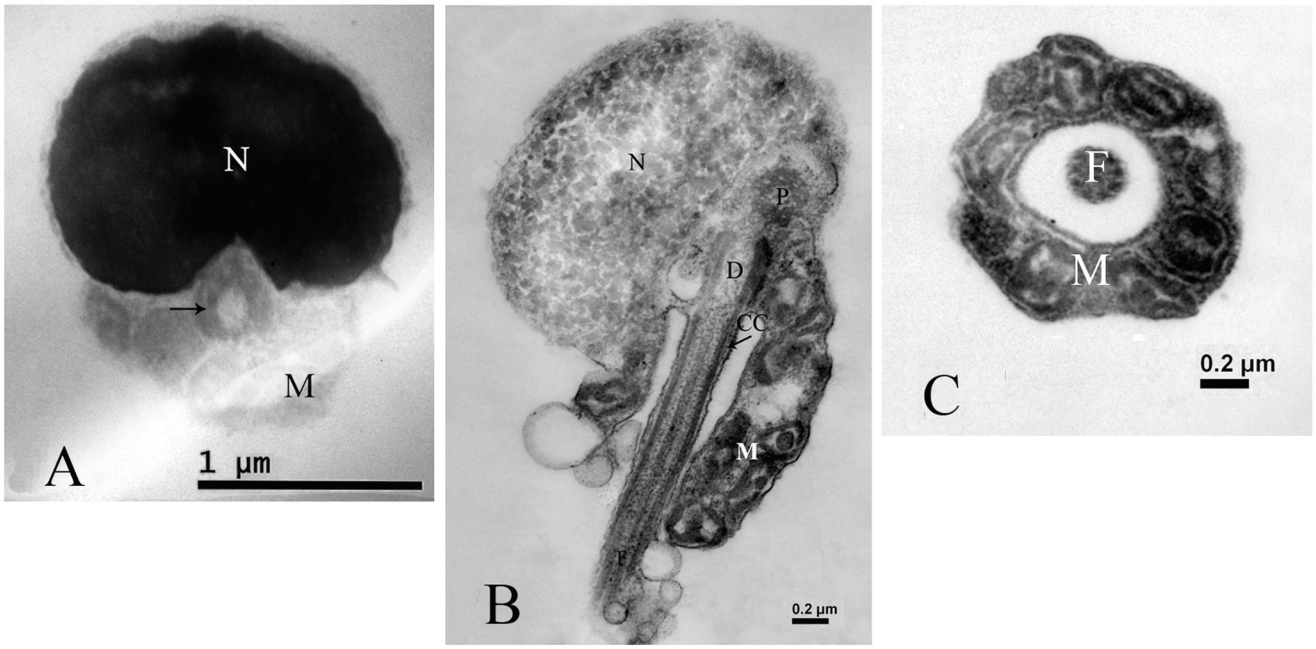

Fig. 2. Transmission electron microscopy photographs of fresh sperm samples: (A) Spermatozoa longitudinal section showing the transitional region of the flagellum and the classic nine doublets of microtubules (9+0), arrow. (B) Longitudinal section of the head and midpiece of a spermatid. (C) Transversal section of the midpiece showing the axoneme surrounded by mitochondria. N: Nucleus; P: Proximal centriole; D: Distal centriole; CC: Citoplasmic Channel; M: Mitochondria; F: Flagellum.

with 1% osmium tetroxide (LADD Research Industries, Willinston, USA) at 4ºC and were dehydrated with an alcohol increasing series and embedded in resin Epoxi (LADD Research Industries, Willinston, USA). Ultrasections (60nm) were put over copper grids, and stained with toluidine blue (Schmid GMBH & CO, Krumbach, Germany). Then, they were stained with 2% uranyl acetate followed by lead citrate, and examined under a TEM Jeol JEM 1200 EX II. Morphological measurements were taken in 20 spermatozoa from S1 using Image Pro Plus 4.5 on the TEM photographs. Abnormalities were observed in the same way in 50 spermatozoa of each sample.

Results

Pejerrey spermatozoon is uniflagellated and differentiated into 3 parts: a small roundish head (1,808 ± 0,060µm in length and 1,673 ± 0,066µm in width), a short midpiece or transitional region (1,115 ± 0,032µm in length and 1,565 ± 0,050µm in width), and a single long tail or flagellum (29,084 ± 1,242µm; Fig. 1 and Table 1). No acrosomal complex is present in the anterior part of the head. At ultrastructural level, the nucleus has a spherical shape with electron dense chromatin, granular and homogeneous in texture (Fig. 2a). The transitional region of the flagellum was the classical nine doublets of microtubules (9+0; Fig. 2a). Posterior to the nucleus it was possible to observe the centriolar complex where the proximal centriole remains apical and perpendicular to the distal centriole (Fig. 2b). In the midpiece it was observe the cytoplasmic channel in which lies the initial segment of the flagellum (Fig 2b). Surrounding this part of the flagellum it was possible to observe 4 to 7 mitochondria arranged in two layers (Fig. 2c).

The flagellum axoneme has the typical 9+2 structure with both dynein arms (Fig. 3a) and it was surrounded by a plasma membrane with lateral extensions like two fins (Fig. 3b). No vesicles were found along its extension (Fig 3c).

Under TEM different types of abnormalities were observed in sperm of both samples. However, these damages occurred at higher frequencies in the frozen thawed sperm (Fig. 4). While in fresh semen, 18% of spermatozoa presented different types of morphological alterations; in thawed sperm this value was higher being around 60%. There were spermatozoa with swollen, ruptured, or absent membranes in the head showing excess cytoplasm (Fig. 4a-c). When disorganize plasma membrane was found in the sperm, it was possible to observe the alteration of mitochondria position and in some cases the absence of these organelles (Fig. 4c).

As in the sperm head, swelling was observed in the flagellum and these often contained cytoplasmic vesicles, which were distributed along the whole length or concentrated in a restricted part of the tail (Fig. 4d). Spermatozoa head encircled by the tail was also seen as an often alteration (Fig. 4e).

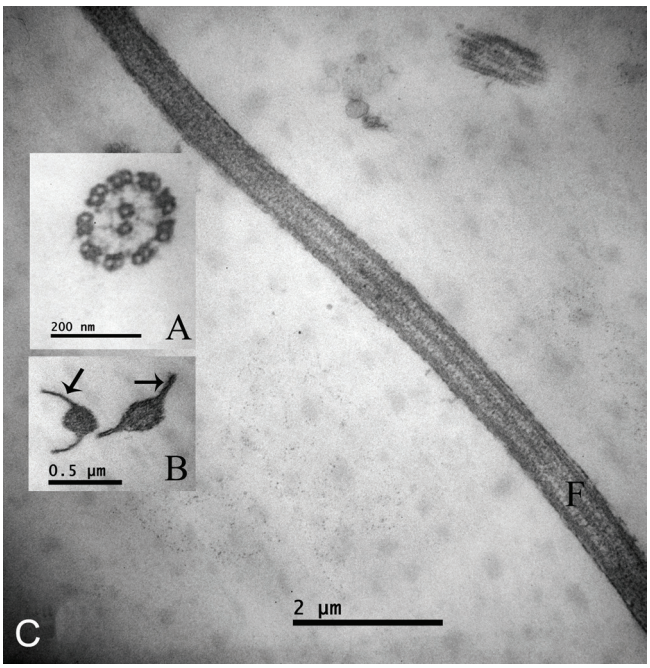

Fig. 3. Transmission electron microscopy photographs of the flagellum. (A) Flagellum axoneme showing the typical 9+2 doublets of microtubules. (B) Transversal section of the flagellum showing the lateral extensions like "fins" in both sides (arrows). (C) Longitudinal section of the flagellum. F: Flagellum.

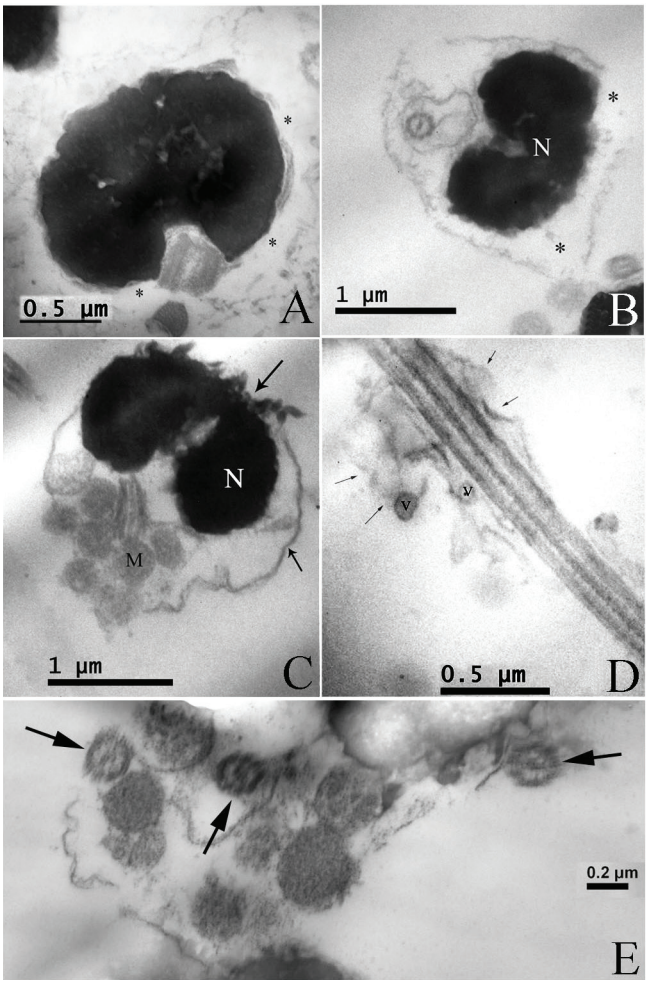

Fig. 4. Transmission electron microscopy photographs of pejerrey post thawed sperm showing different types of alterations. (A) Head with ruptured membranes (*). (B) Head with swollen and broken membrane (*). (C) Broken and swollen nuclear membrane (arrows) and alteration of the spatial orientation of mitochondria. (D) Ruptured and swollen membrane of the flagellum (arrows) with vesicles. (E) Tail around the head (arrows). N: Nucleus; M: Mitochondria; V: Vesicles.

Discussion

To our knowledge this is the first study showing the ultrastructural morphology of *O. bonariensis* sperm using electron microscopy techniques. The characteristics found in pejerrey sperm morphology were common for the Atheriniformes. This group of fish lacks an acrosome in the anterior part of the head, the nucleus is isodiametric with the chromatin usually flocculent, and in the midpiece, the mitochondria form a ring around the axoneme in one or two tier (between four to seven in number). An important characteristic of the spermatozoa of this order is that the flagellum bears two fins in almost all its length and the axoneme has the 9+2 pattern, apparently always with inner and outer dynein arms (Jamieson, 2009). Morphological descriptions of sperm as describe above are very useful to establish synapomorphies and phylogenetic relationships between groups as it was used for Mullidae and Siganidae families (Gwo *et al.*, 2004a), or between species of Doradidae (Quagio-Grassiotto *et al*., 2011). Also, it can be used to describe and compare the spermiogenesis process in some species of Heptapteridae, Pseudopimelodidae, Pimelodidae (Quagio-Grassiotto *et al.*, 2005; Quagio-Grassiotto & Oliveira, 2008) and in some economically important fish species from Sinpercidae family (Luo *et al.*, 2011).

In this context, *Odontesthes* genus has many species with a wide distribution in marine and inland waters of South America (Dyer, 2006) and these kinds of spermatozoa descriptions could be useful to establish better phylogenetic relationships between species of this genus. In pejerrey fresh spermatozoa, some morphological alterations as rupture head membranes, and changes in the position of mitochondria were observed. After cryopreservation treatment, swollen of the head, flagellum which often containing cytoplasmatic vesicles, and the tail surrounding the head were found. In other fish orders, another kind of alterations were reported as lost or dilated mitochondria, swollen midpiece, and broken or double tails (Taddei *et al.*, 2001; He & Woods, 2004; Alavi *et al*., 2008). All these cellular damages could be compromising the sperm motility, but does not necessary mean that the fertilization rate is affected. In this sense, it has been reported

that immotile post thawed spermatozoa of common carp and Atlantic croaker were capable of fertilization (Billard, 1988; Warnecke & Pluta, 2003; Gwo *et al*., 1991). It was also described that the integrity of the cellular membrane is important for the sperm viability and it is the primary site of cooling and warming injury (Morris, 1981). The resistance of spermatozoa to cooling is influenced by the biochemical composition of their membranes (Darin-Bennet *et al*., 1977). In marine fish, spermatozoa were more resistant to freezing than those of freshwater fish due to the higher content of phospholipids and lower polyunsaturated fatty acids (Darin-Bennet *et al*., 1977; Drokin, 1993). In addition to membrane destabilization, physical damage to the nuclear membrane can result from the formation of ice crystals within the cell (Taddei *et al*., 2001) and in the external medium, by osmotic stress or oxidative stress (see Cabrita *et al*., 2010). Cellular osmoregulation also could be compromised resulting in swelling of the head and tail, and secondary injuries to other intracellular organelles could determinate displacement or loss of functionality of mitochondria and outflow of the chromatin (Taddei *et al*., 2001). So the conservation of cell structure and functionality will depend on the cryopreservation protocol (Cabrita *et al*., 2010).

Another cause of this sort of damages could be related to the toxicity of the crioprotector used and its concentration. In pejerrey, it was demonstrated that DMSO is a suitable cryoprotectant for cryopreserving sperm (Lichtenstein *et al*., 2010) as it was also reported in many studies, probable due to its fast penetration into spermatozoa and its interaction with the phospholipids at the sperm membrane (Kerby, 1983; Suquet *et al*., 2000; He & Woods, 2003, 2004). The fertilization rates obtained after cryopreservation using DMSO in pejerrey criopreserved milt were around 60% (Lichtenstein *et al*., 2010). However, some of the morphological alterations observed in this study, cannot be exclusively ascribed to cryopreservation procedure because they are present in fresh sample too, but at lower frequencies. These damages may be also related to the fixation procedures for TEM, especially in the case of the integrity of the sperm head membrane (Taddei *et al*., 2001).

In conclusion, the morphology of pejerrey sperm has been described for the first time showing characteristics of the Atheriniformes. The morphological alterations described in this work could be caused for a combination of cryopreservation and fixation procedures for microscopic observations, and could be related to the diminution in fertility rates found using post thawed sperm.

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