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Effects of cryopreservation on mitochondria of fish spermatozoa

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Abstract

The development of sperm cryopreservation has enabled transcendental changes to occur in the reproductive biotechnology of both mammals and fish; it has become a basic tool for animal improvement. Nevertheless, these protocols cause damage to cell structure and physiology, altering sperm functioning due to cryo-injuries during freezing and thawing. However, studies of the effects on the structural, functional and genomic stability of the mitochondria in fish spermatozoa during cryopreservation are still lacking. The object of this review was to analyse the effect of cryopreservation on mitochondrial metabolic pathways in fish spermatozoa. This effect is related with the bioenergy mechanism for flagellar movement during the activation of sperm motility. In teleost fish, the mitochondria may be cylindrical, spherical or irregular in shape and adhere in a helicoidal or conical pattern to the middle piece. The salmonidae have only a single mitochondrion, but this may vary in other species; the mitochondria provide the flagellum with energy during sperm motility, when sperm respiration is essential. The effects of cryopreservation can induce structural damage to the mitochondria, altering the biochemical process involved in ATP production and thus causing a reduction in sperm motility. Fragmentation damage to nuclear DNA and diminution in sperm motility is mainly associated with damage to the structure and metabolic functioning of the mitochondrion. A direct correlation exists between the mitochondrial membrane potential (transmembrane integrity, $\Delta\Psi_m$) and the motility and fertilizing capacity of the cryopreserved spermatozoa, confirming that this organelle is the energy nucleus of the spermatozoon and that the cessation or prolongation of motility and successful fertilization depend on the availability of ATP in the spermatozoa. Further works are need to incorporate biotechnology studies, at cell and molecular level, of the possible effects of cryopreservation on mitochondrial DNA, enzymatic or metabolic modifications of the citric acid cycle, and the oxidative phosphorylation process in the inner membrane, as well as studies of the mitochondrial ultrastructure. Thus, mitochondrial dynamics could be established as a potential target for therapeutic strategies.

Key words: ATP, cryodamage, fish spermatozoa, mitochondrion, motility.

Introduction

Sperm cryopreservation techniques are widely used in the reproductive management of mammals and fish for both productive and scientific purposes (Merino *et al.* 2011; Figueroa *et al.* 2013). However, despite the growing success of cryopreservation, these techniques cause extensive lethal and sublethal cryodamage to cells. Studies on cryo-injury mechanisms have investigated damage to a wide range of cell structures, showing that cryopreservation implies important cellular and biochemical changes, such as enzyme inactivation, ion alteration and oxidative stress

(Fahy 1986; Carpenter & Crowe 1988; Alvarez & Storey 1992; Gao *et al.* 1997; Labbé *et al.* 2001; Pérez-Cerezales *et al.* 2010; Merino *et al.* 2011; Figueroa *et al.* 2013). However, few investigations have concentrated on the effects of cryopreservation on the structural, functional and genetic stability of the mitochondria in fish spermatozoa (Table 1).

Mitochondria are organelles found in the great majority of eukaryotic cells. They play a fundamental role in cell metabolism and in ATP production through oxidative phosphorylation (Devenish *et al.* 2008). However, this process also is related with oxidative stress produced by a variety of reactive oxygen species (ROS), including the superoxide (SO) anion, hydrogen peroxide (HP) and nitric oxide. However, when ROS production exceeds the sperm's limited antioxidant defences, a state of oxidative stress is induced, characterized by peroxidative damage to the sperm plasma membrane and DNA associated with pathophysiological events such as ageing and apoptosis *in vitro* (De Iuliis *et al.* 2009; Aitken *et al.* 2010; Lahnsteiner & Mansour 2010; Guthrie & Welch 2012).

In mammals and fish, the mitochondrion presents only one intermembrane compartment between the outer and inner membranes, and a mitochondrial matrix characteristic of this type of cell (Lahnsteiner & Patzner 2008). The most specialized zones of this compartment are the inner membrane, where oxidative phosphorylation occurs, and the cytoplasmic matrix, where multiple enzymes are organized into structural and functional subunits. These proteins play an important role in various metabolic pathways necessary for the physiological development of the cell (Mattei 1991; Sickmann *et al.* 2003; Pagliarini *et al.* 2008).

The sperm mitochondria present species-dependent morphological differences, for example they present oval form in the case of the spermatozoa of humans, rhesus monkeys, dogs and other mammals (Gallon *et al.* 2006; Piomboni *et al.* 2012). On the other hand, in fish they may be cylindrical, spherical or irregular – as in the case of *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Thymallus thymallus*, etc. (Lahnsteiner & Patzner 2008). The size and number of mitochondria will depend on the bioenergy

Table 1 Physiological parameters evaluated in the mitochondria of cryopreserved fish sperm

Species	Physiological Parameters	Category	References
Oncorhynchus mykiss	Mitochondrial ultrastructure	Damage to the membranes	Billard (1983)
Micropogonias undulatus			Gwo and Arnold (1992)
Thymallus thymallus			Lahnsteiner *et al.* (1992)
Oncorhynchus mykiss			Lahnsteiner *et al.* (1996)
Macrozoarces americanus		Loss of membrane	Yao (2000)
Paralichthys olivaceus		Damage in the midpiece	Zhang *et al.* (2003)
			Jun *et al.* (2006)
Oncorhynchus mykiss	Mytochondrial integrity	Alterations in potential of membrane	De Baulny *et al.* (1997)
Silurus glanis			De Baulny *et al.* (1999)
Oreochromis niloticus			Segovia *et al.* (2000)
Morone saxatilis			He and Woods (2004)
Sparus aurata			Cabrita *et al.* (2005)
Oncorhynchus mykiss			Merino *et al.* (2011)
			Figueroa *et al.* (2013)
Salmo salar			Figueroa *et al.* (2015)
Clarias gariepinus	Mitochondrial dynamics	Alterations in cellular respiration and bioenergy	Moal *et al.* (1989)
Cyprinus carpio			Perchec *et al.* (1995)
Acipenser fulvescens			Ciereszko *et al.* (1996)
Oncorhynchus mykiss			Lahnsteiner *et al.* (1996)
Oncorhynchus mykiss			De Baulny *et al.* (1997)
Clarias gariepinus			Mansour *et al.* (2003)
Sparus aurata			Cabrita *et al.* (2005)
Perca fluviatilis			Boryshpolets *et al.* (2009)
Sparus aurata	DNA Integrity	Damage to mitochondrial and nuclear genes	Cartón-García *et al.* (2012)
Sparus aurata			Cartón-García *et al.* (2013)
Oncorhynchus mykiss			González-Rojo *et al.* (2014)

requirements of the species. Furthermore, mitochondria contain their own DNA (mtDNA), which encodes proteins necessary for the production of messenger RNA, ribosomal RNA (rRNA) and transfer RNA (tRNA), which in turn sustain the cell's internal metabolism (Piomboni *et al.* 2012).

In mammalian spermatozoa, the mitochondria are located exclusively in the middle piece of the flagellum, around the axoneme, and are closely associated due to the formation of a mitochondrial capsule with multiple disulphide bridges (Calvin *et al.* 1981; Ursini *et al.* 1999). Sperm mitochondria, unlike somatic mitochondria, possess specific isoforms of proteins and isoenzymes, such as cytochrome *c* (Goldberg *et al.* 1977; Hess *et al.* 1993), subunit VI b of cytochrome *c* oxidase (CCO) (Hüttemann *et al.* 2003), lactate dehydrogenase (LDH-X or LDH-C4) (Blanco & Zinkham 1963; Goldberg 1963) and pyruvate-E_1 decarboxylase (Gerez de Burgos *et al.* 1994). In teleost fish, such as *Salmoninae*, *Coregoninae*, *Thymallinae* and *Blennidae*, the mitochondrial complex may adhere to the middle piece in a helicoidal or conical pattern, or it may be contained in invaginations towards the nucleus of the sperm head, or free in the middle piece (Fig. 1). Mitochondrial complex is composed of a variable number of mitochondria. In salmonids, for example, either one mitochondrion or one pair of mitochondria supplies the flagellum with energy during sperm motility (Lahnsteiner *et al.* 2000; Lahnsteiner & Patzner 2008; Figueroa *et al.* 2013).

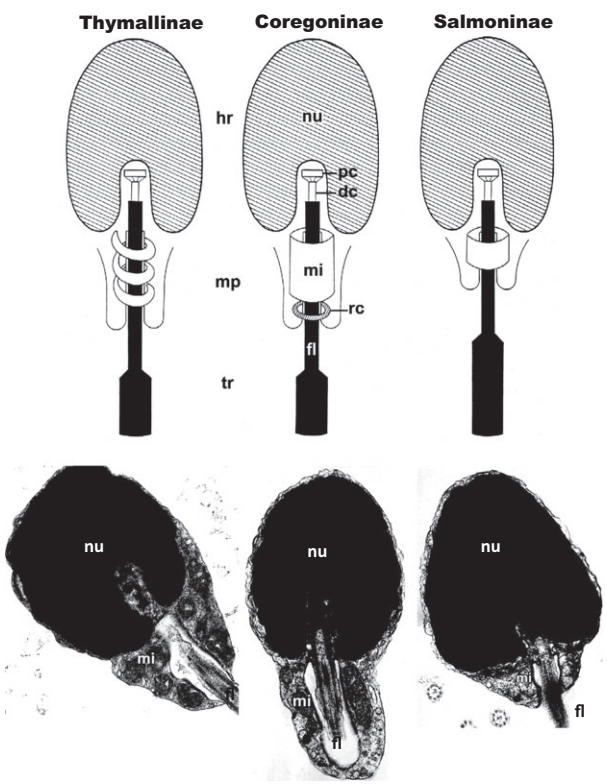

Figure 1 Schematic reconstruction and scanning electron microscopy of types of spermatozoa in salmonid fishes. dc: distal centriole; fl: flagellum; hr: head region; mi: mitochondrium; mp: midpiece; nu: nucleus; pc: proximal centriole; rc: ring of cytoskeletal filaments; tr: tail region (extracted and modified, Lahnsteiner & Patzner 2008).

The mitochondria play a fundamental role in ATP production through oxidative phosphorylation (OXPHOS). Mitochondria use multiple carbon fuels to produce ATP and its metabolites, including pyruvate, which is generated from glycolysis; amino acids such as glutamine; and fatty acids. These carbon fuels feed into the tricarboxylic acid cycle in the mitochondrial matrix to generate the reducing equivalents NADH and FADH2, which deliver their electrons to the electron transport chain (Piomboni *et al.* 2012). At the same time, the mitochondria are actively involved in other processes, such as the generation of free radicals, apoptosis and calcium signalling. Furthermore, the mitochondria play a fundamental role in the mechanism for the activation of flagellar movement during sperm motility (Guthrie & Welch 2012).

Oxidative phosphorylation inside sperm mitochondria requires the coordinated functioning of the two principal components, the respiratory chain and ATP synthase, both located in the inner membrane of the mitochondrion (Brown 2008). The respiratory chain uses the free energy released during this process by the generation of an electrochemical proton (H^+) gradient across the mitochondrial inner membrane; ATP synthase uses this proton gradient to synthesize ATP, thus generating the bioenergy necessary for the activation of sperm motility (Brown 2008; Cosson 2012a; Piomboni *et al.* 2012).

Possible alterations to the structural, functional and genetic mechanisms of the mitochondria due to cryo-injury during cryopreservation, known as cryodamage, have not been studied in detail in the mitochondria of fish spermatozoa, and their relation with motility and fertilizing capacity is not yet understood. The supply of sufficient energy for flagellar movement, in the form of ATP, is fundamental for fertilization. The scope of this work is to propose an updated view of the effects of cryopreservation on mitochondrial functioning in fish spermatozoa, thus providing guidance for future research in the field of mitochondrial bioenergy in cryopreserved spermatozoa.

General effects of cryopreservation

Spermatozoa of many mammal species have been successfully cryopreserved for many years (Isachenko *et al.* 2004). In situations of impaired male fertility, sperm storage will provide the necessary time for a reasonable amount of sperm to be obtained for successful artificial insemination or *in vitro* fertilization. Nevertheless, because of the damage associated with freezing, the motility of cryopreserved sper-

matozoa after thawing is statistically reduced with respect to prefreezing motility; this factor shows wide interindividual variability (Thurston *et al.* 2002). Sperm quality may also be affected by the subsequent slow-thawing process, which induces further cell damage (Mazur *et al.* 1981). Furthermore, the addition and removal of osmotically active cryoprotective agents during freezing and thawing can induce lethal mechanical stress (Perez-Sanchez *et al.* 1994).

The integrity of mammalian sperm DNA is of prime importance for the paternal genetic contribution to normal offspring. Damaged DNA can have a significant negative impact on oocyte fertilization, embryo development rate and live-birth rate. A significant correlation between the presence of nuclear DNA alterations in mature spermatozoa and poor sperm parameters or impaired reproductive efficiency is reported in both humans and animals (Ron-el *et al.* 1991). To date, studies in mammals have shown that the cryopreservation process causes DNA damage to human (Royere *et al.* 1988, 1991; Donnelly *et al.* 2001), boar (Fraser & Strzezek 2004), ram (Peris *et al.* 2004) and mouse sperm (Yildiz *et al.* 2007).

The primary problem in the cryopreservation of mouse sperm cells is that they become very sensitive to cold and osmotic effects (Critser & Mobraten 2000). Significant loss of sperm motility and plasma membrane integrity during cryopreservation affects sperm functioning in mice (Nishizono *et al.* 2004). In human sperm on the other hand, the motility, plasma membrane integrity and mitochondrial function are not significantly affected by cooling and warming during cryopreservation (Henry *et al.* 1993). In general, there is extensive evidence to suggest that the main causes of alterations to sperm physiology after cryopreservation are related with a reduction in motility, as well as alterations to metabolic pathways and enzyme activity in the cells and seminal plasma. Cryopreservation in the fish species *Nibea albiflora* has been shown to cause a decline in the percentage of motile sperm, and the fertilizing capacity of post-thaw sperm was significantly lower than that of fresh sperm. Following cryopreservation, the activities of total ATPase, creatine kinase, succinate dehydrogenase, LDH, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activities increased in seminal plasma and decreased in spermatozoa, while glutathione reductase (GR) activity varied inversely, falling in seminal plasma and increasing in spermatozoa (Huang *et al.* 2014). This process can also affect the mitochondria, which are the main source of energy for sperm.

Characteristics of mitochondria

Mitochondria are cell organelles with a double membrane and their own mitochondrial DNA. The double membrane structure allows the inner membrane to project towards the centre of the mitochondrion, the matrix, producing contoured folds called *mitochondrial crests*, where ATP is produced. The number and morphology of the crests change constantly inside the mitochondria, reflecting cell response to energy demands (Scheffler 1999). In addition to changes in the number and morphology of the crests, the mitochondria may be subject to fission and fusion events within the cell (Bereiter-Hahn & Voth 1994). These fission and fusion events are probably proliferation mechanisms in response to the energy demands of the cell (Brown 2008).

Mitochondrial DNA is unique among cell organelles and is found in multiple copies within each mitochondrion. Multiple individual copies of the mitochondrial genome are found in DNA groups known as nucleoids, with each mitochondrion containing multiple nucleoids (Jacobs *et al.* 2000). This unique structure of the mitochondrion goes back to its origins. Mitochondria are thought to have originated from a symbiotic relationship between a host prokaryote and an alpha proteobacterium or proto-mitochondrion (Andersson *et al.* 2003; Dyall *et al.* 2004) which was able to capture oxygen (Kurland & Andersson 2000; Gabaldon & Huynen 2003) or was a facultative producer of anaerobic hydrogen (Martin *et al.* 2001). This relationship marked the first cooperative stages of eukaryotic life. The benefits of this symbiosis led to energy production through the process of OXPHOS in almost all cells. Over time, this symbiosis converted the proto-mitochondria into an inclusion in the cytoplasmic organelle system of the host. As part of this inclusion process, mitochondrial genes whose functions were duplicated in the two symbionts were transferred to the nuclear genome or lost completely (Gray *et al.* 1999; Timmis *et al.* 2004). This transference and loss of mitochondrial genes have continued to the point where there is only one noncoding zone in the sequence of the mitochondrial genome of vertebrates. This zone is responsible for initiating replication of the genome, tRNA and rRNA coding sequences, and genes responsible for OXPHOS which are unable to pass through the mitochondrial membranes. This reduction in genome size amount has been translated into the conversion of mitochondrial genomes into a model of efficiency for the superimposition of coding sequences, between the heavy and light strands of various genes, and a complete lack of introns.

The origins of this symbiosis also explain the circular nature observed for mtDNA, and the use of an independent genetic code for the production of mitochondrial polypeptides (Barrell *et al.* 1979). The mitochondrial genome of a vertebrate generally presents a single, noncoding control region (D-loop) responsible for initiating replication. Moreover, the coding regions of 13 polypeptides, 22 tRNA,

two rRNA and one light initiation chain for short-sequence replication are contained in the typical structure of circular DNA (Attardi 1985; Brown 2008).

The 13 encoded mitochondrial polypeptides present in vertebrate genomes are used exclusively for ATP production by oxidative phosphorylation. These polypeptides include seven NADH components for complex I, cytochrome b for complex III, cytochrome oxidase, subunits of complex IV and two ATPase subunits of complex V (Fig. 2) (Brown 2008).

Mitochondrial physiology

The mitochondria have a physiological requirement for an integral, impermeable inner membrane to maintain the proton concentration potentials in the intermembrane space, thus ensuring sufficient supply of substrates for various metabolic events which occur inside or outside the mitochondrion (Pagliarini & Rutter 2013).

Inside the mitochondria, the oxidative metabolism of glucides, fats and proteins is frequently divided into three stages: in the first stage, the catabolic pathways of amino acids (e.g. oxidative deamination), the beta oxidation of fatty acids, and glycolysis give rise to two-carbon molecules of acetyl-CoA; the second stage comprises the citric acid cycle, which depends on the formation of malate and acetyl-CoA; and the third stage is that of oxidative phosphorylation, in which the reductive power of the NADH and $FADH_2$ generated is used for the synthesis of ATP or metabolic bioenergy (Brown 2008).

Mitochondria are intracellular energy-producing units found in almost all cells. Thousands of these organelles can be found in a single living cell, depending on its type and function (Brown 2008); they are responsible for producing ATP, through the OXPHOS process, thus providing energy for cell activities, including the Na^+/K^+ ATPase pump, endocytosis, protein synthesis and many other cell processes. Each mitochondrion is an autonomous, energy-producing unit with an inner and an outer membrane. ATP is produced through a process of electron transfer between the five enzyme complexes included in the inner membrane of the mitochondrion (Chan 2006). The first two complexes, NADH dehydrogenase and succinate dehydrogenase (complexes I and II, respectively), receive NADH and succinate electrons independently, which they transfer to the Q coenzyme, resulting in the formation of ubiquinol (Fig. 2). The ubiquinol then transfers electrons to the bc_1 complex (complex III). The transfer of electrons to complex III leads to the formation of cytochrome *c*, which then cedes its electrons to the CCO of complex IV. Complex IV is produced as a result on conclusion of the transfer of electrons to the molecular oxygen and the formation of water (H_2O) (Fig 2). Electron transfer results from hydrogen ions (H^+) being pumped from complexes I, III and IV of the mitochondrial inner membrane to the intermembrane space, where the H^+ ions are stored. This ion transfer results in the establishment of a pH gradient and an electrical potential across the inner mitochondrial membrane. This potential difference allows the protons to flow back by virtue of the gradient by means of an enzyme, in a process known as chemiosmosis (Pagliarini & Rutter 2013). This concentration gradient is used by a fifth enzyme complex, ATP synthase (complex V), to combine adenosine diphosphate (ADP) and inorganic phosphate to form ATP (Brown 2008), through the integrated functioning of the five complexes (Fig. 2).

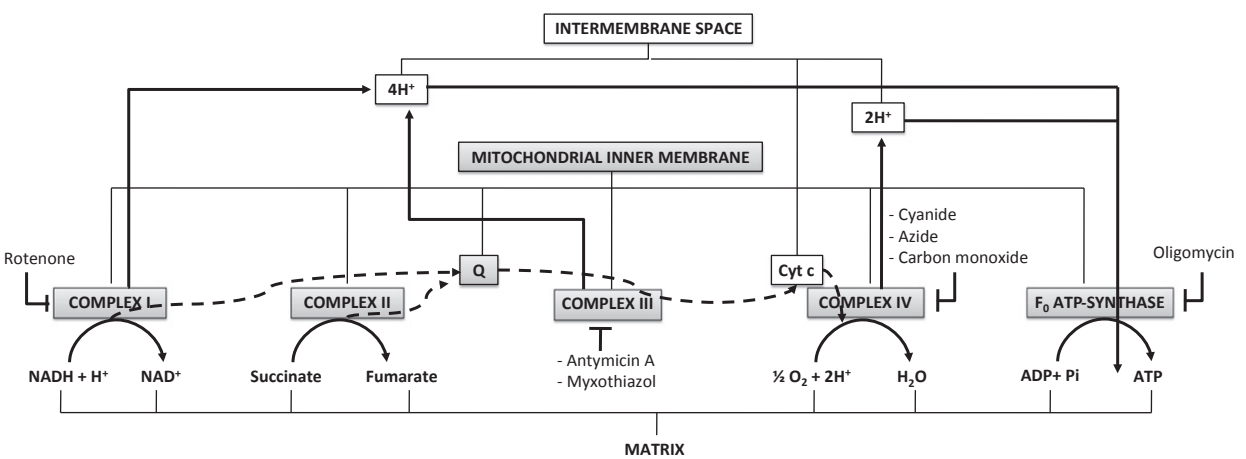

Figure 2 The oxidative phosphorylation process in the mitochondrial inner membrane. The principal protein complexes inserted in the membrane (I, II, III and IV) are shown; their function is the formation of protons (H^+) in the intermembrane space. ATP synthase produces ATP by mobilizing protons to the mitochondrial matrix by energy difference ($F_0 - F_1$). Mitochondrial respiration inhibitors cytochrome *c* and coenzyme.

Oxidative phosphorylation is an important part of metabolism, producing ROS such as superoxide ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), hypohalous acids (HOX, where X = Cl^-, Br^-, I^- or SCN^-), nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$). These molecules are highly disruptive to cellular function, and an increase in ROS production contributes significantly to several diseases, including some that may compromise reproduction and fertility. The mitochondrial antioxidant enzymes that effect these metabolic pathways, such as SOD, catalase, GPx and GR, have been studied to evaluate the possible effects in the form of alterations in cell bioenergy and respiration (Halliwell & Gutteridge 1989; Agarwal & Allamaneni 2004; Guthrie *et al.* 2012).

General characteristics of sperm motility in fish

Motility is the condition which enables the spermatozoon to reach the oocyte in order to fertilize it successfully, and has been considered to be one of the principal variables of sperm quality in fish (Rurangwa *et al.* 2004), as it is an integrated quality variable, combining various cell components responsible for the activation and sustainability of the motility and progressive movement of the spermatozoon (Bobe & Labbé 2010). Fish spermatozoa, unlike those of mammals, are immobile before ejaculation; the osmolarity and ion content of the aquatic medium are central factors in activating motility (Figueroa *et al.* 2014). The mechanisms involved in activating sperm motility are considered to be of vital importance in regulating processes such as artificial fertilization and cryopreservation. In fish with external fertilization, the activation of sperm motility is triggered by ionic changes (K^+ and Ca^{+2}) producing reduced osmolality in freshwater species and increased osmolality in saltwater species (Morisawa *et al.* 1999; Cosson 2010; Dzyuba & Cosson 2014). This, combined with variations in the concentration of intracellular ATP, leads to a possible general scheme which may explain how changes in the internal ionic concentration occurring in response to external osmolality could control fish sperm motility (Fig. 3). These changes occur when the spermatozoa come into contact with water after their release during the reproduction process (Linhart *et al.* 2002; Cosson 2012b). Furthermore, various glycoproteins have been

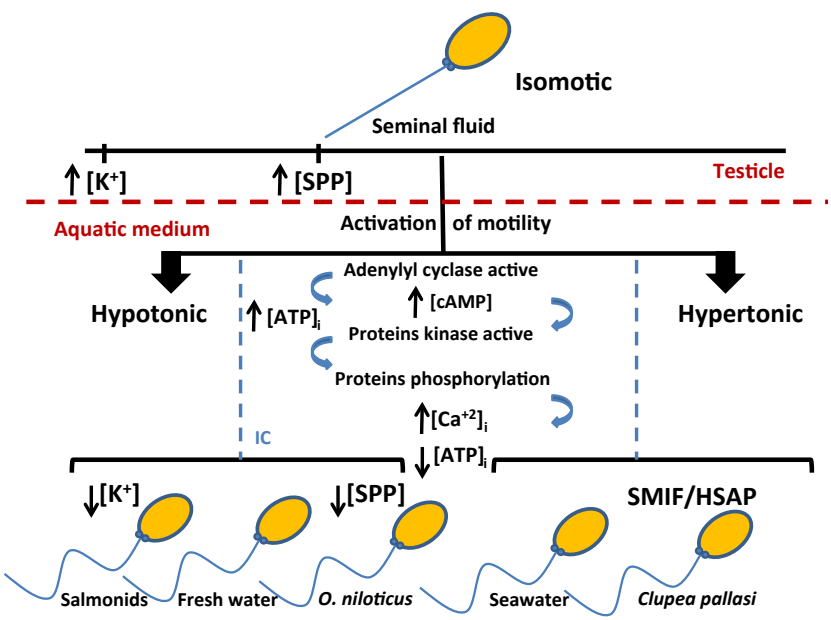

Figure 3 General model of sperm activation in salmonids and other freshwater and marine fish species. The spermatozoa are immobile in the seminal fluid (testicle), an isosmotic medium, and presence of specific proteins (SPP: seminal plasma protein fraction that motility inhibited). Motility activation occurs (aquatic medium) at a low potassium concentration in salmonids, and in other species in response to a reduction (freshwater species) or increase (marine species) in the osmolality of the medium, and presence of specific proteins secreted by the oocyte (SMIF: sperm motility initiation factor/HSAP: herring sperm activating protein). Overall changes intracellular (IC) occurring in fish sperm activation are by the modification of the osmolality of the external medium that lead to the activation cascade of molecular events that increase the concentration of cAMP and intracellular Ca^{+2}. The rise of internal ionic concentration reaches values where dynein-ATPase activity is optimal, and consequently, motility is at high velocity. Later, the ATP content becomes lower because the renewal by mitochondrial phosphorylation is too slow; this combines with a further decrease in internal ionic concentration to values where the dynein activity stops, and therefore, flagellar waves are fully arrested several minutes later (extracted and modified, Cosson 2004; and Figueroa *et al.* 2014).

reported to be motility activators. These proteins are synthesized in the follicular cells and transferred to the outer layer of the mature oocyte; during reproduction, they reach sperm receptors in the plasma membrane, inducing intracellular signalling pathways and loss of membrane integrity (Fig. 3) (Oda *et al.* 1998).

Motility is one of the parameters most frequently used to assess semen quality after cryopreservation and generally presents a positive correlation with fertilizing capacity.

Effects of cryopreservation on mitochondrial functioning

Two types of damage may be caused to the mitochondrial membrane potential during cryopreservation, which may affect the spermatozoa motility: direct damage to the DNA or alterations to the inner or outer membrane; and indirect damage provoked by the fragmentation of nuclear DNA, on which the mitochondrion depends for certain proteins which are not coded by its genome (Kurland & Andersson 2000). During cryopreservation of the spermatozoa of some fish, for example *Polyodon spathula*, damage occurred to nuclear DNA, leading to a marked reduction in sperm motility post-thawing, apparently caused by the unsuitable osmolality of the diluent and the concentration of the cryoprotector (Li *et al.* 2008; Dzyuba *et al.* 2013).

In work by Irvine *et al.* (2000), it was observed that semen with a high level of DNA damage presented low motility and velocity. It is possible that the DNA affected in these cases may be nuclear DNA, which is related to mitochondrial function. In most animal cells, only the mitochondria contain coding DNA for the aerobic cell respiration proteins which trigger ATP production. Although these relations between DNA, mitochondrial function and sperm motility have not been fully explained, some authors assert that, apart from the fragmentation damage caused to nuclear DNA, the diminution in sperm motility must be associated also with damage to the structure and metabolic functioning of the mitochondrion (Perchec *et al.* 1995; Zilli *et al.* 2014) (Table 1). Figueroa *et al.* (2013) show experimentally that a direct correlation exists between the mitochondrial potential and the motility and fertilizing capacity of the cryopreserved spermatozoa, confirming that this organelle is the energy pivot of the spermatozoon and that the cessation or prolongation of motility and successful fertilization depend on the availability of ATP in the spermatozoa (Fraser & Strzezek 2007; Bondarenko *et al.* 2013).

Damage to the genome would hinder the replication and transcription of mitochondrial or nuclear target genes and would consequently impair efficient synthesis of key proteins for energy production. This in turn would lead to a diminution of sperm motility; however, in some cases this damage might be repaired by DNA repair systems in the oocyte. Damage to the proteome translates into interruption of the phosphorylation cascade and enzyme activation, affecting the flagellar movement and fertilizing capacity of the spermatozoon.

Interventions with antioxidants

Spermatozoa are protected by various antioxidants and antioxidant enzymes, found in the seminal plasma or in the spermatozoa itself, which prevent oxidative damage (Kim & Parthasarathy 1998; Lahnsteiner & Mansour 2010; Li *et al.* 2010; Shaliutina *et al.* 2013; Dzyuba *et al.* 2014). An antioxidant that reduces oxidative stress and improves sperm motility could be useful in cryopreservation protocols (Lahnsteiner *et al.* 2011; Gazo *et al.* 2014). Antioxidants are the agents which break the oxidative chain reaction, thereby reducing oxidative stress (Martínez-Páramo *et al.* 2013; Shaliutina-Kolešová *et al.* 2014). Recent studies demonstrate that supplementation of cryopreservation extenders with antioxidants provides a cryoprotective effect on bull, ram, goat, boar, canine, human and fish sperm quality, thus improving semen parameters such as sperm motility, membrane integrity and $\Delta\Psi_m$ after thawing (Bucak *et al.* 2010; Cabrita *et al.* 2011; Martínez-Páramo *et al.* 2012; Figueroa *et al.* 2015; Viveiros *et al.* 2015).

With the rapid development of sperm cryopreservation, some researches have been conducted on antioxidant supplementation *in vitro* to improve techniques for sperm storage and cryopreservation (Cabrita *et al.* 2011; Hagedorn *et al.* 2012; Gadea *et al.* 2013). However, the effect of each antioxidant was species specific (Cabrita *et al.* 2011) and depended on the type of molecule and concentration used for each species. In the case of ROS-derived damage, the cascade of lipid peroxidation (LPO) in the sperm membrane has been shown to correlate with decreased sperm motility and membrane damage (Storey 1997; Liu *et al.* 2015). Lipid peroxidation probably promoted sperm membrane alterations, which could be avoided by the use of an antioxidant (Peris *et al.* 2007). A great variety of antioxidant substances, including vitamins, enzymes, trehalose, taurine and other free radical scavengers, have been used in sperm cryopreservation (Pena *et al.* 2003; Hagedorn *et al.* 2012; Gadea *et al.* 2013). Vitamin E (antioxidant) may directly quench free radicals such as peroxyl and alkoxyl ($ROO^{\bullet}$) generated during ferrous ascorbate-induced LPO and is therefore suggested as a major chain-breaking antioxidant (Bansal & Bilaspuri 2009; Martínez-Páramo *et al.* 2012). In the case of attenuation of mitochondrial ROS sources, the use of flavonoids and derived compounds such as lycopene and resveratrol has shown to be efficient

in reducing DNA damage, maintaining mitochondrial activity. However, the bioavailability of these compounds is questionable in other models of oxidative damage (Bucak *et al.* 2015). Moreover, the mechanism of the protective effect of antioxidants in fish sperm is not yet fully understood. Further studies are required to obtain more information on the mechanism of these antioxidant scavenging ROS and how the normal structure and function of the sperm are maintained.

Supplementation with these antioxidants prior to cryopreservation may be recommended to as a way of enhancing sperm cryopreservation for the fish breeding industry (Cabrita *et al.* 2011).

Perspectives

This review will allow to support the mechanistic bases for carrying out studies oriented towards gaining an understanding of the effects of cryopreservation on the mitochondrial physiology of fish spermatozoa. Currently, there are few studies that investigate possible alterations in the mitochondria of fish spermatozoa as a result of cryodamage. Further work will enable researchers to incorporate biotechnology oriented towards cell and molecular-level studies of the possible effects of cryopreservation on mitochondrial DNA, enzymatic or metabolic modifications of the citric acid cycle, and the oxidative phosphorylation process in the inner membrane, as well as studies of the ultrastructure of the mitochondrion. All this knowledge is necessary in order to optimize the design of pharmacological targets that may help to mitigate this type of damage. Development and progress in this area of research will contribute to scientific knowledge and provide standardized tools to ensure successful use of cryopreserved fish spermatozoa in the salmon-farming industry and aquaculture in general.

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