Original Article

Use of melatonin as an inhibitor of apoptotic process for cryopreservation of zebrafish (*Danio rerio*) embryos

Uso da melatonina como inibidor do processo apoptotico para a criopreservação de embriões de zebrafish (*Danio rerio*)

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Abstract

This study investigated the use of melatonin to arrest the effects of apoptosis in vitrified zebrafish (*D. rerio*) embryos. Dechorionated embryos at 22-24 somite-stage were divided (n = 60/treatment) into a non-vitrified (Control Group, 0 M melatonin) and vitrified treatments with 0 M (T1), 1 μM (T2) and 1 mM of melatonin (T3). For vitrified treatments, a solution methanol/propylene glycol based was used and the embryos stored in -196 °C for a week. After thaw, survival rate, scanning electron microscopy, expression of anti (*bcl-2*) and pro-apoptotic (*bax/caspase-3*) genes, reactive oxygen species (ROS) formation and DNA fragmentation analyses were performed. No live embryos were obtained from vitrified treatments, observing a rapid degeneration immediately after thawing, with the vitelline layer rupture and leakage of its content, followed by breakdown of epithelial cells and melanisation of the tissue. Regarding the apoptotic process, T3 had the highest relative gene expression, for the three genes (P < 0.05) furthermore, T2 had similar expression of pro-apoptotic genes to CG (P < 0.05). ROS formation revealed that CG presented lower percentage of embryo surface area affected (3.80 ± 0.40%) (P < 0.05), in contrast, no differences were found among the other groups. T1 was most significantly (P < 0.05) damaged by DNA fragmentation. The vitrified groups with melatonin had similar damage levels of CG (P > 0.05). The inclusion of 1 μM of melatonin in the vitrifying solution, countered the effects of apoptotic process in post-thaw embryos, suggesting its utility in cryopreserving fish embryos.

Keywords: cryoinjuries, DNA fragmentation, gene expression, reactive oxygen species, vitrification.

Resumo

Este estudo investigou o uso da melatonina para conter os efeitos da apoptose em embriões vitrificados de zebrafish (*D. rerio*). Embriões descorionados no estágio de 22-24 somitos foram divididos (n = 60 / tratamento) em tratamento não vitrificado (Grupo Controle, melatonina 0 M) e tratamentos vitrificados com 0 M (T1), 1 μM (T2) e 1 mM de melatonina (T3). Para os tratamentos vitrificados, utilizou-se uma solução à base de metanol/ propilenoglicol e os embriões foram armazenados em -196 °C por uma semana. Após o descongelamento, foram realizadas análises de taxa de sobrevivência, microscopia eletrônica de varredura, expressão dos genes anti (*bcl-2*) e pró-apoptóticos (*bax/caspase-3*), formação de espécies reativas de oxigênio (EROS) e análises de fragmentação de DNA. Não foram obtidos embriões vivos a partir dos tratamentos vitrificados, observando uma rápida degeneração imediatamente após o descongelamento, com ruptura da camada vitelina e vazamento de seu conteúdo, seguida de quebra das células epiteliais e melanização do tecido. Em relação ao processo apoptótico. T3 apresentou expressão gênica relativa alta para os três genes (P <0,05), além disso, T2 apresentou expressão semelhante as dos genes pró-apoptóticos de GC (P <0,05). A formação de EROS revelou que GC apresentou menor percentual de área de superfície embrionária afetada (3,80 ± 0,40%) (P <0,05), ao contrário, não foram encontradas diferenças entre os outros grupos. T1 foi mais significativamente (P <0,05) danificado pela fragmentação do DNA. Os grupos vitrificados com melatonina apresentaram níveis de dano semelhantes ao do GC (P> 0,05). A inclusão de 1 μM de melatonina na solução de vitrificação, contrariou os efeitos do processo apoptótico em embriões pós-descongelamento, sugerindo sua utilidade na criopreservação de embriões de peixes.

Palavras-chave: crio injúrias, fragmentação do DNA, expressão gênica, espécies reativas de oxigênio, vitrificação.

1. Introduction

Development of effective techniques for cryopreservation of fish eggs and embryos could provide a valuable tool for the propagation of economically important and endangered species alike. The access to cryopreserved embryos in periods when natural spawning does not occur, could allow an increase in commercial production and conservation of rheophilic (Bozkurt, 2018; Machado et al., 2019) or any species of fish.

Cryopreservation of fish embryos has been attempted in many species, with a particular focus on the zebrafish (*Danio rerio*) one of the most important animal models in genetic and reproductive studies for vertebrates and for the development of technologies in fish farming, its wide use being justified by its small size, easy maintenance, prolificity, rapid growth, transparent embryo characteristics and complete genome sequencing (Amsterdam and Hopkins, 2006; Silveira et al., 2012). However, cryoinjuries are commonly encountered with only a few studies reporting survival of cryopreserved embryos. Zebrafish embryos at 50% epiboly stage cooled to 0 °C and stored for 6 and 18h suffered numerous macro- and microscopic malformations, observing that only a few morphologically normal individuals were able to develop to the adult stage (Paes and Nakaghi, 2018). Studying the vitrification of *D. rerio* embryos, Connolly et al. (2017) found that embryos dehydrated during or after segmentation (20-22 somites) survived for 3 hours post-thaw, exhibiting developmental delay, edema and trunk necrosis by 2-4 days post-treatment. Tian et al. (2015) observed continuity in the development of only 2.96% *Epinephelus septemfasciatus* embryos, post vitrification. More recently, survival rates of 5.15-9.7% have been obtained for cryopreserved embryos of *Epinephelus moara* (Tian et al., 2017, 2018). Thus far, the most promising results have been obtained for the Persian sturgeon (*Acipenser persicus*) embryos vitrificated using DMSO-based solution at segmentation stage (20-22 somites), with hatch rates of about 45.45% (Keivanloo and Sudagar, 2016).

Unfortunately, the fish eggs and embryos present greater challenge for cryopreservation compared to that of their sperm. Primarily, the large fat-rich vitelline sac is osmotically distinct to the rest of the tissue (Lahnsteiner, 2008). Further, their large size, multiple semipermeable membranes and complex compartmentalization hinder the exchange of cryoprotectants and water (Bozkurt, 2017). This limits uniform cryoprotection and rehydration during freezing and thawing respectively, leading to heightened 'freeze-thaw' sensitivity and susceptibility to intracellular ice formation causing membrane rupture easily (Fornari et al., 2012). Also, the metabolic pathways to counter toxic effects and osmotic shock of cryoprotective agents are inadequate and reduction of intracellular ATP stocks during storage cause irreversible damage to mitochondria and other vital energy components of the cells (Herrárez, 2009).

It is therefore necessary to make technical advances on multiple aspects of cryopreserving fish embryos, including cryogenic techniques, choice of cryoprotectants and use of most appropriate embryonic developmental stage (Isayeva et al., 2004). Nonetheless, vitrification at large is widely employed for cryopreservation of human embryos that are relatively small and void of yolk, providing template for cryopreserving embryos of other mammals (Caetano et al., 2010). Despite this progress, it is common to encounter abnormal increase in reactive oxygen species (ROS) in the intracellular environment during vitrification. This causes oxidative DNA damage due to disruption of mitochondria and release of cytochrome C to cytoplasm (Simon et al., 2000; Favetta et al., 2007), reducing viability of the cryopreserved embryos. At a molecular level, abnormal elevation of intracellular ROS levels leads to increase in relative expression of *bax* and *caspase-3* (i.e. pro-apoptotic genes) with concurrent decrease of *bcl-2* (an anti-apoptotic gene) in vitrified mouse (Jang et al., 2014) and bovine (Chen et al., 2014) oocytes.

One way to prevent the excessive formation of ROS is to incorporate antioxidants in the cryoprotectants such as the melatonin (N-acetyl-5-hydroxytryptamine), a hormone found in all vertebrate animals that modulate the circadian rhythm and reproduction (Stehle et al., 2011; Hwang et al., 2013). Melatonin and its metabolites are potent free radical scavengers and antioxidants (Chen et al., 2006; Manchester et al., 2015). Thus far, researchers have successfully used melatonin to improve the cryopreservation success of bovine (Zhao et al., 2016), pigs (Shi et al., 2009), sheep (Succu et al., 2011) and humans (Espino et al., 2010; Ortiz et al., 2011) embryos. However, studies on the efficiency of this antioxidant on cryopreservation of fish embryos have not been tested.

As a step towards advancing cryopreservation of fish embryos, this study investigated the use of melatonin to arrest the effects of ROS formation and apoptosis during cryo-storage of vitrified zebrafish embryos.

2. Materials and Methods

All experiments were approved by the University of Tasmania Animal Ethics Committee (No. A0016597).

2.1. Breeding and procurement of embryos

Fifty adult zebrafish, 25 males and 25 females, were purchased from commercial suppliers and maintained in an aquarium recirculation system. Constant water temperature (24 to 26 °C) and photoperiod (14 hours of light per day) were maintained throughout the experiments. Fish received flocculated commercial feed (API Tropical Flakes, Nebraska, Kansas, USA) three times daily and was supplemented with live feed (Artemia) once a day.

To obtain embryos, the night before spawning, males and females were selected based on secondary sexual characteristics and placed (1:1 sex ratio) in breeding aquariums with mesh at the bottom. After spawning, the breeders were removed and embryos incubated till 22-24 somite-stage (approximately 22 hours after fertilization) and used for the treatments (n = 60/treatment, Total 240). The experimental groups were: Non-vitrified (Control Group, 0 M melatonin - Sigma-Aldrich, Saint Louis, MO, USA) and vitrified with 0 M (T1), 1 µM (T2) and 1 mM of melatonin (T3).

2.2. Dechorionation of embryos

To improve permeability of the cryoprotectants and replacement of water from the embryos, the chorion was removed (Sorrells et al., 2013) about an hour before they reached 22-24 somites stage. Briefly, the embryos were placed in a petri dish containing 10 mL of pronase E 10 mg/mL solution (Sigma-Aldrich, St. Louis, Missouri, USA) in phosphate buffered saline (PBS), followed by incubation at 27 °C for 30 minutes in a conventional incubator. The chorion was removed by rinsing with PBS followed by gentle pipetting and the dechorionated embryos were incubated in fresh solution of PBS, until use.

2.3. Inclusion of melatonin and embryo cryopreservation

The vitrifying solution (Table 1) was prepared based on those described previously for Persian sturgeon (*Acipenser persicus*) (Keivanloo and Sudagar, 2013) and zebrafish (*Danio rerio*) (Guan et al., 2010) embryos, with minor modifications following toxicity tests of the components. The chosen melatonin doses (1 µM and 1 mM) were based on a bovine study (Zhao et al., 2016).

For the vitrification process in a small petri dish, dechorionized embryos were exposed to an increasing concentration gradient of the vitrifying solution, one minute at each concentration (0%, 25%, 50%, 75% and 100% L-15 culture medium), totaling five minutes of exposure. Subsequently, they were immediately placed in 0.5 ml cryopreservation straws and immersed in liquid nitrogen in a Styrofoam box. After 20 seconds, the samples were transferred to liquid nitrogen storage (-196 °C), where they were held one week (Caetano et al., 2010).

Thawing was performed by immersion in a water bath at 26 °C for 8 seconds, and gradual rehydration was carried out in decreasing concentrations of the vitrifying solution (100%, 75%, 50%, 25% and 0%) that contained 0.1 M sucrose to reduce osmotic shock. During thawing, images were recorded in order to evaluate the sequence of cryoinjuries. Control group embryos were stored or fixed as required for analysis at the time of cryopreservation. After thawing, the embryos were rinsed twice with PBS, and incubated at 26 °C to observe their development and possible survival rates. The embryo survival rate was obtained by dividing the number of live embryos (which were not deformed or white) by the total number of evaluated embryos.

Table 1. Vitrifying solution used for cryopreservation of zebrafish (*D. rerio*) embryos.

Component	Concentration
Propylene glycol	3 M
Methanol	1.5 M
Penicillin	0.09 mM
Dihydrostreptomycin	0.06 M
Melatonin	0 M, 1 µM or 1 mM
Egg yolk	15 mL
Culture medium (Leibowitz L-15)	q.s. 100 mL

2.4. Scanning microscopy

Six embryos per treatment were fixed in 2.5% glutaraldehyde solution with addition of 0.1 M cacodylate buffer at pH 7.2, remaining refrigerated until dehydration (using 30, 50, 70, 80 90, 95 and 100% ethanol baths, serially for 30 minutes each). For drying a BAL-TAC CPD 030 critical point liquid CO_2 dryer was used. Subsequently, the embryos were transferred to an aluminum base (stubs), then metallized with palladium gold ions in the Desk II Denton Vacuum Metallizer. The samples were then examined and electron micro graphed on a JEOL Scanning Electron Microscope (JSM-5410) to evaluate the damage caused by vitrification (Rawson et al., 2001).

2.5. Gene expression

Immediately after thawing, four embryos per treatment were placed into 1.5 mL tubes containing 1 mL RNA later (Sigma-Aldrich, St. Louis, Missouri, USA) and stored at 4 °C. For RNA extraction, individual embryos were placed in new 1.5 mL tubes and traces of RNA later removed. Total RNA extraction was performed using RNA minikit (BioLine, London, UK) according to manufacturer instructions. After extraction, the total RNA samples were aliquoted (2µL) for quantitation and the remainder stored in an ultra-freezer at -80 °C.

RNA integrity was evaluated on 1% agarose gel stained by SYBR Safe DNA Stain (Invitrogen, Carlsbad CA, USA) and concentration obtained using Qubit® Fluorometer (Invitrogen, Carlsbad CA, USA). The cDNA was then synthesized using the Super Script III Kit (Invitrogen, Carlsbad CA, USA) and stored at -20 °C until real-time PCR (qRT-PCR) quantification. The qRT-PCR primers for *bax, bcl-2*, *caspase-3* and β-actin (reference gene) were designed based on publicly available sequence data (Table 2).

For qRT-PCR reactions, the Itaq Universal Syber® Green Supermix fluorescent dye (BioRad, California, USA) was used as recommended by the manufacturer. All analyzes were performed in triplicate with a final reaction volume of 10 µL using the BioRad CFX (BioRad, California, USA). Optimal primer and cDNA concentrations were determined by efficiency tests using three primer concentrations (100, 200 and 400 nM) and four cDNA concentrations (10, 100, 200 and 400 ng).

2.6. Quantification of oxygen reactive species (ROS)

Intracellular reactive oxygen levels were quantified using the 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) molecular probes (Eugene, OR, USA) according to manufacturer instructions. Immediately after vitrification, ten embryos per treatment were washed twice in PBS, incubated for 60 minutes in 10 mM of H_2DCFDA solution at room temperature, washed again in PBS and photographed using a Leica DM750 phase contrast microscope equipped with a Leica DFC420 camera. The wavelengths for excitation and emission were 492-495 nm and 517-527 nm, respectively. The percentage of surface area of the fluorescence-emitting embryo (affected by the formation of ROS) was measured using the software Image J (Schneider et al., 2012).

Table 2. Forward (F) and reverse (R) primer sequences of β-actin, caspase-3, *bcl-2* and *bax* and their annealing temperature used in qRT-PCR assays.

Gene	Sequence (5´ – 3´)	Temperature (°C)	Genbank ID
β-actin	F: AAA GCC TTT GCT GGA GAT	63	AF025305.1
	R: CCC ACA TAG GAG TCT TTC TGT CC		
caspase-3	F: ATG AAC GGA GAC TGT GTG GA	63	AB047003.1
	R: GTA TCT GAA GGC ATG GGA TTG A		
bcl-2	F: GGA TGA CTG ACT ACC TGA ACG G	63	570772
	R: GTA TGA AAA CGG GTG GAA CAC A		
bax	F: TGC CTT TTA TTA GAA AGA CCT GCA T	63	NM001328066.1
	R: TCC AGC AAG GAA AAC TCC AAC T		

2.7. DNA fragmentation (TUNEL)

DNA fragmentation was assessed by the TUNEL assay using the In-Situ Fluorescence Cell Death Detection Kit (Roche, Grenzach-Wyhlen, Germany) according to manufacturer instructions. After thawing, ten embryos per treatment underwent two washes in PBS solution, fixed in 4% paraformaldehyde solution, treated with 0.1% Triton X-100 for 40 min and exposed to overnight blocking solution at 4 °C. Subsequently, the embryos were incubated in TUNEL solution at 37 °C for one hour in the dark, washed in PBS and photographed using a Leica DM750 phase contrast microscope equipped with a Leica DFC420 camera. The wavelengths for excitation and emission were 450-500 nm and 515-565 nm, respectively. Similar to ROS analysis, the percentage of surface area of the embryo emitting fluorescence (i.e., showing DNA fragmentation) was measured using Image J software. For the positive control, a sample was treated with DNAse I (50 IU/mL; Promega, Southampton, UK) for 15 minutes at 37 °C before incubation.

2.8. Statistical analysis

Data were expressed as mean ± standard error (SE). The data were tested by Shapiro-Wilk for normality, if data did not present the normal distribution, the transformation square was applied. For significant differences, ANOVA was used, followed by Duncan test. The level of significance for all statistical tests was set to 5% (p < 0.05). For gene expression, the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used. Statistical analyses were conducted with the R software version v. 3.5.1 (R Core Team, 2019).

3. Results

3.1. Survival rate and cryoinjuries

While the non-vitrified treatment had an embryonic survival rate of 92.30 ± 4.90%, no live embryos were obtained after thawing the vitrified embryos. In general, the embryos of the vitrified groups retained their morphological structures intact during liquid nitrogen storage (-196 °C), however, immediately after thawing a rapid degeneration was observed, with the rupture of the vitelline syncytial layer (VSL) and leakage of its content, followed by breakdown of epithelial cells and melanisation of the tissue.

Scanning electron microscopy analysis verified the major cryoinjuries caused by the vitrification process. As observed, the most affected region was the VSL, which had irregular shapes with signs of rupture in all vitrified embryos. In addition, lesions were also observed in the epidermal layer of the head and tail in all vitrified embryos, such as invagination, wrinkling and perforation (Figure 1).

3.2. Expression of apoptotic genes

Amplification efficiency was similar for the genes of interest, ranging from 90 to 110%. The analysis of dissociation curves did not show nonspecific products or primer dimer formation. The level of β-actin was not different (P > 0.05) between treatments, suggesting the assays were robust.

Differences in relative gene expression were observed between treatments. The relative gene expression levels of the *bcl-2, bax* and *caspase-3* genes are shown in Figure 2. The group of cryopreserved embryos that received the highest melatonin inclusion (1 mM) had the highest relative gene expression (P < 0.05) for all the three genes studied (P < 0.05). Embryos with 0 M and 1 μM melatonin obtained similar relative gene expression for *bcl-2*, *bax* and *caspase-3* (P > 0.05). Furthermore, 1 μM equaled (P < 0.05) its relative gene expression levels for pro-apoptotic genes (*bax/caspase-3*) with those of non-cryopreserved embryos (Control group).

3.3. Reactive oxygen species and DNA fragmentation

Analysis of ROS formation revealed that the control group presented a lower percentage of surface area of the embryo affected (3.80 ± 0.40%) (P < 0.05). In contrast, among the groups of vitrified embryos, 0 M (47.35 ± 8.80%), 1 μM (54.22 ± 12.47%) and 1 mM (65.98 ± 7.90%), no differences (P > 0.05) were found (Figure 3).

Based on DNA fragmentation analysis (TUNEL assay), vitrified embryos without melatonin (0 M) were the most significantly (P < 0.05) damaged with 79.74 ± 1.00% of their area affected (Figure 3). The groups of vitrified embryos with melatonin addition, 1 μM (45.41 ± 10.20%) or 1 mM (53.93

± 18.50%) had similar damage levels to the group of non-vitrified embryos (Control group, 47.47 ± 10.82%) (P > 0.05) (Figure 3). Both ROS and TUNEL assays encountered auto fluorescence particularly associated with the yolk sac. The areas of embryos affected by DNA fragmentation were more intense, with greater fluorescence than ROS signals (Figure 4).

4. Discussion

4.1. Cryoinjuries

The full causes and effects of cryoinjuries during vitrification are still not completely known, but it is a consensus that the success of cryopreservation is closely linked to the sensitivity of each cell type to cold shock and the toxicity of cryoprotectants. Thermal stress and the formation of ice crystals in the intracellular environment induce apoptosis that block the muscle development and promote membrane rupture causing tail deformities following thaw in zebrafish embryos (Li et al., 2018). Solutions required for vitrification have high concentration of cryoprotectants and usually include high molecular weight cryoprotective agents that may be toxic for embryos of gilthead seabream (*Sparus aurata*) (Cabrita et al., 2006).

The observed maintenance of intact morphological structure during storage, with onset of apoptotic process only post thaw ratified the observations that vitrification is a promising option for cryopreservation of this type of
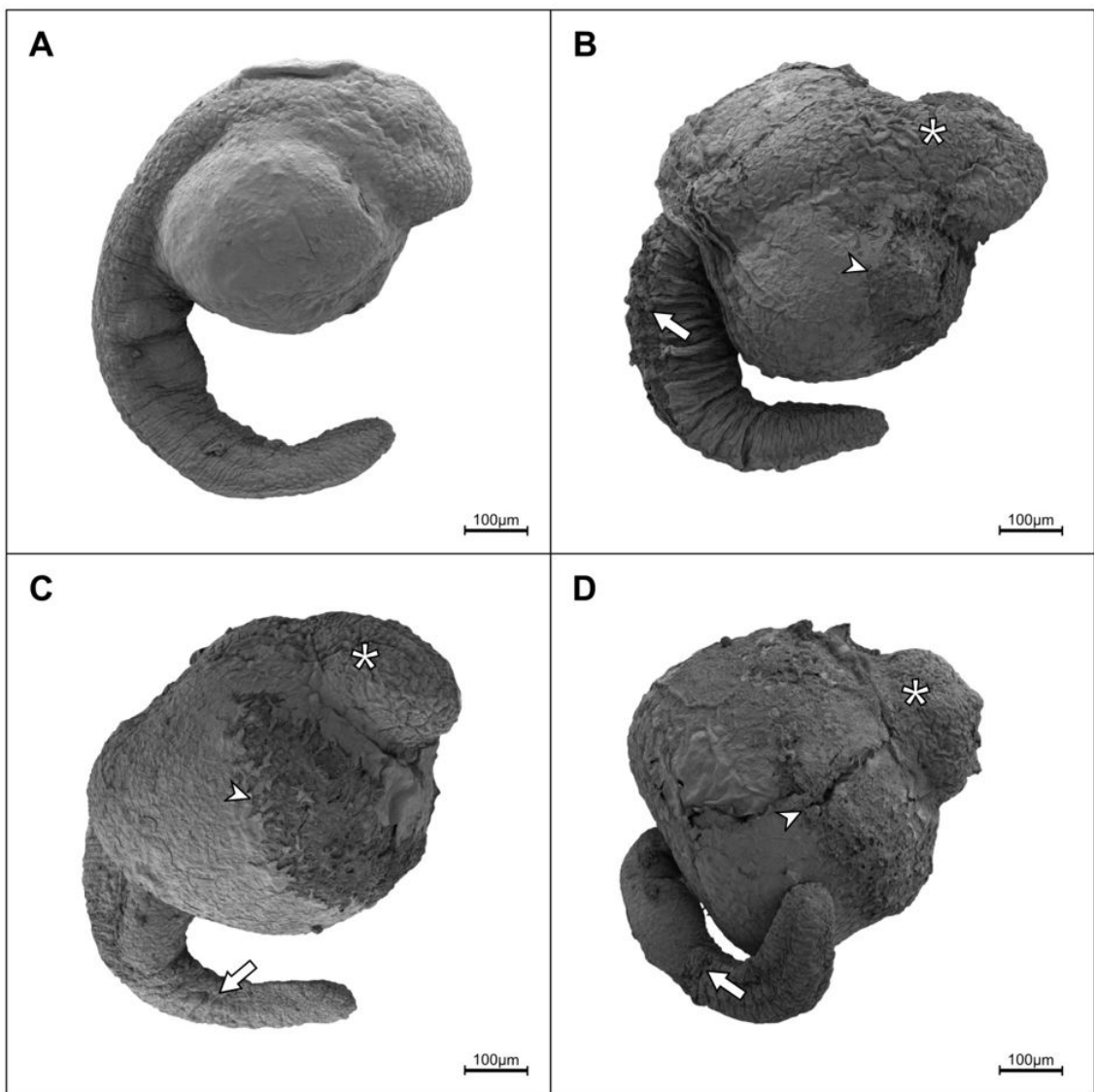

Figure 1. Representative scanning electron micrographs of vitrified and non-vitrified zebrafish (*Danio rerio*) embryos. (A) Control group; (B-D) Vitrified with 0, 1 μM and 1 mM melatonin respectively. Arrows, arrow head and asterisk point to invaginations and perforations, rupture of the vitelline membrane and wrinkling of the epidermis respectively.

material and may be the path to developing an effective protocol (Keivanloo and Sudagar, 2013, 2016).

Regardless, the cell membranes suffer the first and most severe damage during the freeze-thaw process

Figure 2. *Bax, bcl-2* and *caspase-3* mRNA expression levels in zebrafish (*Danio rerio*) embryos for the control group and vitrified treatments with 0, 1 µM and 1 mM melatonin respectively obtained by the $2^{-\Delta\Delta CT}$ method. Boxes with same letter are not significantly different from one another (P > 0.05).

Figure 3. Percentage area of zebrafish (*Danio rerio*) embryos affected by reactive oxygen species (ROS) formation and DNA fragmentation after vitrification for the control group and vitrified treatments with 0, 1 µM and 1 mM melatonin respectively. Bars with different letter within the same assay were significantly different from one another (P < 0.05).

(Herrárez, 2009). In situations where the vitrifying solution is not efficient, the cell plasma membrane may become disorganized during liquid-solid transition, losing its typical fluid state, which affects the permeability and composition of its membrane proteins. Among them, those that form permeable channels and cellular connections, cause damage to the cytoskeleton resulting in extravasation of intracellular material and the rupture of fragile tissues such as the vitelline syncytial layer (VSL) (Harvey, 1983).

The cryoinjuries encountered in this study indicated that VSL was considerably affected, with irregular shapes and thicknesses, causing them to break easily post thaw. Similar cryoinjuries, leaking of yolk and presence of damaged blastoderm, have been reported for *Piaractus mesopotamicus* embryos, making their post-thaw survival unviable (Fornari et al., 2011; Neves et al., 2012). Ultra-structural changes such as the wrinkling and leaking of the yolk, culminating in the complete mortality have also been reported for cryopreserved *Prochilodus lineatus* embryos (Ninhaus-Silveira et al., 2009).

Vitelline syncytial layer has been considered one of the main obstacles to the successful cryopreservation of fish embryos, having unique characteristics (i.e. presence of microvilli, large cytoplasmic mass, high concentration of mitochondria, vacuoles and ribosomes, and complex networks of endomembrane and euchromatic nuclei in the region of yolk/endoderm interphase) in relation to the rest of the embryonic tissues in addition to an intense metabolic activity i.e. the function of sustaining, separating and making the yolk available to the embryo (Ninhaus-Silveira et al., 2006). VSL is known to limit permeability of cryoprotectants in zebrafish (Hagedorn et al., 2004) an aspect possibly highlighted by the loss of VSL contents post thaw that was observed in this study. Thus, it is essential that future studies carry out prior tests to determine the efficacy of cryoprotectant permeability and protection of this specific layer.

Cryoinjuries to the outer epithelial layers of the embryos such as invaginations and wrinkles (Fornari et al., 2011) are also common due to ineffective dehydration and rehydration processes but can be more readily prevented by adjusting the concentrations of cryoprotectants. This generally manifests in darkening of the tissue assumed to be related to the thawing stress caused by the formation of intracellular ice crystals due to insufficient rates of heating or over-dehydration (Zhang et al., 2020). Embryo darkening after thawing has been observed in *Sparus aurata* (Cabrita et al., 2006) and *Pagrus Major* (Ding et al., 2007) as was also the case in this study.

4.2. Gene expression

Cryopreservation and thawing can alter the expression of genes involved in development and metabolism of *D. rerio* embryos, such as *sox2, sox3* and *sox19a*, interfering with their normal development (Riesco and Robles, 2013; Desai et al., 2011). However, studies related to the expression of the genes involved in the apoptotic process have not been reported so far. Our research has studied in an unprecedented way the relative expression of the anti-apoptotic gene *bcl-*
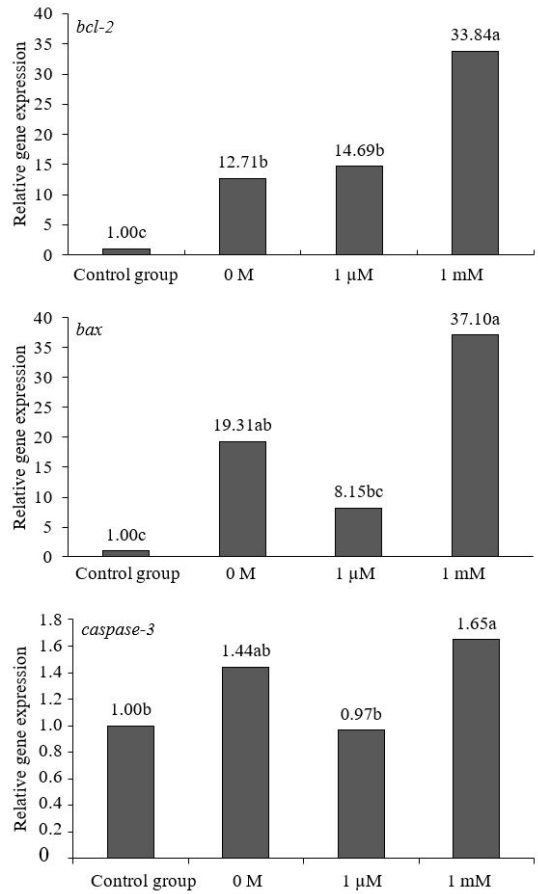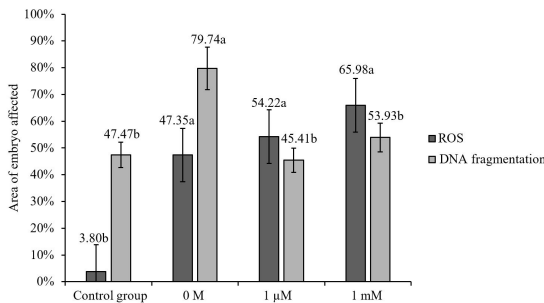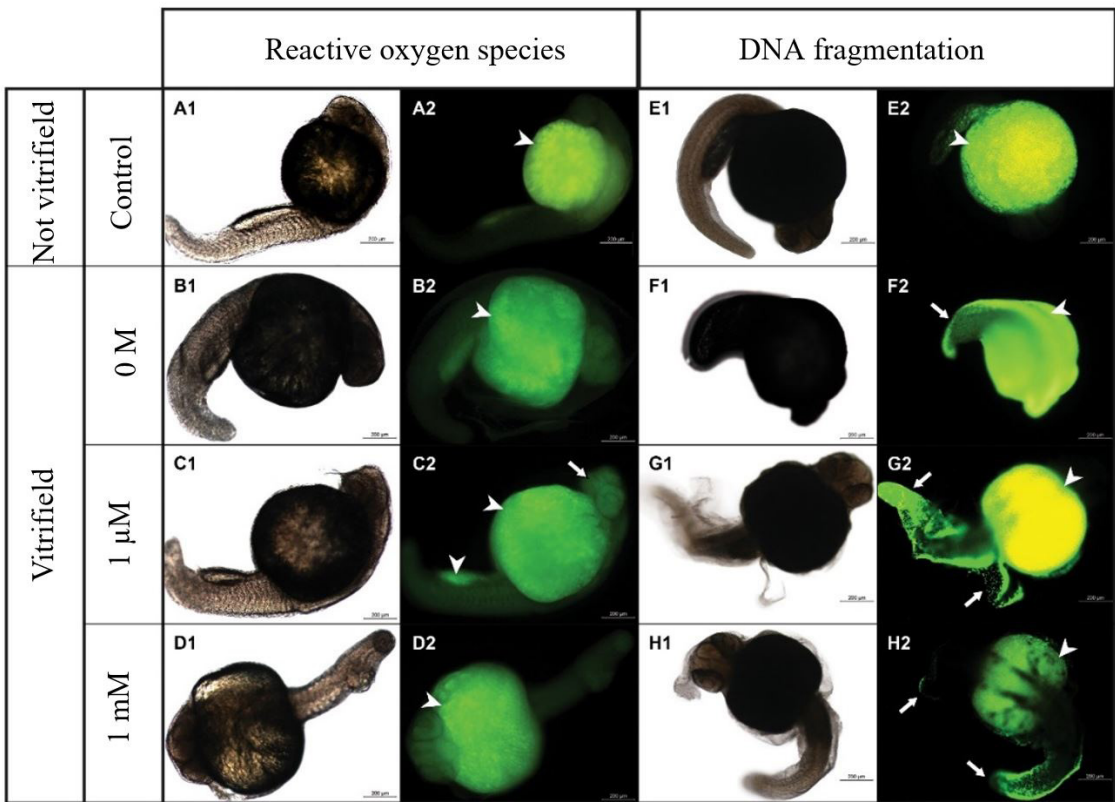
Figure 4. Representative images of zebrafish (*Danio rerio*) embryos showing reactive oxygen species (ROS) formation (A-D) and DNA fragmentation (E-H) following vitrification for the control group and vitrified treatments with 0, 1 µM and 1 mM melatonin respectively. Arrows and arrow heads point to tissues affected in the body axis and those in close proximity to the yolk respectively.

2 and the pro-apoptotic genes *bax* and *caspase-3* in cryopreserved fish embryos.

Healthy embryos have proportionally greater *bcl-2* expression in relation to *bax/caspase-3*, whereas in embryos affected by apoptotic events this proportion is decreased, with an increase in *bax/caspase-3* (Zhao et al., 2016). Sun et al. (2002), when comparing fragmented and normal mouse blastocysts, found that *bcl-2* levels tended to be lower in fragmented ones. In bovine oocytes and embryos produced *in vitro*, *bcl-2* expression was higher in good quality samples and lower in fragmented embryos, whereas *bax* expression was found in all types of oocytes and embryos, but with greater expression in fragmented samples (Yang and Rajamahendran, 2002). In addition, the inclusion of melatonin (1 mM) in the vitrifying solution of bovine embryos improved the post-thaw quality, decreased the relative expression of the *bax* and *caspase-3* and increased *bcl-2* (Zhao et al., 2016).

In this study, the three studied genes (*bcl-2*, *bax* and *caspase-3*) were expressed at high levels in the vitrified embryos. Zhu et al. (2008) cite that the increase in the expression of these genes occurs after stress induction in the embryos, suggesting that higher the expression of these three genes, higher is the apoptotic process. Among the vitrified embryos with inclusion of melatonin, those

that received the highest (1 mM) level of inclusion also had the highest levels of *bcl-2* and *bax/caspase-3* expression, suggesting a higher apoptotic activity. We speculate that the inclusion of 1 mM melatonin in the vitrifying solution may have been involved in a process of overloading that negatively affects cells. Felici et al. (1999) obtained similar results when examining the expression of *bcl-2* and *bax* proteins in primordial germ cells and fetal oocytes of mice, noting that both proteins were overloaded when they passed through the apoptotic process in culture. In contrast, the good results with the inclusion of 1 µM melatonin (pro-apoptotic genes expression similar to those of non-cryopreserved embryos) highlights the need to carryout studies using lower levels of melatonin (10^{-9} M and/or 10^{-12} M), to confirm these results.

4.3. Reactive oxygen species and DNA fragmentation

Vitrification is associated with elevated levels of reactive oxygen species (ROS) and apoptotic events in embryos (Zhao et al., 2016); however, little information is available on the effect of melatonin as inhibited from the formation of these components and its antioxidant effects. Although cryopreservation has increased the formation of ROS in relation to non-cryopreserved embryos, the levels of melatonin inclusion did not influence among the vitrified groups. Different results

from those observed by Zhao et al. (2015, 2016), who demonstrated that the inclusion of melatonin in the vitrifying solution significantly decreased the formation of ROS's in bovine embryos. This divergence may have been caused by the ineffectiveness of vitrifying solution, which did not protect the large amount of yolk from the formation of ROS, considerably increasing the fluorescence emitted by the samples, may have impaired the finding of differences between treatments.

Likewise, the vitelline yolk sac was also a problem for the analysis of DNA fragmentation by Tunel, showing natural fluorescence to the reagent since this layer does not have DNA to be fragmented, however, it was possible to find differences between treatments. The groups cryopreserved with melatonin (1 µM and 1 mM) showed a reduction in the presence of fragmented DNA in relation to the cryopreserved group without melatonin, reaching a level comparable to the non-cryopreserved embryos. Results that corroborate those found by Yabu et al. (2001), who observed that the induction of apoptosis by environmental stress (thermal shock at 39 °C for 1h and ultraviolet light irradiation) in zebrafish embryos produced more Tunel-positive cells, from the formation of extensive embryo damage, increased caspase-3 activity and levels of DNA fragmentation. An alternative to be tested in the future to the solution of problems related to vitelline reserve is its partial manual removal (50%) before vitrification, a fact that facilitates the performance of fluorescence analyzes in addition to increasing resistance to cryoinjuries (Liu et al., 2001).

Although the vitrifying solution used did not adequately protect zebrafish embryos (especially the vitelline membrane) the inclusion of 1 µM of melatonin in the vitrifying solution, was able to partly counter the apoptotic process, suggesting its utility to improve cryopreservation of fish embryos. The results therefore could contribute to future development of an adequate protocol for the vitrification of macrolecithal eggs and embryos of teleosts such as the zebrafish, that remain a challenge in cryobiology.

Unlike sperms, the cryopreservation of fish embryos has remained a challenge that require significant technical advances to overcome intracellular crystallization during freezing and the damage they cause during thawing processes (Tian et al., 2018; Isayeva et al., 2004). This is further compounded by formation of ROS and DNA damage. While the study used a promising cryopreservation medium (Guan et al., 2010; Keivanloo and Sudagar, 2013; Zhao et al., 2016), the results indicate that the cryoprotectant was not effective, as no embryos survived. However, the embryos remained intact during storage with tissue damage commencing only post thaw. The incorporation of melatonin appears to minimize apoptotic process and DNA fragmentation.

Acknowledgements

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