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REDUCED GLUTATHIONE AND ATP IN THE SEMINAL CRYOPRESERVATION OF TAMBAQUI

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Abstract

BACKGROUND: The aim of this study was to analyze the effect of reduced glutathione (GR) and adenosine triphosphate (ATP) in cryopreserved sperm tambaqui, *Colossoma macropomum*.. **OBJECTIVE:** Semen samples were frozen at different concentrations of GR (2, 4, and 6 mM) and ATP (7.5, 15, 30 mM) evaluated separately. **MATERIALS AND METHODS:** Different concentrations of GR and ATP were added to the diluent Beltsville Thawing Solution 10 % of DMSO (extender control). The thawed sperm was evaluated as the kinetic parameters for Computer Assisted Semen Analysis (CASA) and cell feature parameters by flow cytometry. **RESULTS:** All concentrations of GR and ATP had gradual reduction in the production of reactive oxygen species, mainly ATP 15; 22.5 mM and 30 mM, which decreased by 50% or more when compared to the control. All concentrations of GR and ATP did not change (P> 0.05) DNA fragmentation and lipid peroxidation. The membrane fluidity GR in concentrations 4 and 6 mM were better than control; and the concentrations of 2 and 4 mM maintained more efficiently to motility period, total and progressive motility. **CONCLUSION:** Thus, it is indicated the use of BTS extender with 10% DMSO added 4 mM GR thawed sperm Tambaqui *C. macropomum*.

Keywords: Antioxidant; conservation; sperm; fish; ROS

INTRODUCTION

The Tambaqui (Colossoma macropomum, Cuvier 1818) originates from the Amazon basin and is widely distributed in its tributaries. The species performs reproductive migration and spawning in summer which coincides with the full season (Godinho 2007). In natural environments it feeds on fruits and seeds, especially during the breeding season, which requires energy intake for maturation (Vieira et al 2011). Thus it has great ecological importance in the Amazonian ecosystems, and it helps in spreading seeds over long distances (Anderson et al 2011). However, increased human activities on deforestation of riparian vegetation (Claro Jr et al 2004), construction of dams and hydroelectric decrease the genetic diversity and impair reproduction (Isaac and Ruffino 1996).

Cryopreservation is a great tool for creating germplasm banks enabling restoration and preservation of endangered species through biodiversity conservation (Cabrita et al, 2010, Martínez-Páramo et al 2009). Furthermore, the freezing and thawing causes damage due to thermal shock (Bucak et al 2008, Wang et al 1997) and rapid restoration of the metabolism of the sperm cells by rehydration (Holt 2000). During the procedure, there is the occurrence in the formation of intra- and extracellular ice crystals generating mechanical injury to the organelles (Cabrita et al 2005) and decrease of sperm motility (Chatterjee et al 2001). Moreover, in thawed sperm there is increased production of reactive oxygen species (Gadea et al 2011) and reduction of the antioxidant system, which are lower due to dilution in freezing extenders (Cabrita et al 2011).

The increased production of reactive oxygen species (ROS) suppresses the ability of the seminal plasma defense system and leads to oxidative stress (Bansal and Bilaspuri 2011). All cellular components are subject to oxidative damage; however, the cell membranes are particularly susceptible because of their high composition of polyunsaturated fatty acids (Martinez-Dato et al 2012, Agarwal et al 2005). The destabilization of phospholipids leads to a loss of selective permeability (Hazel et al 1984), the viability of the gametes, and fertilization of the oocyte capacity (Stejskalet al, 2008, Aitken 2006). Among all antioxidants glutathione, in the reduced and non-reduced forms, is widely found in different cell types (Bucak et al 2008), however it was shown that after freezing / thawing a 64% reduction in the content of glutathione content in bovine sperm occurs (Stradaiolii et al, 2007, Bilodeau et al 2001), 32% in humans (Gadea et al 2011) and 78% in pigs (Gadea et al 2004).

In recent years, antioxidants have been added the cryopreserved sperm in mammals, however, the results were promising (Buranaamnuay et al 2011 Gadea et al 2004). For this reason, Karaji et al (2014) suggested the use of combinations of different concentrations and reduced of glutathione and superoxide dismutase in the observed improvement in total and progressive motility of bovine sperm. In rainbow trout, the use of antioxidant uric acid, methionine, superoxide dismutase, reduced glutathione, tocopherol and isolated carnitine increased motility in the control period (Kutluyer et al 2014). Up to date, the use of antioxidants isolated on freezing spermatic in Brazilian native fish is not performed.

Sperm motility is crucial to ensure reproductive success, and depends on aspects such as intracellular ATP production and flagellar structure (Cabrita et al 2010). The ATP content is the main source to generate the movement plague and ensure reproductive success. However, storage of gametes reduces intracellular ATP, and consequently, the time and speed of sperm motility (Aramli et al 2013). The addition of exogenous ATP increased the movement of sperm and artificial fertilization rates in mice (Rodríguez-Miranda et al 2008), cattle (Luria et al 2002) and human (Foresta et al 1992). The selective permeability reduction caused by the quiescent process and restoration of normal metabolism stored by cryopreservation leads to loss of ATP. Thus, the extracellular ATP enters the sperm cell, minimizes their depletion and increases cell viability (Blanco et al 2011). Up to date, no research added extracellular ATP in sperm teleost fish storage. Thus, the aim of this study was to add the reduced glutathione antioxidant (GR) and exogenous ATP in different concentrations in supplementing the sperm freezing extender Tambaqui, C. macropomum.

MATERIALS AND METHODS

Animals

This study was developed according to the guidelines of the ethics of the Federal University of Rio Grande committee (CEUA 015/2015). The collection and initial analyzes of fresh

semen and seminal freezing were carried out at Embrapa Tabuleiros Costeiros in Aracaju (SE Brazil) and thawing and subsequent analysis at the Federal University of Pelotas. All chemicals used in the experiments were obtained from Sigma Chemical Company® (St. Louis, MO, USA).

During the breeding season of the species C. macropomum, total 15 males (8.0 ± 5.0 kg; CT: 70 ± 66 cm) sexually mature fish farming were selected in Santa Clara, located in the municipality of Propriá (SE Brazil). Males were hormonally induced with an intramuscular injection of carp pituitary extract (2 mg / kg body weight), to increase seminal volume. The animals were kept in the tank with water at a controlled temperature of 28°C. After 10 hours each male was removed from the tank and dried with towel, the urogenital pore was blotted dry. Through abdominal massage samples were collected in 15 mL conical tubes, and packed in Styrofoam ice (avoiding their direct contact) at a temperature of 8°C. Samples which were contaminated by feces, urine, blood or water were discarded.

All males have motility higher than 80% before freezing, being assessed by light microscopy with phase contrast in magnification of 200X (BX - Olympus 41). The sperm activation was made with 1μL sample and 4μL NaHCO3 solution (Carneiro et al 2012), at a depth under the cover slip.

Samples stored at 8°C were transported to the laboratory Embrapa Tabuleiros Costeiros (Aracaju, SE) for freezing in the different treatments. An aliquot of semen was diluted at a 1: 2000 in 4% formalin for evaluation in a Neubauer chamber optical phase contrast microscope (Olympus BX 41) for determining sperm concentration.

Cryopreservation

The base extender Beltsville Thawing Solution (BTS: 320 mOsm; pH 7.2) is composed of 37 g of glucose, 6 g sodium citrate, 1.25 g sodium bicarbonate, 1.25 EDTA, 0.9 g of potassium chloride in 1L Distilled water (Pursel 1975). The different concentrations of GR isolated and ATP were added to the extender (base and control) BTS with 10% dimethylsulfoxide (DMSO) (Varela Junior et al 2012). This experiment showed a total of 8 treatments (control; GR 2; 4; 6 mM ATP and 7.5, 15, 22.5 and 30 mM). Semen samples were diluted and homogenized in each treatment at a final concentration of 800 million cells per ml (Varela Junior et al 2015) .The treatments counted with 4 replications totaling 480 samples. After, they were potted in vane 250 μL, sealed with polyvinyl alcohol and wrapped in aluminum racks. The racks remained at room temperature (22°C) for 20 min to stabilize the action of the GR and ATP on sperm cells. Then the samples were expressed in dryshipper nitrogen vapor canister (LVM ™, SC4 / 2V, GA, USA) for 12 hours, and transferred to liquid nitrogen container (LVM ™, TURN 34, GA, USA) to -196°C, being stored for at least 30 days (Varela Junior et al 2012).

The samples were thawed in a water bath at 45°C for 8 s were placed into microtubes 1.5 uL (Streit DP Jr. et al 2006). After it sperm kinetics analysis and cell structures were carried out

Sperm analysis

The thawed sperm was activated and recorded by Computer Assisted Semen Analysis (CASA) (Dziewulska et al 2011). For the evaluation 10 fields with least 1000 cells were captured, activation to the capture of the fields was repeated 3-5 times. The parameters evaluated were total motility (%), progressive motility (%), average distance traveled DAP (mM), distance curvilinear DCL (mM), DSL straight away (mM), average speed of VAP path (μm / s), speed curvilinear VCL (μm / s), rectilinear speed VSL (μm / s), straightness STR (VSL / VAP,%), LIN linearity (VSL / VCL%), oscillation (WOB), lateral displacement of the head ALH (mM) , cross beat frequency BCF (Hz) (Dziewulska et al 2011). The motility time was evaluated at the time of activation to stop the forward movement of sperm (Sorensen1979).

For evaluations of membrane fluidity, DNA fragmentation, lipid peroxidation, membrane integrity and mitochondrial, cell disruption and reactive oxygen species, the Attune Acoustic Focusing ® equipment was used (Life Technologies) equipped with blue laser (Argon 488 nm) and laser violet (UV 405 nm). Analyses were performed using version 2.1 software (Life Technologies). For detection of all evaluations violet laser of 405 nm was used (450/40, VL-1). The populations of sperm cells stained with Hoechst 33342 at a concentration of 16.2 mM, excluding the DNA fragmentation was not used (Martinez-Alborcia et al 2012). Non sperm events were discarded by graphics FSC x SSC and Hoechst 33342 negative dispersion. To read

all parameters, the cells stained with fluorophores were added to calcium-free PBS (80 g NaCl, KCl 11.5 g KCl, 24g Na2HPO4, 2g KH2PO4 in 1L Milique water), using a total of 10,000 sperm per analysis.

The membrane integrity was verified through Sybr14 fluorophores and propidium iodide (PI) (MiniTube, Tiefenbach, Germany). The thawed semen aliquot was incubated for 5 min fluorescent probe containing 0.25 mM of Sybr14 and 7.5 µM IP according to the manufacturer's instructions - MiniTube. The sperm were classified as not damaged and functional membrane (Sybr + / IP) and injured and / or non-functional membrane (Sybr + / IP +; Sybr- / IP +; Sybr- / IP) (Figueroa et al 2015). To check the percentage of cell disruption, the cells that had been classified as IP + disrupted, the cells that had IP - were classified as broken.

The check Fluidity hydrophobic membrane used 2.7 µM merocyanine 540 (M540) and 0.1 µM YO PRO-1 (Invitrogen - Eugene, OR, USA) in 10 µL sample thawed for 5 min. The cells were classified as high fluidity (M540 high concentration) and low fluidity (low concentration M540) only for the intact spermatozoa (negative YO-PRO) (Fernandez-Gago et al 2013). The flow rate of the membrane was calculated from the number of sperm with low fluidity / number of sperm with low fluidity + spermatozoa with high fluidity * 100.

The mitochondria functionality was observed with 3.1 mM of Rhodamine 123 (green fluorescence) and IP 7.5 µM in 10 µL sample thawed for 5 min. The sperm cells were classified as the high functionality (high fluorescence by the accumulation of Rhodamine) and low functionality (low fluorescence, low accumulation of Rhodamine), which assessed only intact spermatozoa (negative IP) (Liuet al 2015) .The functionality rate mitochondria was calculated by the number of sperm with high mitochondrial membrane potential / high number of sperm mitochondrial membrane potential + low sperm mitochondrial membrane potential * 100.

The DNA integrity was assessed by chromatin structure assay (SCSA). To check this parameter, 10µL of thawed sperm was added to 5µL TNE (0.01 M Tris-HCl; 00:15 M NaCl; 0.001 M EDTA; pH 7.2), 10µL of 1X Triton (Triton X-100, 1%) (v / v) at 30 second intervals. The dye acridine orange is added and incubated for 30 s not exceeding the time of 2 min, to do the reading. The sperm was classified integrate DNA (green) and fragmented (orange / red) (Jenkinset al 2015). The DNA fragmentation rate was calculated by the number of sperm with DNA integrate / number of sperm DNA integrate + sperm with fragmented DNA * 100.

The concentration of sperm in ROS cells was performed by 1.0 µM of the fluorophore 2'7' dichlorofluorescein diacetate (H2DCFDA) (emits green fluorescence when oxidized) and IP 7.5 µM. The median intensity of green fluorescence was used only for live sperm (IP-) (Domínguez-Rebolledo et al 2011).

Lipid peroxidation of sperm was evaluated just after thawing. It was added to a final concentration of 1 µM Bodipy C11 (Hagedornet al 2012) in 10 µL sample, and incubated for 2 hours at room temperature (20°C) and analyzed only live spermatozoa. A sperm lipid peroxidation rate was calculated from the intensity median green fluorescence (peroxidated lipid) / medium intensity of green fluorescence + medium intensity of red fluorescence (no lipid peroxidated) * 100.

Statistical analysis

The variables were analyzed for normality by the Shapiro-Wilk test followed by analysis of variance (ANOVA) by Tukey test. Treatments were considered independent variables, and the motility time parameters, DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, WOB, ALH, BCF, membrane integrity, membrane fluidity, mitochondria functionality, index DNA fragmentation, ROS, lipid peroxidation, cell disruption were considered dependent variables. All analyzes were done by Statistix® software 9.0 (Statistix 2008).

RESULTS

The total volume of fresh semen was 4.0 ± 0.5 mL, sperm motility was 96 ± 10% and motility period was 130 ± 7 s.

In the total and progressive motility analysis, 4 mM GR treatment was superior to the control ($P < 0.05$) and ATP concentrations (7.5, 15, 22.5, 30 mM) and 6 mM control showed no difference ($P > 0.05$), as GR2 0 was superior only in motility progressive and motility period (Tab. 1).

In membrane fluidity analysis, the best result was in the GR 4 and GR 6 treatments that differed significantly ($P < 0.05$) from the control

(Tab. 2). In DNA fragmentation parameters, lipid peroxidation and mitochondrial integrity treatments did not differ from control (P> 0.05) (Tab. 2). Regarding the integrity of membrane treatment ATP 22.5 differed from the control and achieved the highest average intact membranes (60%) (P <0.05) (Tab. 2). The remaining ATP treatments (7.5, 15, 30 mM) and GR (2 mM) were similar to the control (P> 0.05) (Tab. 2).

The frozen samples with 15mM ATP (P> 0.05), 22.5 mM and 30 mM (P <0.05) decreased ROS production by more than 50% compared to control. Treatments ATP 7.5 mM, 4 mM, 6 mM did not differ and were not greater than the control (P> 0.05) (Tab. 2). Treatment with 22.5 mM ATP and 30 mM ATP better maintained its integrity in the frozen samples when compared with controls (P <0.05) (Tab. 2).

DISCUSSION

This is the first study using ATP and GR in seminal cryopreservation C. macropomum, Brazilian native fish. In this study the addition of antioxidants reduces the availability of reactive oxygen species, stabilizing the fluidity and integrity of the cytoplasmic membrane. Among the GR treatments, concentrations 2 and 4 mM showed superior performances in total ratings motility, progressive motility and time of motility compared to control. The frozen samples with 15 22.5 and 30 mM decreased ROS production by more than 50% compared to control.

Glutathione reduced in 6 mM and 4 mM concentrations improved flow ability compared to the control membrane, and the concentrations of 2 and 4 mm, were more effective as cryoprotectant substances in the sperm motility characteristics of the period, total and progressive motility. Likewise, these treatments have improved handling parameters for stabilizing cell structures ensuring viability of the gametes. Thus, the GR reduced the oxidative damage caused by the imbalance of the antioxidant system and the formation of reactive oxygen species (Bansal and Bilaspuri 2011). The stabilization of cytoplasmic membrane retained the metabolic pathways, such as oxidative phosphorylation, allowing production ATP for generation flagellar beat and, consequently, higher motility rates.

Motility is the most used parameter to evaluate the quality of gametes between species, because the sperm movement ensures oocyte fertilization. It is evident that the quality of frozen sperm is lower when compared to spermatozoa in nature, especially by reducing the antioxidant content as reduced glutathione and increased reactive oxygen species (Gadea et al 2011). In this regard, several studies supplementing freezing with GR remained handling charges corroborating this study (Gadea et al 2013).

Kutluyer et al (2014) by adding reduced glutathione in isolation in freezing diluent for rainbow trout observed an increase in the duration and thawed sperm motility rate. These studies show effectiveness of adding antioxidants alone giving higher quality in the thawed sperm cells.

The results of ROS showed the same pattern in higher concentrations of ATP GR and gave lower production of ROS in the control. As a result, there was a reduction in ROS attack on the polyunsaturated fatty acids, which make up the plasma membrane (Lahnsteiner et al 2009). Thus, membranes suffer less from the cryopreservation process due to stabilization of the phospholipid. With this, the number of moving cells increased, which was visible in total and progressive motility increased, however it did not act directly on the metabolism of every cell, increasing the non VCL, VSL or BCF.

Even with the addition of ATP concentrations in 22, 5 and 30 mM were effective in reducing the production of reactive oxygen species, keeping unchanged organelles, but this exogenous energy available was not used to that obtain an increase of flagellar beats.. Probably, disorganization of lipid bilayer derived from the freezing enables exogenous ATP input (Blanco et al 2011), which is normally impermeable to the plasma membrane, reducing the production of ROS, but it may be suggested that sperm cell does not use this source of energy to increase their motility. The addition of exogenous GR promoted a balance between ROS and antioxidant system, which is reduced by dilution after freezing in the cryopreservation solutions (Cabrita et al 2011).

In addition, GR and ATP promoted protection of DNA due to the high integrity of percentages (99.4; 97.2). In studies adding antioxidants freezing extenders similar results to this research were obtained, as

Table 1: Sperm kinetic analysis thawed C. macropomum with GR and ATP (mM). Data are expressed as mean and standard error of the mean.

	Tratamentos							
	Control	ATP 7,5	ATP 15	ATP 22,5	ATP30	GR 2	GR4	GR6
TM(%)	30,3 ± 1,11^{b}	31,7 ± 1,46^{b}	28,9 ± 1,33^{b}	21,8 ± 1,14^{c}	19,1 ± 0,99^{c}	34,6 ± 1,45^{ab}	38,1 ± 1,73^{a}	30,7 ±1,54^{b}
PM(%)	20,6 ± 0,89^{c}	22,8 ± 1,28^{bc}	19,4 ± 1,11^{cd}	15,6 ± 0,97 ^{de}	12,9 ± 0,83 ^{e}	25,7 ± 1,36^{ab}	29,4 ± 1,49^{a}	21,6 ±1,28^{bc}
MotP (s)	50,9 ± 4,09^{b}	52,5 ± 3,32^{b}	11,6 ± 2,93^{c}	1,0 ± 0,3^{c}	1,1 ± 0,4^{c}	74,4 ± 4,05^{a}	62,9 ± 3,84^{ab}	60,9 ± 4,39^{ab}
DAP (µm)	14,3 ± 0,28^{ab}	14,1 ± 0,30^{ab}	13,4 ± 0,34^{bc}	12,3 ± 0,31^{c}	13,8 ± 0,36^{ab}	14,7 ± 0,26^{ab}	15,0 ± 0,32^{a}	14,4 ± 0,37^{ab}
DSL	10,7 ± 0,21^{ab}	10,0 ± 0,23^{bc}	9,38 ± 0,21^{c}	9,13 ± 0,20^{c}	9,74 ± 0,18^{c}	11,1 ± 0,21^{a}	10,9 ± 0,23^{ab}	11,1 ± 0,33^{a}
DCL (%)	18,9 ± 0,41^{a}	19,4 ± 0,47^{a}	18,5 ± 0,55^{ab}	16,6 ± 0,58^{b}	18,5 ± 0,60^{ab}	19,2 ± 0,35^{a}	19,6 ± 0,52^{a}	18,5 ± 0,44^{ab}
VAP (µm/s)	33,3 ± 0,69^{a}	33,1 ± 0,71^{a}	32,0 ± 0,83^{ab}	29,0 ± 0,81^{b}	32,8 ± 0,94^{a}	33,8 ±0,64^{a}	34,7 ±0,81^{a}	33,3 ± 0,83^{a}
VSL (µm/s)	24,9 ± 0,50^{a}	23,4 ± 0,52^{ab}	22,4± 0,51^{b}	21,3 ± 0,50^{b}	23,2 ± 0,50^{ab}	25,5 ± 0,48^{a}	25,2 ± 0,57^{a}	25,6 ± 0,73^{a}
VCL (µm/s)	43,8 ± 1,04^{ab}	45,7 ± 1,11^{a}	44,3 ± 1,35^{a}	39,0 ± 1,50^{b}	43,9 ± 1,51^{ab}	44,3 ± 0,90^{a}	45,4 ± 1,28^{a}	42,6 ± 1,00^{ab}
STR	0,8 ± 0,07^{ab}	0,70 ± 0,07^{c}	0,70 ± 0,08^{c}	0,74 ± 0,01^{ab}	0,72 ± 0,01^{bc}	0,75 ± 0,07^{ab}	0,73 ±0,09^{abc}	0,76 ± 0,07^{a}
LIN	0,58 ± 0,01^{a}	0,52 ± 0,08^{c}	0,52 ± 0,01^{bc}	0,58 ± 0,01^{a}	0,55 ± 0,01^{abc}	58 ± 0,09^{a}	0,57 ± 0,01^{ab}	0,60 ± 0,01^{a}
WOB	0,76 ± 0,09^{ab}	0,73 ± 0,07^{b}	0,73± 0,01^{b}	0,76 ± 0,01^{ab}	0,75 ± 0,09^{ab}	0,80 ± 0,06^{ab}	0,77 ± 0,08^{a}	0,78 ± 0,08^{a}
ALH	2,6 ± 0,08 ^{abc}	2,93 ± 0,10^{a}	2,89 ± 0,11^{ab}	2,85 ± 0,13^{abc}	2,97 ± 0,13^{a}	2,62 ± 0,08^{abc}	2,50 ± 0,09^{bc}	2,42 ± 0,07^{c}
BCF	23,8 ± 0,48^{abc}	22,1 ± 0,44^{c}	22,5 ± 0,41^{bc}	22,4± 0,55^{c}	24,0 ± 0,50^{abc}	23,4 ± 0,36 ^{abc}	24,4 ± 0,43^{ab}	24,9 ± 0,46^{a}

* Total motility (TM), progressive motility (PM), motility period (MotP) traveled an average distance (DAP), straight away (DSL), curvilinear distance (DCL), route medium speed (VAP), straight line velocity (VSL), curvilinear velocity (VCL), straightness (STR), linearity (LIN), oscillation (WOB), lateral displacement of the head (ALH), cross flagellar beat frequency (BCF). Different letters in the same row have statistical difference test Tukey (P <0.05).

Table 2: Analysis of flow cytometry: fluidity cytoplasmic membrane (FM), cytoplasmic membrane integrity (MI), cellular integrity (CI), reactive oxygen species (ROS), DNA fragmentation (DNA), lipid peroxidation (LPO), Potential mitochondrial membrane (PMit) of thawed sperm C olossoma macropomum with GR isolated and ATP (mM).

	Trataments			
	Control	ATP 7,5	ATP 15	ATP 22,5
FM (%)	56,7 ± 2,2^{bcd}	66,0 ± 2,8^{ab}	63,6 ± 2,1^{abc}	55,0 ± 3,2^{cd}
MI (%)	40,0 ± 3,0^{c}	54,1 ± 2,3^{ab}	51,1 ± 3,5^{abc}	60,9 ± 3,0^{a}
IC (%)	61,5±3,2^{b}	67,9±2,5^{ab}	68,3±3,1^{ab}	76,2±2,0^{a}
PMit (%)	59,8 ± 3,8^{a}	65,4 ± 3,9^{a}	63,5 ± 3,6^{a}	63,3 ± 3,2^{a}
ROS (%)	1142,8 ± 180,0^{a}	780,2 ± 86,3^{abcd}	507,5 ± 117,5^{bcd}	394,3 ± 47,7^{cd}
LPO (%)	17,6 ± 2,7^{a}	17,5 ± 2,2^{a}	18,2 ± 2,1^{a}	18,7 ± 2,1^{a}
DNA (%)	98,5 ± 0,3^{a}	98,7 ± 0,2^{a}	98,9 ± 0,2^{a}	99,4 ± 0,1^{a}
	ATP30	GR 2	GR4	GR6
FM (%)	48,3 ± 3,2^{d}	65,7 ± 2,7^{abc}	70,0 ± 2,0^{a}	70,7 ± 1,5^{a}
MI (%)	55,6 ± 3,6^{ab}	47,9 ± 2,8^{abc}	51,0 ± 2,6^{abc}	47,0 ± 3,4^{bc}
IC (%)	73,8±2,1^{a}	69,3±2,0^{ab}	69,5±2,6^{ab}	64,9±3,5^{ab}
PMit (%)	60,5 ± 3,4^{a}	63,8 ± 3,3^{a}	62,9 ± 3,8^{a}	65,5 ± 2,9^{a}
ROS (%)	323,8 ± 70,6^{d}	1045,7 ± 178,9^{ab}	888,2 ± 133,5^{abc}	720,7 ± 134,0^{abcd}
LPO (%)	16,7 ± 2,1^{a}	16,1 ± 3,2^{a}	12,0 ± 1,3^{a}	13,0 ± 1,5^{a}
DNA (%)	99,2 ± 0,2^{a}	98,8 ± 0,2^{a}	98,5 ± 0,2^{a}	98,9 ± 0,3^{a}

Data are expressed as mean and standard error of the mean. Different letters in the same row have statistical difference test Tukey (P <0.05).

fragmentation (Ogretmen et al 2015) and the damage (Cabrita et al 2011) in DNA. Gadea et al (2005) observed low level of chromatin condensation with the addition of glutathione and suggested that the presence of this tripeptide reduced oxidative stress, oxidation of thiols and hypercondensation of sperm chromatin. Likewise, this may have occurred in this study as it showed low fragmentation rates (2.8%, 0.6%).

In search of better information allowing an extender able to preserve sperm quality, the addition of GR did not improve indicative of cellular components. But it maintained more efficient sperm kinetics of fresh semen, promoting greater total motility, progressive motility and period when compared to the control treatment. Thus, it is indicated the use of BTS extender with 10% DMSO added 4 mM GR thawed sperm C. macropomum.

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