An Acad Bras Cienc (2021) 93(3): e20191229 DOI 10.1590/0001-3765202120191229
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Effect of different cryopreservation extenders added with antioxidants on semen quality and *in vitro* embryo production efficiency in cattle

NATALIA C. SILVA, KAREN M. LEÃO, JOÃO T. PÁDUA, THAISA C. MARQUES, FRANCISCO R.A. NETO, MARGOT A.N. DODE & ANDRIELLE T.M. CUNHA

Abstract: To evaluate the addition of antioxidants in extenders on post-thaw bovine semen quality and in vitro embryo production efficiency. Six semen samples were collected from five Holstein bulls. In the experiment I, the samples were diluted with AndroMed® and Bovimix® and added antioxidants glutathione (1.5 and 2.5 mM) and melatonin (0.5 and 1.0 mM). In the experiment II, the best treatments obtained in experiment I were used for in vitro fecundation. Glutathione did not improve sperm viability. Melatonin had a negative effect on semen characteristics. Andromed® showed better results in sperm kinetics parameters. Bovimix® was more efficient in maintaining cell integrity parameters. Significant correlation was found between sperm kinetics parameters and between cell integrity parameters. For in vitro embryo production, after oocyte selection, maturation, fertilization and cultivation were performed using the four treatments previously evaluated. Andromed® was more efficient in the cleavage rate, no effect of the addition of glutathione. However, the addition of 2.5 mM glutathione in the Bovimix® improved the cleavage rate. There was a significant moderate correlation between cleavage rate and sperm kinetic characteristics. Glutathione did not improve sperm viability. Melatonin reduced the maintenance of sperm characteristics. Andromed® was more efficient in in vitro embryo production and no effect of glutathione was found in this extender. Addition of 2.5 mM glutathione in the Bovimix® extender provided a higher cleavage rate.

Key words: antioxidants, *Bos taurus*, sperm, fertilization, oocytes.

INTRODUCTION

Semen quality is of utmost importance for the success of cattle artificial insemination programs. Thus, it is essential to maintain the characteristics of sperm through an adequate cryopreservation process (Schober et al. 2007), which includes the use of appropriate extenders and the addition of antioxidants to prevent the excessive production of reactive oxygen species (ROS) (Raheja et al. 2018).

Egg yolk is one of the most used substances for freezing bovine semen. However, its use is discussed because it contains constituents that inhibit sperm cell respiration and may lead to reduced motility, and the great risk of microbiological contamination, as it is an animal product (Crespilho et al. 2012). As a consequence, the World Organization for Animal Health (OIE), recommended in the 2003 Terrestrial Animal Health Code, that animal origin products used in semen processing should be free of any biological risk or processed in order to ensure the safety of such compounds (Marco-Jiménez et al. 2004). As a result, substitution of egg yolk by products of plant origins, such as soybean

lecithin in cryopreservation extenders, has been currently evaluated for the dilution of bull semen and it was shown efficient in conserving post-thaw motility and fertility (Murphy et al. 2018).

Antioxidant substances have also been added to the extender media to minimize the deleterious effects caused by ROS on sperm of various species, including cattle. Antioxidants, such as glutathione and melatonin, slow the oxidation rate by maintaining ROS production of at physiological levels (Ashok et al. 2014), resulting in high quality semen samples and consequent increase in the number of *in vitro* produced embryos (Pang et al. 2016).

Shah et al. (2017) indicated that glutathione at 0.5 mM can be effectively used as an additive in bull semen egg yolk tris glycerol extender for freezing and thawing, to protect the sperm from deleterious effects of cryopreservation. Other study found that the addition of 1 mM glutathione in the lecithin-based extender provided better quality to bovine semen and better in vitro fertility (Mousavi et al. 2019).

Supplementation of 2 or 3 mM concentration of melatonin in the bull semen extender improved the quality of post-thawed bull semen (Ashrafi et al. 2013). Pang et al. (2016) concluded that 0.01 mM and 1 mM melatonin improved sperm quality but only 1 mM increased blastocyst development rate of in vitro-produced bovine embryos.

In this context, this study aimed to evaluate the effect of extenders based on egg yolk (Bovimix®) and soybean lecithin (Andromed®), and the addition of glutathione (1.5 and 2.5 mM) and melatonin (0.5 and 1.0 mM) on the quality of post-thawed bovine semen. In addition, checking the fertility of these samples on the bovine *in vitro* embryo production efficiency and the correlation between sperm characteristics with *in vitro* embryo production (IVEP).

MATERIALS AND METHODS

Experiment I - semen cryopreservation and evaluation

Animals, semen collection and evaluation

The study was conducted at the Animal Reproduction Laboratory of the Instituto Federal Goiano, Rio Verde, Brazil, after approval by the Ethics Committee of the same institution, protocol 011/2014.

Five Holstein bulls were selected by andrological examination. A total of 30 ejaculates (six ejaculates/bull) were collected from bulls using electroejaculator (Boijector®, model 65A). For collection, the animals were kept in a containment trunk and the prepuces were cleaned with paper towels. After collection, the percentages of total motility (%), progressive motility (%) and vigor (0-5) were evaluated by optical microscopy of a semen drop between slide and coverslip preheated to 37°C. Only the ejaculates that met the criteria recommended by the andrological examination and semen evaluation manual of the Colégio Brasileiro de Reprodução Animal (Brasil 2009), were used in the experiment.

Preparation of the extenders

The ejaculates were divided into two portions, diluted to a concentration of $30x10^6$ motile sperm per mL, with egg yolk (Bovimix®, Nutricell, Campinas-SP) or soybean lecithin (AndroMed®, Minitub, Hauptstrasse-Germany) based extenders, added with the antioxidants melatonin (Sigma Co., St. Louis, MO, USA - M5250) or glutathione (Sigma Co., St. Louis, MO, USA - G6013-10G) according to experimental treatments.

Each portion was fractionated into five 15-mL polystyrene conical tubes, resulting in ten treatments: Bovimix® Control (BC); Bovimix® +

0.5 mM melatonin (BM0.5); Bovimix® + 1.0 mM melatonin (BM1.0); Bovimix® + 1.5 mM glutathione (BG1.5); Bovimix® + 2.5 mM glutathione (BG2.5); AndroMed® Control (AC); AndroMed® + 0.5 mM melatonin (AM0.5); AndroMed® + 1.0 mM melatonin (AM1.0); AndroMed® + 1.5 mM glutathione (AG1.5) and AndroMed® + 2.5 mM glutathione (AG2.5).

Semen freezing

The samples were homogenized, stored in 0.5 mL plastic straws and sealed, placed on a stainless steel tray and refrigerated in a Minitub® refrigerator (Porto Alegre, State of Rio Grande do Sul) at 5°C for five hours. The trays were then placed 3 cm above the nitrogen level in a 40 L Styrofoam box. After 15 minutes in nitrogen vapor, straws were immersed in liquid nitrogen, racked and stored in cryogenic device for further analysis.

Thawing of semen

The straws cryopreserved were removed from liquid nitrogen and thawed in water bath at 37°C for 30 seconds. This procedure was used for all sperm evaluations.

Sperm evaluation

Sperm kinetics analysis

Sperm kinetics analyses of semen samples were performed by the CASA software (Sperm Analysis System, Ivos Ultimate 12's, Hamilton Thorne Bioscences, Beverly, MA, USA), previously adjusted for bovine sperm analysis. An aliquot 10 µL of thawed semen sample ($1{,}2x10^6$ motile sperm) was mounted on the reading slide (Makler, Santa Ana, CA, USA) heated at 37ºC. Then three fields were selected for reading and analysis of the samples.

Sperm kinetics variables analyzed were: total motility (MT%); progressive motility (MP%); average path velocity (VAP-µm/s); straight-line velocity (VSL-µm/s); curvilinear velocity (VCL-µm/s); lateral head displacement (ALH-µm); flagellar beat cross frequency (BCF-Hz); linearity (LIN%) and straightness (STR-%).

Flow Cytometry Assessment

Membrane integrity, acrosomal integrity, and membrane stability were evaluated according to the manufacturer's recommendations for analysis of bovine semen in a flow cytometer (FlowSight®-Amnis Imaging Flow Cytometers, Merck Milipore, Darmstadt, Germany). An aliquot 10 µL of thawed semen sample ($1{,}2x10^6$ motile sperm) was incubated at 37°C for 10 minutes in a conical microtube in a fluorescent probe solution according to the analysis, protected from light. For each thawed sample, were performed membrane integrity, acrosomal integrity and membrane stability analyzes.

Membrane integrity analysis

Membrane integrity analyses were determined by combining SBER-14 fluorescent probes (100 nM/mL) and propidium iodide (2 mg/mL, Sperm Viability Kit L-7011, Molecular Probes Inc, Eugene, OR, USA), to check intact and damaged cells.

Acrosomal integrity analysis

For acrosomal integrity analysis, the probes fluorescein-5-isothiocyanate conjugated to peanut agglutinin - FITC-PNA (100 mg/mL, Sigma, Louis, MO, USA) in combination with propidium iodide (0.5 mg/mL, Molecular Probes, Eugene, OR, USA) were used. Thus, cells with reacted or intact acrosome were determined.

Membrane stability analysis

Membrane stability was analyzed by combining the fluorescent probes Merocyanine 540 (540 mM/mL, M540, Molecular Probes, Eugene, OR, USA) and YOPRO-1 (25 µM/mL Molecular Probes, Eugene, OR, USA). Stable live, dead, unstable live and dead cells were counted.

Experiment II - *in vitro* embryo production (IVEP)

Six replicates of IVEP were performed with four best treatments obtained in Experiment I. To avoid bull effect, for *in vitro* fertilization, the best bull was selected according to *in vitro* analyzes of post-thaw semen by CASA method and flow cytometer obtained in Experiment I.

We evaluated the cleavage rate three days (D3) after IVF, blastocyst rate seven days (D7) after IVF and hatching rate eight (D8) and nine (D9) days after IVF. The blastocyst rate was calculated on the cleavage rate, and the hatching rates calculated on the blastocyst rate.

Oocyte selection and *in vitro* maturation

Crossbred cow ovaries from animals of the slaughterhouse were collected and transported in thermos containing physiological saline solution (0.9% NaCL) at 35°C to the laboratory and processed within 4 hours.

In the laboratory, the ovaries obtained were washed with the same solution and the cumulus-oocyte complexes (COC, n=1983, six replicates per treatment) aspirated from 2 to 8 mm diameter follicles with the aid of 10 mL syringes and 40x12 mm needles. Soon after, COCs were stored in 15 mL conical tubes and kept in a water bath at 35°C for 10 minutes for decantation.

Next, the oocytes were screened, according to Stojkovic et al. (2001) by depositing follicular fluid containing the pellet with COCs in a 35 mm Petri dish under a stereomicroscope (Nikon SMZ745). Groups of 30 to 35 COCs with grade I and II quality, according to Stojkovic et al. (2001), were washed and incubated in 200 µL droplets of maturation medium (TCM- 199 with Earle's salts and L-glutamine (Gibco®; Invitrogen Co., Grand Island, NY, USA) supplemented with 10% foetal bovine serum (v/v), 0.2 mM pyruvate, 5 mg/mL luteinizing hormone (Lutropin-V®; Bioniche Co., Belleville, ON, Canada), 1 mg/mL follicle-stimulating hormone (Folltropin®; Bioniche Co.), 75 µg/mL amikacin and 1 mM cystamine) covered with mineral oil (Sigma, Corning, New York, USA) in a 96x21 mm Petri dish for 22 to 24 hours in an incubator at 38.8°C in a 5% CO_2 atmosphere and saturated relative humidity without condensation.

In vitro fertilization

After the maturation period, COCs were washed in the fertilization medium (FEC) TALP- FERT (Tyrode's albumin lactate pyruvate (TALP) supplemented with 6 mg/mL bovine serum albumin (BSA)-fatty acid-free, 0.2 mM pyruvate, 30 µg/mL heparin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine and 75 mg/mL amikacin) added with antibiotics (penicillin + streptomycin) according to the manufacturer's recommendation (10 µL for 1 mL FEC medium). They were then transferred to Petri dishes containing 200 µL drops of the same medium, covered with mineral oil.

For *in vitro* fertilization, semen samples were thawed in a water bath at 37°C for 30 seconds. Total motility (%)> 60%, progressive motility (%) and vigor (0-5) were evaluated under an optical microscope (Olympus, CX21). Sperm cells were selected by discontinuous Percoll gradient centrifugation (90% and 45%) as previously described (Parrish et al. 1995) and capacitation medium (CAP, buffered medium with Tyrode's HEPES, 0.2 mM pyruvate and 75

mg/mL amikacin). Soon after, the supernatant was taken and added with 50 µL FEC medium.

In a conical microtube, 95 µL distilled water was added with 5 µL semen resuspended in FEC medium for counting sperm concentration in a Neubauer chamber. Immediately afterwards, the inseminating dose to be added to the fertilization drops was calculated to give a final concentration of $1x10^6$ SPTZ/mL.

In vitro embryo cultivation

Sperm and oocytes were incubated for 10 hours at 38.8°C in 5% CO_2 and saturated relative humidity without condensation. *In vitro* insemination day was considered as day zero (D0). Zygotes from fertilized oocytes were washed and transferred to 200 µL droplets of culture medium (modified synthetic oviduct fluid (Holm et al. 1999) supplemented with 2.7 mM myo-inositol, 0.2 mM pyruvate, 2.5% foetal bovine serum (v/v), 5 mg/mL BSA-fatty acid-free and 75 µg/ mL amikacin) and incubated at 38.8 °C in 5% CO_2 for seven days. The cleavage rate was evaluated 72 hours after insemination (D3), and half of the culture medium of each drop was replaced. Structures were considered cleaved when the cellular division with at least 2-4 cells was present. The morphological quality of the embryos was measured according to International Embryo Transfer Society (IETS) guidelines (Bó and Mapletoft 2013). According to IETS, the quality of the embryos is classified in Grade 1 to 4 (1-excellent/good, 2-regular, 3-poor, 4-unviable). Embryos are graded regarding the number of physical characteristics including shape, colour and density of cytoplasm or inner cell mass, number and compactness of cells, area of perivitelline space, number of extruded or degenerate cells and frequency and size of cytoplasmic vacuoles. The blastocyst stage was observed seven days (D7) after IVF. Hatching rates were assessed eight (D8) and nine (D9) days after IVF.

Statistical analysis

Data analyzed were randomly distributed in blocks, with the bull being the block. As for the analysis of the characteristics of sperm kinetics, different antioxidants and extenders evaluated, data were log transformed, because the data did not have normal distribution, and subsequently tested by nonparametric Friedman's test ($P<0.05$). To compare the percentage of cleavage rate, blastocysts and hatching between treatments, the Chi-square test was applied.

It was also estimated the correlation between the variables of *in vitro* semen quality and the efficiency of *in vitro* embryo production, seeking to evaluate the association between characteristics, using the Spearman correlation coefficient (r).

For the interpretation of the correlation coefficients, values of r between 0.90 and 0.70 were considered as high magnitude correlations, between 0.69 and 0.40, moderate correlations, between 0.39 and 0.20, low correlations, and between 0.19 and 0.00, weak correlations (Silva 2011).

The analyses were run using the R Project for Statistical Computing program through R Project version 3.2.2 (R Core Team 2015).

RESULTS

Experiment I

Andromed® extender without the addition of glutathione at different concentrations was found to improve sperm kinetic characteristics of bovine semen. Melatonin reduced semen viability. Bovimix® extender obtained better results in terms of cell integrity.

The total motility percentage in the AG 1.5 treatment was equal to the AC treatment and

higher than the other treatments. Progressive motility was similar between the AC, AG 1.5 and AG 2.5 treatments, but higher than the other treatments (Table I). These results are similar to those observed for the variables VAP, VSL, VCL and ALH.

Among the sperm kinetic variables evaluated, BCF was higher in the AG1.5 treatment when compared to the BM0.5, BM1.0, BG1.5 and AM1.0 treatments.

The STR was similar between BC, BG1.5 and BG2.5 treatments; in BC, it was higher than in the

Table I. Mean values and standard error of the sperm kinetic characteristics of cryopreserved Holstein semen with the extenders based on egg yolk (Bovimix®) and soybean lecithin (AndroMed®) added with the antioxidants glutathione and melatonin at different concentrations.

Groups	MT%	MP%	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR%	LIN%
BC	37.0±4.29^{b}	33.5±5.54^{bc}	24.0±1.79^{de}	25.0±4.11^{d}	22.0±7.41^{cd}	28.0±1.29^{bc}	30.5±4.22^{abc}	46.0±2.78^{a}	41.0±1.22^{a}
BM0.5	16.0±2.31^{d}	15.0±2.02^{e}	16.0±2.54^{ef}	16.0±4.43^{e}	16.0±7.76^{de}	16.0±1.28^{de}	20.0±4.47^{cde}	27.5±3.50^{cd}	30.0±2.34^{ab}
BM1.0	7.01±1.23^{e}	6.0±0.67^{f}	8.0±3.91^{f}	8.0±5.35^{f}	7.0±7.82^{f}	10.0±1.19^{e}	11.0±4.80^{de}	19.5±5.82^{d}	22.0±4.07^{b}
BG1.5	25.5±2.80^{C}	27.0±3.55^{cd}	25.0±1.49^{de}	25.0±4.78^{d}	25.0±7.04^{bc}	20.0±1.22^{cde}	25.0±4.06^{bcd}	41.0±2.91^{ab}	40.5±1.67^{a}
BG2.5	35.5±5.68^{b}	33.0±7.12^{bcd}	27.0±1.49^{d}	24.0±4.43^{d}	24.0±7.11^{cd}	25.0±1.33^{cd}	31.5±4.22^{abc}	38.0±2.62^{abc}	31.0±1.32^{ab}
AC	42.5±3.19^{ab}	43.0±3.88^{a}	46.0±2.60^{ab}	45.0±7.38^{ab}	45.0±10.9^{a}	43.0±1.26^{a}	36.0±3.35^{ab}	18.0±3.21^{d}	24.5±0.78^{b}
AM0.5	21.5±2.10^{cd}	24.5±1.85^{d}	32.0±2.35^{cd}	37.0±6.98^{C}	33.0±9.57^{b}	37.0±1.29^{ab}	33.0±3.12^{abc}	29.5±3.77^{bcd}	29.5±1.41^{ab}
AM1.0	8.0±1.08^{e}	9.0±1.37^{ef}	11.0±5.03^{f}	7.0±6.44^{f}	13.0±10.1^{ef}	13.0±1.19^{e}	9.0±4.17^{e}	5.0±6.50^{e}	5.0±4.35^{c}
AG1.5	47.0±2.49^{a}	46.0±2.91^{a}	48.0±2.43^{a}	48.0±7.46^{a}	47.0±10.8^{a}	42.0±1.18^{a}	41.0±3.37^{a}	28.0±3.22^{cd}	27.5±1.22^{ab}
AG2.5	35.0±2.37^{b}	38.0±2.76^{ab}	38.0±2.55^{bc}	40.0±6.89^{bc}	43.0±10.3^{a}	41.0±1.15^{a}	38.0±3.30^{ab}	22.5±3.27^{d}	24.0±0.93^{b}

***Different letters in the same column indicate significant differences (P <0.05) by Friedman test. Total motility (MT-%), progressive motility (MP-%), average path velocity (VAP-µm/s), progressive linear velocity (VSL-µm/s), curvilinear velocity (VCL-µm/s), amplitude of lateral head displacement (ALH-µm/s), flagellar beat cross frequency (BCF- Hz), straightness (STR-%) and linearity (LIN-%). BC: Bovimix® extender without antioxidant (control), BM0.5: extender added with 0.5mM melatonin, BM1.0: extender added with 1.0mM melatonin, BG1.5: extender added with 1.5mM glutathione, BG2.5: extender added with 2.5mM glutathione, AC: AndroMed® extender without antioxidant (control), AM0.5: extender added with 0.5mM melatonin, AM1.0: extender added with 1.0mM Melatonin, AG1.5: extender added with 1.5mM glutathione, AG2.5: extender added with 2.5mM glutathione.**

other seven treatments. The linearity observed in BC was higher than in BM1.0, AC, AM1.0 and AG2.5.

Plasma membrane integrity (IM) results were higher in BC, BG1.5 and BG2.5 treatments. Acrosomal integrity (PNA) had the same result profile as the IM, being higher in BC, BG1.5 and BG2.5 treatments (Table II). Membrane stability (EST) of BC treatment was similar to BM0.5; BG1.5; BG2.5 and AC treatments and higher than BM1.0; AM0.5; AM1.0; G1.5 and AG2.5 treatments.

Regarding the use of different extenders, a difference was found in both treatments in relation to sperm kinetic characteristics. Andromed® extender showed better results in maintaining sperm kinetic characteristics. However, Bovimix® extender obtained better results regarding the membrane integrity (IM), acrosomal integrity (PNA) and membrane stability (EST), as presented in Tables I and II.

Considering the addition of melatonin, it can be observed that in the two concentrations tested, melatonin reduced the maintenance of sperm viability after freezing, as observed in membrane integrity (IM) and acrosomal integrity (PNA). This is because the concentration used is toxic to sperm, which reduced their viability.

Table III lists the correlations between the semen quality characteristics evaluated. High and significant positive correlations were found between MT and MP, VAP and VCL, between MP and VSL, VSL and ALH, STR and LIN, IM and PNA, IM and EST, and PNA and EST.

Moderate and significant positive correlation estimates were observed between MT and VAP, VSL, VCL and ALH. As well as between MP and the characteristics VAP, ALH, and BCF. Moderate and significant positive correlation estimates were also verified between VSL and BCF, ALH and BCF, ALH and STR parameters.

Table II. Mean values and standard error of cell integrity characteristics of cryopreserved Holstein semen with the extenders based on egg yolk (Bovimix®) and soybean lecithin (AndroMed®) added with the antioxidants glutathione and melatonin at different concentrations.

Groups	IM (%)	PNA (%)	EST (%)
BC	43.0±3.07^{a}	45.0±3.08^{a}	43.0±3.51^{a}
BM 0.5	31.0±3.72^{b}	31.0±2.22^{b}	32.0±2.70^{abc}
BM 1.0	12.0±2.29^{cd}	13.0±2.32^{c}	14.0±2.30^{ef}
BG 1.5	44.0±2.68^{a}	44.0±2.69^{a}	35.0±3.53^{abc}
BG 2.5	45.0±3.45^{a}	45.0±3.42^{a}	41.0±3.88^{ab}
AC	25.0±2.20^{b}	29.0±2.26^{b}	32.0±2.10^{abc}
AM 0.5	13.0±1.40^{c}	14.0±1.37^{c}	18.0±1.19^{def}
AM 1.0	5.0±0.63^{d}	5.0±0.80^{d}	5.0±0.61^{f}
AG 1.5	26.0±2.36^{b}	24.0±2.21^{b}	27.0±2.24^{cde}
AG 2.5	31.0±2.00^{b}	25.0±1.88^{b}	28.0±1.63^{bcd}

***Different letters in the same column indicate significant differences (P <0.05) by Friedman test. Plasma membrane integrity (IM), acrosomal integrity (PNA) and membrane stability (EST) in flow cytometer. BC: Bovimix® extender without antioxidant (control), BM0.5: extender added with 0.5mM melatonin, BM1.0: extender added with 1.0mM melatonin, BG1.5: extender added with 1.5mM glutathione, BG2.5: extender added with 2.5mM glutathione, AC: AndroMed® extender without antioxidant (control), AM0.5: extender added with 0.5mM melatonin, AM1.0: extender added with 1.0mM Melatonin, AG1.5: extender added with 1.5mM glutathione, AG2.5: extender added with 2.5mM glutathione.**

The correlations between sperm kinetic characteristics and cell membrane integrity were significant, with low magnitude.

Experiment II

Based on the best *in vitro* analyzes of post-thaw semen by CASA method and flow cytometer obtained in Experiment I, the groups BC, BC2.5, BG and BG2.5 were selected for *in vitro* fertilization.

Regarding the *in vitro* embryo production, the effect of using different cryopreservation extenders on the cleavage rate was observed, with a difference between the treatment with

Table III. Correlation between sperm kinetic characteristics and cell integrity characteristics, total motility (MT-%), progressive motility (MP-%), average path velocity (VAP-µm/s), progressive linear velocity (VSL-µm/s), curvilinear velocity (VCL-µm/s), amplitude of lateral head displacement (ALH-µm/s), flagellar beat cross frequency (BCF-Hz), straightness (STR-%), linearity (LIN-%), membrane integrity (IM%), acrosomal integrity (PNA%) and membrane stability (EST) of Holstein bull semen.

	Characteristics											
Characteristics	MT	MP	VAP	VSL	VCL	ALH	BCF	STR	LIN	IM	PNA	EST
MT	--	0.9087*	0.5624*	0.4868*	0.4693*	0.4127*	0.2340*	0.0956	0.0127	0.3904*	0.3964*	0.3195*
MP	--	--	0.4782*	0.7351*	0.2940*	0.6050*	0.4060*	0.0940	0.0592	0.2499*	0.2725*	0.1684*
VAP	--	--	--	0.3390*	0.9046*	0.1730*	0.0779	0.3470*	0.0662	0.1051*	0.1259*	0.1110*
VSL	--	--	--	--	0.0697	0.7624*	0.6433*	0.1981*	0.0339	0.0885	0.0436	0.1275*
VCL	--	--	--	--	--	0.0663	0.2271*	0.3678*	0.0220	0.1374*	0.1390*	0.2001*
ALH	--	--	--	--	--	--	0.5845*	0.4483*	0.3665*	0.0285	0.0244	0.0072
BCF	--	--	--	--	--	--	--	0.1794*	0.0357	0.0540	0.0157	0.0510
STR	--	--	--	--	--	--	--	--	0.8017*	0.3336*	0.3449*	0.2662*
LIN	--	--	--	--	--	--	--	--	--	0.3021*	0.3317*	0.1633*
IMF	--	--	--	--	--	--	--	--	--	0.2907*	0.3094*	0.2180*
IM	--	--	--	--	--	--	--	--	--	--	0.8974*	0.8159*
PNA	--	--	--	--	--	--	--	--	--	--	--	0.7296*
EST	--	--	--	--	--	--	--	--	--	--	--	--

*Spearman correlation between sperm characteristics. (P <0.05).

soybean lecithin (Andromed®) and egg yolk (Bovimix®). Soybean lecithin-based extender showed higher cleavage rate, but without difference between treatments for blastocyst rate and hatching rate on days eight (D8) and nine (D9) after IVF (Table IV).

The addition of glutathione in soybean lecithin-based cryopreservation medium (Andromed®) did not provide higher cleavage rates when compared to Andromed® treatment without this antioxidant. However, the addition of 2.5 mM glutathione in the egg yolk-based medium (Bovimix®) for bovine sperm cryopreservation improved the cleavage rate when compared to Bovimix® treatment without glutathione.

Regarding blastocyst and hatching rates on day eight (D8) and nine (D9) after IVF, no difference was detected in the evaluated treatments.

Table V presents the correlation values between sperm kinetic parameters of bovine semen and cell integrity analyzed by a cytometer, as well as cleavage rate, blastocyst and hatching rates on day eight (D8) and nine (D9) after IVF.

There was a significant moderate positive correlation between the characteristics of velocity (VAP, VCL and ALH) and the cleavage rate. The variables LIN, IM and PNA showed significant moderate negative correlations with the cleavage rate. Regarding blastocyst and hatching rates on day eight (D8) and nine (D9) after IVF, no correlation was detected with the evaluated sperm characteristics.

DISCUSSION

Our findings suggest further research on the appropriate concentration of antioxidants to be used to combat the deleterious effects of reactive oxygen species and improve sperm quality. The concentration used may cause reduction in semen viability due to the higher cytotoxic effect caused by the high concentration, mainly melatonin. As for extenders, Andromed® showed better results in maintaining the kinetic characteristics of bovine semen, regardless of the addition of antioxidants, and can be used instead of egg yolk-based extender (Bovimix®).

In this research, melatonin reduced sperm cell viability at the evaluated concentrations (0.5 and 1.0 mM) and in the different extenders tested, negatively influencing the sperm quality of the samples. It is believed that melatonin at

Table IV. Percentage values of cleavage rate (TC), blastocyst rate (TB) and hatching rate on day eight (TE - D8) and nine (TE - D9) after fertilization with cryopreserved Holstein semen with extender based on soybean lecithin (AndroMed®) and egg yolk (Bovimix®), with and without the addition of glutathione at different concentrations.

Groups	Number of oocytes	TC (%)	TB (%)	TE - D8	TE - D9
BC	484	35.12 (170/484) ^{c}	35.29 (60/170) ^{a}	36.67 (22/60) ^{a}	46.67 (28/22) ^{a}
BG2.5	508	43.31 (220/508) ^{b}	30.45 (67/220) ^{a}	41.79 (28/67) ^{a}	44.78 (30/28) ^{a}
AC	489	51.33 (251/489) ^{a}	37.45 (94/251) ^{a}	42.55 (40/94) ^{a}	47.88 (45/40) ^{a}
AG1.5	502	52.99 (266/502) ^{a}	34.96 (93/266) ^{a}	40.86 (38/93) ^{a}	50.54 (47/38) ^{a}

***Different letters in the same column indicate significant differences (P <0.05) by the Chi-square test. Bovimix® control, Bovimix® added with 2.5 mM glutathione, AndroMed® control, AndroMed® added with 1.5 mM glutathione.**

the evaluated concentrations may have caused cytotoxic effect on sperm cells. Nevertheless, when 0.01 mM and 1 mM melatonin was added in thawed bovine semen and incubated for only 1 h at 38.5°C in humidified air with 5% CO_2 before in vitro fertilization, led to higher plasma membrane integrity, mitochondrial activity and acrosome integrity, and significantly decreased intracellular ROS levels; increases the blastocyst rate and it decreases apoptosis rate (Pang et al. 2016). This protective effects of this antioxidant on spermatozoa are associated with a reduction in lipid peroxidation as a consequence of increasing the total antioxidant capacity and antioxidant enzymes activity like catalase (Ashrafi et al. 2013).

According ChaithraShree et al. (2019), the addition of 0.1 mM melatonin protected the plasma membrane and acrosome region and maintained the ultrastructure integrity of the cryopreserved bovine spermatozoa when compared to control group, whereas the electron micrography of spermatozoa treated with 0.2 and 0.25 mM melatonin illustrated highest damage to the plasma and acrosome membrane.

Table V. Correlation and confidence interval estimates between different sperm variables of bovine semen with cleavage rate, blastocyst rate and hatching rate on day eight (D8) and nine (D9) after *in vitro* fertilization.

Variables	Cleavage rate	Blastocyst rate	Hatching rate	
D8		**D9**		
MT	0.278 (-0.105;0.590)	0.091 (-0.291;0.448)	- 0.107 (-0.462;0.276)	- 0.235 (-0.559;0.150)
MP	0.3712 (-0.002;0.653)	0.124 (-0.261;0.475)	- 0.048 (-0.413;0.330)	- 0.165 (-0.507;0.221)
VAP	0.433* (0.071;0.694)	0.155 (-0.230;0.499)	0.136 (-0.248;0.485)	0.081 (-0.300;0.441)
VSL	0.325 (-0.053;0.623)	0.213 (-0.173;0.543)	0.091 (-0.291;0.449)	0.030 (-0.346;0.399)
VCL	0.506* (0.164;0.739)	0.096 (-0.286;0.453)	0.135 (-0.250;0.484)	0.943 (-0.288;0.451)
ALH	0.483* (0.134;0.725)	0.020 (-0.354;0.390)	0.187 (-0.199;0.523)	0.112 (-0.271;0.466)
BCF	0.255 (-0.129;0.573)	- 0.128 (-0.478;0.257)	0.113 (-0.271;0.466)	0.100 (-0.283;0.456)
STR	- 0.358 (-0.645;0.016)	0.100 (-0.283;0.456)	- 0.204 (-0.536;0.182)	-0.122 (-0.474;0.262)
LIN	- 0.452*(-0.706;0.095)	0.021 (-0.354;0.391)	- 0.087 (-0.446;0.295)	- 0.053 (-0.418;0.325)
IM	- 0.421* (-0.686;0.057)	0.057 (-0.322;0.421)	- 0.191 (-0.527;0.195)	- 0.283 (-0.593;0.100)
PNA	- 0.420* (-0.685;0.055)	- 0.091 (-0.449;0.291)	- 0.182 (-0.520;0.204)	- 0.103 (-0.458;0.280)
EST	- 0.128 (-0.478;0.256)	0.093 (-0.289;0.450)	- 0.136 (-0.487;0.249)	- 0.152 (-0.497;0.233)

***Pearson correlation between sperm characteristics and embryonic development rates (P <0.05). Total motility (MT%), progressive motility (MP%), average path velocity (VAP-µm/s), progressive linear velocity (VSL-µm/s), curvilinear velocity (VCL-µm/s), amplitude of head lateral displacement (ALH-µm/s), flagellar beat cross frequency (BCF-Hz), straightness (STR%), linearity (LIN%), membrane integrity (IM%), acrosomal integrity (PNA%) and membrane stability (EST%) in flow cytometer.**

It may be that the negative results presented by melatonin´s groups in this study were due to toxicity of the solvent, either by type or concentration or by not being included in the control group. The melatonin stock solution was based on ethanol, obtaining a final concentration of 2% in the samples. Pang et al. (2016), the final concentration of ethanol used was 0.01% and they were not used in the control because obtained the same results without this solvent. Ashsafi et al. (2013) used dimethyl sulfoxide in the stock solution with a final concentration of 0.1%, including in the control group. ChaithraShree et al. (2019) used deionized water to prepare melatonin.

Thus, addition of melatonin during sperm freezing at the concentrations used during the present study did not maintain the sperm characteristics. However, its beneficial effects appear to depend on the solvent used in the preparation of the stock solution, melatonin concentration and timing of its use.

No effect of glutathione was observed on ovine sperm characteristics cryopreserved using tris-egg yolk extender supplemented with 0, 1, 5 and 10 mM glutathione (Perez et al. 2012) nor with 100, 200, and 400 mM (Câmara et al. 2011). On the other hand, Mata-Campuzano et al. (2014) observed that 5 mM glutathione had a positive impact on ovine semen quality because improved mitochondrial activity compared with 0.2 and 1 mM, but did not see increase in farrow rates sheep after artificial insemination. However, addition of 1 mM glutathione in the lecithin-based extender provided better quality to bovine semen compared with 0.5, 2 and 3 mM, promoting increase blastocyst and hatching rate at *in vitro* produced embryo (Mousavi et al. 2019), a result contrary to that observed in this study.

Thereby, adding glutathione at the correct concentration in bull semen preservation, maintain of the membrane integrity, important for sperm metabolism, motility, capacitation, acrosome reaction and the binding of spermatozoa to the egg surface (Yadav et al. 2019).

Due to the possible microbiological contamination caused by extenders based on animal products, Andromed® has been widely used in cryopreservation of bovine semen. In this study, better results were observed regarding sperm kinetic characteristics when using soybean lecithin-based extender. Similar result was reported by Aires et al. (2003) who found greater motility in semen of bulls cryopreserved with soybean lecithin-based extender (AndroMed®)" compared with the egg yolk-based extender (73.54% and 59.57%, respectively). In turn, Salmani et al. (2014) evaluated different concentrations of soybean lecithin (0.5, 1, 1.5, 2 and 2.5%) and egg yolk (15%) in goat semen cryopreservation and found no difference in the characteristics of sperm kinetics and membrane integrity of semen diluted with both extenders.

Samples of sperm with high velocity, as observed with Andromed® (VAP, VCL, VSL, and ALG) and low percentage of STR and LIN are indicative of cell hyperactivation, not by the type of antioxidant used but by the extender evaluated. Thus, this feature is considered undesirable since sperm activation should occur near the fertilization site. Thus, this early activation results in shorter sperm life span, reducing the fecundation capacity of the animal (Verstegen et al. 2002). Therefore, it is believed that Bovimix® extender was more efficient in maintaining sperm characteristics, as it provided better percentages of membrane integrity and acrosomal integrity with lower velocities.

Thus, it is assumed that there was hyperactivation of cryopreserved cells with soybean lecithin-based extender, since higher mean values of velocity and low percentage of

STR and LIN were registered when compared to treatments with Bovimix®.

Regarding *in vitro* embryo production, Andromed® extender was better in maintaining sperm kinetic characteristics of bovine semen and resulted in a higher cleavage rate when compared to egg yolk-based extender, due to the higher percentages of velocity of samples cryopreserved with Andromed® and correlation of these characteristics with fertilization rate. This is positive for *in vitro* embryo production, as sperm are deposited with mature oocytes suitable for fertilization. Thus, it cannot be transposed to *in vivo* fertilization, which requires longer sperm life for fertilization, since this time is reduced when there is hyperactivation due to the higher metabolism of cells.

Prado et al. (2012) found no difference between the extenders Andromed® and egg yolk-based on bovine blastocyst rate. Florez-Rodriguez et al. (2014) reported no difference between egg yolk- and soybean lecithin-based extender on cleavage, blastocyst and hatching rates, similar to the present study. In turn, Crespilho et al. (2012) observed that bovine semen samples cryopreserved with Tris-fructose extender had higher conception rates (59.26%) than Botubov® (both 20% egg yolk) and Botubov®-L (1% soybean lecithin).

Regarding the addition of glutathione in soybean-based medium (Andromed®), no higher cleavage rates were observed. However, the addition of 2.5 mM glutathione in Bovimix® medium for bovine semen cryopreservation improved the cleavage rate when compared to the control treatment. However, there was an increase in the number of embryos in the 2.5 mM glutathione treatment compared to the control treatment in the same extender. This result is numerically representative as to the outcome of pregnancy, considering that pregnancy rates of bovine recipients with *in vitro*-produced embryos range from 30 to 51% (Andrade et al. 2012).

Simões et al. (2013) pointed out that bovine semen is resistant to changes caused in the sperm membrane during cryopreservation and IVEP procedures. However, damage to the membranes results in modifications in chromatin and DNA of the cell, not blocking *in vitro* fertilization and not changing the cleavage rate. However, after the first cleavages, it induces apoptosis of cells with low blastocyst rate. Thus, these changes may have occurred in the samples used in this study, because the concentration of the antioxidant studied had no positive effect on sperm cell protection against ROS, resulting in low blastocyst rate.

CONCLUSIONS

It was concluded that glutathione did not improve bovine sperm viability. Melatonin reduced the maintenance of sperm characteristics. Andromed® was more efficient in *in vitro* embryo production independent of glutathione addition. The addition of 2.5 mM glutathione in Bovimix® extender for sperm cryopreservation provided a higher cleavage rate in *in vitro* embryo production.

Acknowledgments

Special thanks to Universidade Federal de Goiás, Embrapa Recursos Genéticos e Biotecnologia, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Instituto Federal Goiano de Educação Ciência e Tecnologia Goiano for support and structure enabled to the project execution.

REFERENCES

AIRES VA, HINSCH KD, MUELLER-SCHLOESSER F, BOGNER K, MUELLER-SCHLOESSER S & HINSCH E. 2003. *In vitro* and *in vivo* comparison of egg yolk-based and soybean

lecithin-based extenders for cryopreservation of bovine semen. Theriogenology 60(2): 269-279.

ANDRADE GA, FERNANDES MA, KNYCHALA RM, BONATO GL & SANTOS RM. 2012. Fatores que afetam a taxa de prenhez de receptoras de embriões bovinos produzidos *in vitro*. Rev Bras Reprod Anim 36(1): 66-69.

ASHOK A, GURPRIYA V, CHLOE O & DU PLESSIS SS. 2014. Effect of Oxidative Stress on Male Reproduction. World J Mens Heal 32(1): 1-17.

ASHRAFI I, KOHRAM H & ARDABILI FF. 2013. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim Reprod Sci 139(1-4): 25-30.

BÓ GA & MAPLETOFT RJ. 2013. Evaluation and classification of bovine embryos Anim Reprod 10(3): 344-348.

BRASIL. 2009. Ministério da Agricultura Pecuária e do Abastecimento. Portaria n° 109, de 25 de maio de 2009. Institui Grupo de Trabalho no âmbito do Departamento de Fiscalização de Insumos Pecuários - DFIP/SDA, com a finalidade de atualizar o Manual de Procedimentos para Exame Andrológico e Avaliação de Sêmen Animal. Diário Oficial da União, Brasília, DF. Seç. 1, p. 5.

CÂMARA DR, MELLO-PINTO MMC, PINTO LC, BRASIL OO, NUNES JF & GUERRA MMP. 2011. Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen. Small Rumin Res 100(1): 44-49.

CHAITHRASHREE AR, INGOLE SD, DIGHE VD, NAGVEKAR AS, BHARUCHA SB, DAGLI NR, KEKAN PN & KHARDE SD. 2019. Effect of melatonin on bovine sperm characteristics and ultrastructure changes following cryopreservation. Vet Med Sci 6(2): 177-186.

CRESPILHO AM, SÁ FILHO MF, DELL'AQUA JA, NICHI M, MONTEIRO GA, AVANZI BR, MARTINS A & PAPA FO. 2012. Comparison of *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. Livest Sci 149(1-2): 1-6.

FLOREZ-RODRIGUEZ SA, ARRUDA RP, BIANCHI-ALVES MR, AFFONSO FJ, CARVALHO HF, LEMES KM, LANÇONI R, DE ANDRADE AFC & CELEGHINI ECC. 2014. Morphofunctional characterization of cooled sperm with different extenders to use in equine-assisted reproduction. J Equine Vet Sci 34(7): 911-917.

HOLM P, SCHIMIDT MH, GREVE T & CALLESEN H. 1999. High bovine blas- tocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo- inositol with or without without serum- proteins. Theriogenology 52(4): 683-700.

MARCO-JIMÉNEZ F, PUCHADES S, MOCE E, VIUDES-DE-CARTRO M, VICENTE J & RODRIGUEZ M. 2004. Use of Powdered Egg Yolk vs Fresh Egg Yolk for the Cryopreservation of Ovine Semen. Reprod Dom Anim 39(6): 438-441.

MATA-CAMPUZANO M, ÁLVAREZ-RODRÍGUEZ M, TAMAYO-CANUL J, LÓPEZ-URUEÑA E, DE PAZ P, ANEL L, MARTÍNEZ-PASTOR F & ÁLVAREZ M. 2014. Refrigerated storage of ram sperm in presence of Trolox and GSH antioxidants: Effect of temperature, extender and storage time. Anim Reprod Sci 151 (3-4): 137-147.

MOUSAVI SM, TOWHIDI A, ZHANDI M, AMOABEDINY G, SANGCHESHMEH AM, SHARAFI M & HUSSAINI SMH. 2019. Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. Anim Reprod Sci 209: 106171.

MURPHY EM, O'MEARA C, EIVERS B, LONERGAN P & FAIR S. 2018. Comparison of plant- and egg yolk-based semen diluents on in vitro sperm kinematics and in vivo fertility of frozen-thawed bull semen. Anim Reprod Sci 191: 70-75.

PANG YW, SUN YQ, JIANG XL, HUANG ZQ, ZHAO SJ, DU WH, HAO HS, ZHAO XM & ZHU HB. 2016. Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. Mol Reprod Dev 83(11): 993-1002.

PARRISH J, KROGENAES A & SUSKO-PARRISH L. 1995. Effect of bovine sperm separation by either swim- up or percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44(6): 859-869.

PEREZ EGA, NICHI M, VIANA CH, SILVA ROC, DALMAZZO A, GÓES PAA, GURGEL JRC, BARNABE RC, BARNABE VH & BARNABE RC. 2012. Efeito da adição de glutationa na função e estresse oxidativo em sêmen ovino criopreservado. Braz J Vet Res Anim Sci 49(4): 262-268.

PRADO RB, KOIVISTO MB, CARREIRA JT, PERRI SHV, RODRIGUES LH, NETTO HA, TORREGROSSA TLG, VICENTE WRR & FELICIANO MAR. 2012. Efeito da utilização de diferentes diluidores para a produção *in vitro* de embriões bovinos. Arq Bras Med Vet Zootec 64(5): 1118-1126.

R CORE TEAM. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

RAHEJA N, CHOUDHARY S, GREWAL S, SHARMA N & KUMAR N. 2018. A review on semen extenders and additives used in cattle and buffalo bull semen preservation. J Entomol 6(3): 239-245.

SALMANI H, TOWHIDI A, ZHANDI M, BAHREINI M & SHARAFI M. 2014. *In vitro* assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. Cryobiology 68(2): 276-280.

SCHOBER D, AURICH C, NOHL H & GILLE L. 2007. Influence of cryopreservation on mitochondrial functions in equine spermatozoa. Theriogenology 68(5): 745-754.

SHAH N, SINGH V, YADAV HP, VERMA M, CHAUHAN DS, SAXENA A, YADAV S & SWAIN DK. 2017. Effect of reduced glutathione supplementation in semen extender on tyrosine phosphorylation and apoptosis like changes in frozen thawed Hariana bull spermatozoa. Anim Reprod Sci 182: 111-122.

SILVA S. 2011. Interpretação do coeficiente de correlação. Disponível em: http://www.leg.ufpr.br/~silvia/CE055/node102.html.

SIMÕES R, FEITOSA WB, SIQUEIRA AFP, NICHI M, PAULA-LOPES FF, MARQUES MG, PERES MA, BARNABE VH, VISINTIN JA & ASSUMPÇÃO MEO. 2013. Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo *in vitro* development outcome. Reproduction 146(5): 433-441.

STOJKOVIC M, MACHADO SA, STOJKOVIC P, ZAKHARTCHENKO V, HUTZLER P, GONÇALVES PB & WOLF E. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture. Biol Reprod 64(3): 904-909.

VERSTEGEN J, IGUER-OUADA M & ONCLIN K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57(1): 149-179.

YADAV D, SINGH V, PATEL A, YADAV SS, OJHA AK, KUMAR A, YADAV S, KUMAR B, YADAV B & SAXENA A. 2019. Effect of glutathione on membrane integrity of bovine spermatozoa during cryopreservation of extended semen in tris based dilutor. Pharma Innovation 8(6): 734-738.

How to cite

SILVA NC, LEÃO KM, PÁDUA JT, MARQUES TC, NETO FRA, DODE MAN & CUNHA ATM. 2021. Effect of different cryopreservation extenders added with antioxidants on semen quality and *in vitro* embryo production efficiency in cattle. An Acad Bras Cienc 93: e20191229. DOI 10.1590/0001-3765202120191229.

Manuscript received on October 8, 2019;
accepted for publication on June 7, 2020

NATALIA C. SILVA[1]
https://orcid.org/0000-0003-3709-9844

KAREN M. LEÃO[2]
https://orcid.org/0000-0001-5236-7558

JOÃO T. PÁDUA[1]
https://orcid.org/0000-0003-0888-7972

THAISA C. MARQUES[2]
https://orcid.org/0000-0003-1112-6699

FRANCISCO R.A. NETO[2]
https://orcid.org/0000-0003-1064-5614

MARGOT A.N. DODE[3]
https://orcid.org/0000-0002-1096-0457

ANDRIELLE T.M. CUNHA[3]
https://orcid.org/0000-0001-7272-468X

[1]Universidade Federal de Goiás, Escola de Veterinária e Zootecnia, Rua Esperança, s/n, Campus Samambaia, 74690-900 Goiânia, GO, Brazil

[2]Instituto Federal Goiano, Rua Sul Goiânia, Km 01, Zona Rural, 75901-970 Rio Verde, GO, Brazil

[3]Universidade de Brasília, Instituto de Biologia, Campus Universitário Darcy Ribeiro, s/n, Asa Norte, 70910-900 Brasília, DF, Brazil

Correspondence to: **Natalia do Carmo Silva**
E-mail: nataliazootec@hotmail.com

Author contributions

Natalia do Carmo Silva: Responsible for the execution of the project and development of the research article; Karen Martins Leão: Project collaborator, providing support and assisting in its execution; João Teodoro Pádua: Project collaborator, assisting in the execution of the same; Thaisa Campos Marques and Andrielle Cunha: Responsible for assisting in the execution of the project and laboratory activities; Francisco Neto: Responsible for the statistical analysis of the project; Margot Dode: Responsible for assisting in the execution of the project, as well as the availability of laboratories.