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Addition of insulin-like growth factor I (IGF-I) and reduced glutathione (GSH) to cryopreserved boar semen

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ABSTRACT

The objective of this study was to evaluate the effect of adding reduced glutathione (GSH) to a boar semen freezing extender supplemented with insulin-like growth factor I (IGF-I) or anti-IGF-I. Eight ejaculates from eight boars were extended to obtain insemination doses, which were supplemented with either recombinant human IGF-I (30 ng/mL) or anti-IGF-I (60 ng/mL) shortly after extension. After 24 h of liquid storage at 17 °C, the semen was frozen with or without GSH (5 mM) in the freezing extender for a total of six treatments. Osmotic resistance and acrosome integrity was greater in fresh semen ($P < 0.05$) soon after adding IGF-I or the anti-IGF-I antibody. After 24 h of cooling, the supplementation with these compounds resulted in an increased ($P < 0.05$) percentage of sperm with relatively greater mitochondrial activity and reduced the percentage of cells with relatively greater concentrations of superoxide. After thawing, there was a reduction ($P < 0.05$) in the percentage and fluorescence intensity of sperm with greater quantities of superoxide and peroxide only in samples treated with GSH + IGF-I and GSH + anti-IGF-I. The addition of GSH (alone or in combination with IGF-I or anti-IGF-I), however, reduced the percentage of sperm with an intact acrosome ($P < 0.05$). The same effect was not observed with IGF-I or anti-IGF-I alone. In conclusion, the addition of IGF-I or anti-IGF-I improved the quality of fresh or liquid-stored semen. Using GSH in the freezing extender improved the antioxidant potential of frozen semen only in combination with IGF-I or an anti-IGF-I antibody.

1. Introduction

Compared with liquid-stored semen, cryopreservation is an effective method to preserve gametes for long periods of time (Yeste, 2016). This technology, however, is not yet widely used in swine reproduction, primarily due to the difficulty of freezing boar semen (Watson, 2000). Compared with liquid-stored semen, the primary problem associated with semen cryopreservation is the marked reduction in semen quality after thawing, which results in a decrease in both the parturition rate (10%–20%) and the number of piglets (2–3) per litter (Roca et al., 2011).

The reduction in boar semen quality that occurs during the cryopreservation process is primarily related to damage to the sperm membranes, which have an essential function in the fertilization process (Cuasnicu et al., 2001). This membrane damage probably occurs because boar sperm contain greater quantities of unsaturated phospholipids and lesser quantities of cholesterol than other species, as well as an asymmetric distribution of cholesterol (greater in the inner monolayer than in the outer monolayer) (Casas and

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Flores, 2013). According to Yeagle (1985), among other characteristics, cholesterol profoundly affects the physical properties of membranes, including fluidity, membrane lipid and protein chain positioning, and permeability, and has a direct relationship with cellular resistance to heat shock (Meyer and Smit, 2009). During semen cooling, the organization of membrane phospholipids, proteins and cholesterol is modified due to the transition of the membrane from a liquid-crystalline phase to a gel phase (Drobnis et al., 1993). During the liquid-crystalline phase, the lipid bilayer is relatively fluid, allowing the movement of proteins and lipids within the membrane. In the gel phase, however, the decrease in membrane fluidity decreases the mobility of these components (Parks, 1997), affecting the functional capacity of the membrane and consequently resulting in damage to the cells (Amann and Pickett, 1987; Blanch et al., 2012). Furthermore, changes in the sperm nucleus due to the destabilization of the nucleoprotein structure during the freeze-thaw process may compromise the fertilization capacity of these cells due to DNA fragmentation (Flores et al., 2011; Yeste et al., 2013).

Several studies, therefore, have been conducted to evaluate the addition of substances to the freezing and thawing extenders that are capable of positively affecting the cellular responses to the challenges of cryopreservation. Among these substances, reduced glutathione (GSH) has been widely studied. Due to its antioxidant potential, GSH is vital for the maintenance of intracellular redox balance (Jacob et al., 2003) and helps to maintain the stability of the nucleoprotein structure (Malo et al., 2010; Yeste et al., 2014b). The benefits provided by GSH, however, are variable. Estrada et al. (2014) observed that GSH at 2 mM reduced DNA damage and increased the number of viable spermatozoa in frozen-thawed boar semen. Betarelli et al. (2018) verified that there was a reduced membrane lipid disruption in boar spermatozoa after thawing. According to Yeste et al. (2014b), the beneficial effects of GSH on post-thaw sperm function and survival depend on the intrinsic ejaculate freezing capacity because ejaculates with a lesser freezing capacity require a greater GSH concentration than ejaculates with a relatively greater freezing capacity.

Insulin-like growth factor I (IGF-I) is a natural substance found in semen (Zangeronimo et al., 2013). The IGF-I has marked regulatory effects on protein phosphorylation and its capacity to accelerate the energy metabolism of sperm cells (Macpherson et al., 2002). Results of a previous study in buffalo indicate IGF-1 functions as a metabolic activator of sperm by increasing the carbohydrate metabolism rate (Selvaraju et al., 2009). This results in increased cell movement and, consequently, an increase in the fertilization capacity of the sperm. With greater cellular energy metabolism, however, there can be an increased generation of free radicals (O'Flaherty et al., 1997), which could affect semen quality and the fertilization capacity of spermatozoa. The results from previous studies indicate that the addition of IGF-I to liquid-stored boar semen improved the seminal quality of insemination doses stored for 24 or 72 h (Silva et al., 2011). It was assumed that these results were due to the increase in metabolic rate associated with the antioxidant effects of IGF-I by enzyme activation. In fact, a direct effect of IGF-I on superoxide dismutase (SOD) activity has been reported in wild rats. There, however, have been no published studies on the effect of IGF-I on frozen boar semen. Additionally, the addition of GSH to a boar semen freezing extender supplemented with different quantities of IGF-I has not been evaluated. The central hypothesis of the present study, therefore, is that IGF-I can improve the quality of frozen-thawed boar semen and that the positive effect of adding GSH to the freezing extender is related to the presence of this growth factor in the ejaculate. In this context, the objective of this study was to evaluate the effect of adding GSH to boar semen freezing extender alone or in combination with IGF-I or its antibody (anti-IGF-I) on the quality of frozen boar semen.

2. Material and methods

2.1. Semen collection and processing

The experiment was performed at the Laboratory of Veterinary Physiology and Pharmacology, Federal University of Lavras (UFLA), Lavras, Minas Gerais (MG), Brazil, from January to March 2017. The experiment was approved by the Animal Ethics Committee under case number 043/14.

The eight ejaculates used in this study were obtained from four Duroc and four Large White boars with proven fertility from a commercial farm. The animals were maintained in individual masonry stalls (2.5 m long x2.0 m wide) and were fed 3.0 kg of feed for breeding boars daily divided into two feedings and water was provided *ad libitum*. The semen was collected in the morning, 1 h after feeding, using the "gloved-hand" method. Prior to semen collection, the preputial fluids were evacuated by manually exerting caudo-cranial pressure towards the preputial opening; the preputial opening and the surrounding area were subsequently cleaned. The gel fraction of the semen was separated using a triple layer of gauze/filter paper and was then discarded; only the sperm-rich fraction was collected and used.

After collection, the semen was extended in preheated (37 °C) Beltsville Thawing Solution (BTS; Minitub do Brazil, Porto Alegre, Rio Grande do Sul, Brazil) at a 1:1 ratio and was immediately transported to the laboratory in thermal bottles for semen analysis. The transport time from the collection site to the laboratory did not exceed 60 min. Using a Neubauer chamber, the sperm concentration was adjusted with preheated BTS to obtain 100-mL insemination doses containing three billion sperm each.

Prior to the definition of the experimental groups, increasing amounts (30, 60 and 90 ng/mL) of IGF-I antibody (IGF-I antibody; mouse host 100 µL, Invitrogen, Carlsbad, California, USA) were added to all ejaculates, and then the IGF-I concentrations were defined according to the methodology described by Zangeronimo et al. (2013) using a specific immunoassay kit (IGF-I ELISA Kit 1 × 96 wells; Raybiotech, Norcross, Georgia, USA) according to the manufacturer's protocol.

After determination of the minimum amount of IGF-I antibody required to neutralize all endogenous IGF-I, the extended semen was then divided into three groups: the control group (extended semen with no added substances); the IGF-I group, which was supplemented with recombinant human IGF-I (Recombinant Human IGF-I 50 µg; Sigma-Aldrich, St. Louis, Missouri, USA) at 30 ng/mL (Mendez et al., 2013); and the anti-IGF-I group, which was supplemented with IGF-I antibody (IGF-I antibody; mouse host 100 µL,

Invitrogen, Carlsbad, California, USA) at 60 ng/mL. The IGF-I and anti-IGF-I were added shortly after semen dilution. Samples were subsequently cooled and maintained at 17 °C for 24 h (Yeste et al., 2014a). After this interval, 10-mL aliquots of each semen sample were heated in a 37 °C water bath and evaluated after 120 min of incubation. At this time, total and progressive motility, sperm morphology, osmotic resistance, sperm viability, acrosome integrity, membrane permeability, mitochondrial activity, and intracellular peroxide and superoxide levels were evaluated. All evaluations were performed in triplicate. The experimental design consisted of randomized blocks (ejaculates) with three treatments and eight replicates (ejaculates).

2.2. Cryopreservation and thawing of semen

Cryopreservation was conducted according to the methodology proposed by (Westendorf, 1975) and modified by (Yeste et al., 2013). After 24 h of liquid storage at 17 °C, doses were centrifuged at this same temperature at 600 *g* for 5 min. The pellets were separated from the supernatants and diluted to a concentration of 1.5×10^9 sperm/mL in an LEY extender (80% lactose solution at 11% + 20% egg yolk) using a Neubauer chamber for freezing alone or in combination with GSH at a final concentration of 5 mM (Giaretta et al., 2015). At this time, six experimental groups were set, as follows: T1) control, T2) IGF-I, T3) anti-IGF-I, T4) GSH, T5) GSH + IGF-I, and T6) GSH + anti-IGF-I. The extended samples were then cooled to 5 c°C for 120 min (0.1 °C/min) using a programmable freezer (IceCube 14S; Minitub of Brazil, Porto Alegre, Rio Grande do Sul, Brazil) and subsequently diluted in a LEYGO extender (92.5% LEY, 6% glycerol – Sigma® and 1.5% Orvus ES Paste – OEP, Equex STM; Nova Chemical Sales Inc.; Scituate; MA, USA) to a final concentration of 1×10^9 sperm/mL. The samples were then transferred into 0.5 mL straws for a total of eight straws per treatment. The straws were subsequently transferred to a programmable freezer (IceCube 14S; Minitub of Brazil). The freezing program (SY-LAB software version 1.0; Minitüb Ibérica SL) consisted of 313 s of cooling at the following rates: −6 °C/min from 5 to −5 °C (100 s), −39.8 °C/min from −5 to −80 °C (113 s), maintenance at −80 °C for 30 s, and subsequent cooling at −60 °C/min from −80 to −150 °C (70 s) (Yeste et al., 2014b). At the end of the process, the straws were immersed in liquid nitrogen (−196 °C) and stored for later analysis.

After 15 days of freezing, the contents of the straws were thawed at 37 °C for 20 s. The thawed semen was then extended in BTS at a final ratio of 1:4 (Casas et al., 2010) and incubated in a water bath at 37 °C. The semen evaluations were the same as those performed before freezing, always after 120 min of incubation. All evaluations were performed in triplicate. The experimental design was randomized blocks with six treatments and eight replicates (ejaculates) for each treatment.

2.3. Microscopic evaluations

Sperm motility was evaluated using a computer-assisted sperm analysis system (CASA, Sperm Class Analyzer SCA 5.0; Microptic, Barcelona, Catalonia, Spain) coupled to a phase contrast microscope with a heating plate (Olympus CX31; Olympus, Tokyo, Honshu, Japan). To perform the assessment, 3.0 µL of the semen sample was deposited on a special slide (Leja® 20 µm; Microptic, Barcelona, Catalonia, Spain) preheated to 37 °C. A minimum of 300 cells distributed in five random fields were evaluated in each sample.

To evaluate the osmotic resistance, a 100-µL semen aliquot was deposited in an Eppendorf tube containing 900 µL of hyposmotic sodium citrate solution (1 g of sodium citrate in 100 mL of double-distilled water, 150 mOsm, pH 7.4), and another aliquot was concomitantly deposited in an Eppendorf tube with 900 µL of isosmotic sodium citrate solution (3.2 g of sodium citrate in 100 mL of double-distilled water, 3000 mOsm, pH 7.4). The aliquots were immediately incubated in a water bath at 37 °C for 15 min. A slide smear was then created with a drop of semen mixed with a drop of eosin-nigrosin dye (Blom, 1950). The evaluation was performed using an optical microscope (Olympus CX31; Olympus, Tokyo, Japan) at 400· magnification. The integrity of the acrosome was evaluated for a total of 100 randomly counted sperm and was subsequently quantified as the percentage of acrosomal abnormalities observed in the isosmotic and hyposmotic environments (Rodríguez-Gil and Rigau, 1996).

The total number of sperm abnormalities was evaluated after a 200-µL aliquot of semen was deposited in 700 µL of a 3% formaldehyde-citrate solution (Pursel et al., 1972). After homogenization, 10 µL of the sample was deposited between the slide and coverslip, and 100 random cells were evaluated with oil immersion under a phase contrast microscope (Olympus CX31; Olympus, Tokyo, Honshu, Japan) at 1000· magnification. Abnormalities in the acrosome, head, midpiece and tail were counted. The total number of abnormal cells relative to the total number of counted cells was expressed as a percentage (%).

2.4. Flow cytometry analyses

Flow cytometry was used to determine sperm viability, acrosome integrity, intracellular concentrations of peroxide and superoxide, mitochondrial membrane potential and permeability of the sperm membrane following the recommendations detailed in Lee et al. (2008). In all assessments, the sperm concentration was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL in BTS at 37 °C. The sperm cells were then stained with combinations of each fluorochrome, following the specific protocols for each reagent. The fluorochromes (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were the LIVE/DEAD® Sperm Viability Kit; propidium iodide solution in water (1.0 mg/mL); lectin PNA from *Arachis hypogaea* (peanut), Alexa Fluor® 647 Conjugate; 2′,7′-dichlorofluorescin diacetate (H_2DCFDA); dihydroethidium (hydroethidine); 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1); and YO-PRO-1 iodide (491/509) 1 mM solution in DMSO. All reagents were prepared according to the manufacturer's specifications. A correction procedure consisting of differentiating particles in the presence or absence of DNA was performed in tests (YO-PRO-1/PI; JC-1; H2DCFDA/PI; and HE/YO-PRO-1) in which the presence of unusual particles could overestimate the percentages of intact spermatozoa (Petrunkina et al., 2010; Yeste et al., 2013). Samples were evaluated in a flow

cytometer (Guava® EasyCyte 8 H T; Merck-Millipore, Darmstadt, Germany) equipped with a 488 nm and a 640 nm laser.

Three fluorescence intensity channels (FL-1, FL-2 and FL-3) were used. The channels had the following characteristics: FL-1 (green fluorescence): Dichroic/Splitter, DRLP 550 nm and bandpass filter (BPF) 525 nm; FL-2 (orange fluorescence): DRLP 600 nm and BPF 575 nm; and FL-3 (red fluorescence): longpass filter 670/730 nm. The signals were logarithmically amplified, and the photomultiplier configurations were adjusted for staining methods.

The cytometer provided the electronic volume (EV) and the side scatter (SS) for each event. With all analyses, the sheath flow rate was set at 4.17 µL/min, and EV and SS were recorded in a linear mode (in EV vs. SS dot plots) for a minimum of 10,000 events per replicate. Each variable was evaluated in triplicate in independent tubes. The analyzer threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter < 7 µm) and cell aggregates (particle diameter > 12 µm). Compensation was used to minimize fluorescence spillover into a different channel.

Information about the events was collected in the list-mode data files (LMD), and the GuavaSoft software (version 3.1, Merck-Millipore, Hayward, CA, USA) was used to analyze cytometric histograms and dot plots.

2.5. Sperm viability (SYBR-14/PI)

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/PI) (Molecular Probes, Eugene, USA) and propidium iodide (PI), according to the protocol described by Garner and Johnson (1995). In a 96-well cell culture plate, 600 µL of semen was mixed with SYBR®-14 at a final concentration of 100 nM. After 10 min at 38 °C in a water bath in a darkened area, PI was added at a final concentration of 10 µM. After 5 min of incubation, samples were analyzed using flow cytometry. The fluorescence of SYBR®-14 was measured in FL-1, and the PI fluorescence was measured in FL-3. Viable, green-stained spermatozoa (SYBR-14 + /PI-) and nonviable, red-stained spermatozoa (SYBR-14-/PI +) sperm were registered. Single-stained samples were used to set EV gain and FL-1 and FL3 PMT voltages and to compensate for SYBR-14 spillover into the FL-3 channel (2.45%).

2.6. Acrosome integrity (PNA-FITC/PI)

The acrosome integrity (percentage of viable spermatozoa with intact acrosomes) was determined by costaining of spermatozoa with lectin from *Arachis hypogaea* (peanut agglutinin) conjugated with fluorescein isothiocyanate (FITC-PNA) and PI (Nagy et al., 2003). In a 96-well cell culture plate, 600 µL of semen sample was added with FITC-PNA at a final concentration of 2.5 µg/mL and PI at 10 µM. The samples were incubated at 38 °C for 5 min in the dark. FITC-PNA was measured by FL-1, and PI was measured by FL-3. Spermatozoa were divided into four populations: viable with intact acrosome (PNA-FITC-/PI-); viable with acrosomal exocytosis (PNA-FITC + /PI-); and nonviable cells with damaged acrosome (PNA-FITC + /PI +). Only viable spermatozoa were analyzed.

2.7. Intracellular concentrations of peroxides and superoxides (H2DCFDA/PI and HE/YOPRO-1)

Intracellular peroxide (H_2O_2) and superoxide ($O_2^{-\cdot}$) concentrations were determined using the modified protocol described by (Guthrie and Welch, 2006). For analysis of peroxides, the semen samples were stained with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) at a final concentration of 200 µM and PI at 10 µM and incubated in a water bath at 25 °C for 60 min in a darkened area. The fluorescence from H_2DCFDA was measured on the flow cytometer by FL-1, and the fluorescence from PI was measured by FL-3. For the superoxide analysis, the semen samples were stained with hydroethidine (HE) at a final concentration of 4 µM and YO-PRO-1 at 40 µM. The sample was incubated at 25 °C for 40 min in the dark. The HE fluorescence was detected by FL-3, and YO-PRO-1 was detected by FL-1 1 as a viability marker. Following staining, the percentages of viable spermatozoa with greater intracellular H_2O_2 concentrations (high DCF + fluorescence) and the percentages of viable spermatozoa with relatively greater $O_2^{-\cdot}$ (high ethidium fluorescence; E +) were recorded.

2.8. Mitochondrial membrane potential (JC-1)

Mitochondrial membrane potential (MMP) was analyzed with tetraethylbenzimidazolcarbocyanine iodide (JC-1), as described by Gillan et al. (2005). Samples were analyzed by flow cytometry using FL1 and FL2. Two sperm subpopulations were determined: the first subpopulation was formed by spermatozoa with high MMP that showed orange staining (JC-1 aggregates), whereas the second population contained the sperm cells with low MMP, which were stained in green (JC-1 monomers). The fluorescence intensity has been registered and the percentage of cells with high MMP and the intensity of cells with relatively greater MMP were calculated.

2.9. Permeability of the sperm membrane (YO-PRO-1 / PI)

For the membrane permeability analysis, the samples were stained with the fluorochromes YO-PRO-1 and PI, according to the modified protocol described by Rathi et al. (2001). Samples were incubated with YO-PRO-1 at a final concentration of 40 µM and IP at 10 µM for 5 min at 38 °C in a darkened area. The fluorescence of PI was measured by FL-3, and YO-PRO-1 was measured by FL-1 as a viability marker. Four different sperm populations were identified: viable spermatozoa with no changes in membrane permeability (YO-PRO-1-/PI-), viable spermatozoa with early changes in membrane permeability (YO-PRO-1 + /PI-), nonspermatozoa (YO-PRO-1 + /PI +) and nonviable spermatozoa (YO-PRO-1-/PI +). Only viable spermatozoa were analyzed.

Table 1
Characteristics of fresh boar semen diluted 120 min after the addition of insulin-like growth factor I (IGF-I) or anti-IGF-I.

Variable	Control	IGF-I	Anti-IGF-I	*P* =	CV
Total motility, %	91.2 ± 1.9	93.9 ± 3.0	90.7 ± 4.4	0.17	2.40
Mean velocity, μm/s	89.2 ± 13.9	88.5 ± 11.1	82.2 ± 10.6	0.27	12.66
Progressive linear velocity, μm/s	22.9 ± 5.1	22.4 ± 5.5	22.1 ± 6.0	0.91	16.30
Curvilinear velocity, μm/s	43.6 ± 8.1	42.5 ± 8.6	41.3 ± 8.8	0.70	10.52
Linearity coefficient, %	27.8 ± 4.7	26.5 ± 3.2	28.0 ± 5.5	0.67	13.02
Straightness coefficient, %	51.3 ± 6.4	50.5 ± 4.1	51.0 ± 4.7	0.93	8.96
Wobble coefficient, %	51.3 ± 5.3	49.5 ± 3.5	51.8 ± 6.0	0.45	7.44
Lateral head displacement, μm	2.45 ± 0.36	2.37 ± 0.32	2.30 ± 0.21	0.36	8.56
Flagellar beat-cross frequency, Hz	8.22 ± 1.52	8.38 ± 0.88	7.60 ± 1.14	0.33	12.73
Sperm abnormalities, %[1]	5.75 ± 4.13	5.50 ± 4.50	4.63 ± 2.88	0.77	23.10
Osmotic resistance, %	72.7 ± 3.6 b	78.6 ± 4.2 a	78.9 ± 4.3 a	0.02	5.16
Viable sperm, %	70.5 ± 4.4	69.1 ± 6.4	69.4 ± 4.6	0.45	2.82
Sperm with intact acrosome[2], %	72.8 ± 4.8 b	76.5 ± 4.2 a	76.0 ± 6.8 a	0.03	–
Sperm with high MA, %	42.8 ± 28.4	48.0 ± 28.7	50.4 ± 29.2	0.39	11.07
Intensity of cells with high MA[2]	16.8 ± 3.6	17.0 ± 3.7	18.8 ± 6.5	0.85	–
Viable sperm with high SO[2], %	58.9 ± 7.0	58.5 ± 6.4	60.2 ± 9.6	0.11	–
Intensity of cells with high SO	148 ± 12	139 ± 12	134 ± 14	0.21	3.65
Viable sperm with high PO, %	58.5 ± 7.1	57.8 ± 10.4	58.9 ± 9.5	0.83	4.99
Intensity of cells with high PO	221 ± 87	252 ± 124	255 ± 144	0.39	9.49
Permeability of intact membrane, %	72.0 ± 3.7	70.8 ± 6.0	73.2 ± 3.8	0.25	3.23

MA: mitochondrial activity; SO: superoxide; PO: peroxide.
[a, b]Means followed by different letters in the column differ according to the SNK test ($P < 0.05$).
[1] Data transformed using Johnson´s transformation.
[2] No significance by Friedman's test ($P > 0.05$).

2.10. Statistical analysis

The data were first tested for normality (Anderson-Darling), homoscedasticity (Breusch-Pagan), and independence of errors (Durbin-Watson). If the results of these tests were not significant, analysis of variance (ANOVA) was performed, and the treatments were compared using the Student-Newman-Keuls (SNK) test at 5%. When the ANOVA assumptions were not met and the Johnson data transformation was not successful in normalizing the data, a nonparametric analysis was used, and the means were compared with Friedman´s test. The level of significance was set at 5% in all cases. All statistical analyses were performed using the Action version 3.5 statistical program (Action Stat, São Carlos, São Paulo, Brazil).

3. Results

3.1. Fresh semen

The amount of free endogenous IGF-I in the extended semen of the experimental animals was 3.46 ± 0.83 ng/mL. The 60 ng/mL concentration of the IGF-I antibody was sufficient to neutralize all of the endogenous IGF-I in the insemination samples.

Adding IGF-I and anti-IGF-I to the extender increased ($P < 0.05$) the osmotic resistance and the number of viable sperm with an intact acrosome (Table 1). There was no effect ($P < 0.05$) of these substances on the other evaluated semen characteristics.

3.2. Effect of IGF-I and anti-IGF-I on semen stored for 24 h

The addition of both IGF-I and its antibody increased ($P < 0.05$) the percentage of sperm with relatively greater mitochondrial activity and reduced ($P < 0.05$) the fluorescence intensity of cells with relatively greater concentrations of superoxides (Table 2). There was no effect ($P > 0.05$) of these substances on the other evaluated semen characteristics.

3.3. Thawed semen after adding GSH to the freezing extender

The percentage of sperm with relatively greater concentrations of superoxide was reduced ($P < 0.05$) by supplementing with only the GSH + IGF-I and GSH + anti-IGF-I (Table 3). The fluorescence intensity of cells with a relatively greater concentration of superoxide was less ($P < 0.01$) when anti-IGF-I and GSH (alone or in combination) were added. There was a greater reduction in the fluorescence intensity of cells with a relatively greater concentration of superoxide when there was supplementation with GSH + IGF-I and GSH + anti-IGF-I. With the reduction in glutathione, there was also a reduced percentage of sperm with greater concentrations of peroxides, and the GSH combinations resulted in greater reductions than that of the control sample. Compared with the control, the addition of IGF-I or its antibody did not affect the percentage of sperm with relatively greater concentrations of peroxides. Adding GSH to the freezing extender, alone or in combination with IGF-I or its antibody, reduced ($P < 0.05$) the percentage of sperm with an intact acrosome. The same effect was not observed with the addition of IGF-I or anti-IGF-I alone. There was no effect

Table 2
Characteristics of boar semen cooled for 24 h after adding insulin-like growth factor I (IGF-I) or its antibody (anti-IGF-I) and evaluated at different incubation times at 37 °C.

Variable	Control	IGF-I	Anti-IGF-I	*P* =	CV
Total motility[1], %	86.4 ± 5.2	84.4 ± 6.6	82.5 ± 6.6	0.22	–
Mean velocity, μm/s	75.1 ± 15.9	77.4 ± 12.6	73.8 ± 12.2	0.73	12.9
Progressive linear velocity, μm/s	19.0 ± 3.2	19.9 ± 5.1	18.7 ± 3.7	0.72	15.9
Curvilinear velocity, μm/s	37.0 ± 6.5	36.9 ± 6.4	35.4 ± 5.9	0.73	12.0
Linearity coefficient, %	27.7 ± 4.7	27.5 ± 5.2	27.5 ± 6.6	0.99	15.8
Straightness coefficient, %	49.3 ± 4.5	50.7 ± 6.4	50.2 ± 8.4	0.88	11.1
Wobble coefficient, %	52.4 ± 5.7	50.4 ± 4.4	50.3 ± 4.9	0.41	6.90
Lateral head displacement, μm	2.10 ± 0.34	2.12 ± 0.28	2.03 ± 0.27	0.70	10.6
Flagellar beat-cross frequency, Hz	7.27 ± 1.03	7.36 ± 1.19	7.42 ± 0.95	0.89	9.10
Sperm abnormalities[1], %	5.90 ± 4.02	6.00 ± 4.02	6.10 ± 3.98	0.50	–
Osmotic resistance, %	70.4 ± 5.1	72.4 ± 7.7	68.7 ± 7.6	0.50	9.27
Viable sperm, %	69.7 ± 8.6	67.4 ± 8.2	68.2 ± 8.7	0.13	3.13
Sperm with intact acrosome, %	74.3 ± 4.3	75.6 ± 2.9	76.6 ± 4.4	0.10	3.23
Sperm with high MA[1], %	57.0 ± 17.3 b	63.9 ± 24.2 a	61.6 ± 26.0 a	0.04	–
Intensity of cells with high MA	17.8 ± 2.1	18.2 ± 3.0	18.2 ± 3.1	0.81	6.57
Viable sperm with high SO[1], %	54.7 ± 9.3	55.9 ± 11.0	58.1 ± 10.3	0.42	–
Intensity of cells with high SO[1]	141 ± 21 a	130 ± 27 b	129 ± 22 b	0.02	–
Viable sperm with high PO, %	61.4 ± 10.7	60.1 ± 12.9	61.6 ± 10.8	0.68	6.00
Intensity of cells with high PO[1]	223 ± 120	207 ± 131	212 ± 108	0.88	–
Permeability of intact membrane, %	70.7 ± 7.4	68.1 ± 8.9	70.1 ± 8.4	0.81	2.72

MA: mitochondrial activity; SO: superoxide; PO: peroxide.
[a,b]Means followed by different letters in the column differ according to the SNK test ($P < 0.05$).
[1] No significance by Friedman´s test ($P > 0.05$).

Table 3
Characteristics of frozen-thawed boar semen after adding insulin-like growth factor I (IGF-I) or anti-IGF-I to the fresh semen (before freezing), adding reduced glutathione (GSH) after cooling (before freezing) and holding at 37 °C for 120 min after thawing.

Variable	Control	IGF-I	Anti-IGF-I	GSH	GSH + IGF-I	GSH + Anti-IGF-I	P =	CV
Total motility, %	39.5 ± 10.1	40.3 ± 12.7	40.6 ± 10.8	43.8 ± 8.0	44.4 ± 7.5	40.7 ± 11.4	0.68	17.3
Mean velocity, μm/s	46.2 ± 6.8	41.5 ± 4.2	44.9 ± 5.7	44.4 ± 8.1	46.2 ± 11.8	48.2 ± 7.5	0.51	11.0
Progressive linear velocity, μm/s	13.4 ± 1.4	12.6 ± 2.0	13.8 ± 1.5	13.1 ± 1.3	13.8 ± 3.8	13.3 ± 2.1	0.86	15.7
Curvilinear velocity[1], μm/s	24.8 ± 3.0	23.9 ± 2.2	25.4 ± 2.4	24.5 ± 2.7	25.4 ± 4.1	25.6 ± 3.5	0.65	–
Linearity coefficient, %	32.3 ± 5.2	35.1 ± 5.7	34.0 ± 3.0	32.7 ± 4.5	33.0 ± 8.9	30.4 ± 6.5	0.55	15.1
Straightness coefficient[1], %	51.4 ± 4.5	52.4 ± 5.3	52.1 ± 3.0	51.0 ± 4.5	50.1 ± 11.1	49.5 ± 5.5	0.85	–
Wobble coefficient, %	58.7 ± 3.5	62.9 ± 4.3	61.8 ± 5.2	60.0 ± 5.0	62.1 ± 5.9	57.5 ± 6.5	0.17	6.6
Lateral head displacement, μm	1.51 ± 0.15	1.45 ± 0.14	1.50 ± 0.14	1.44 ± 0.12	1.44 ± 0.23	1.58 ± 0.17	0.11	7.3
Flagellar beat-cross frequency[1], Hz	4.10 ± 0.62	3.96 ± 0.65	4.35 ± 0.91	4.00 ± 1.02	4.60 ± 1.09	4.20 ± 0.92	0.51	–
Sperm abnormalities[1], %	12.00 ± 4.50	9.55 ± 2.49	10.40 ± 4.64	8.68 ± 2.73	9.20 ± 4.39	8.56 ± 4.14	0.44	–
Osmotic resistance[1], %	65.5 ± 5.4	61.7 ± 4.1	62.9 ± 6.1	62.2 ± 6.3	61.1 ± 4.0	62.5 ± 4.0	0.30	–
Viable sperm, %	47.9 ± 7.4	49.2 ± 10.6	47.4 ± 12.1	42.1 ± 12.0	39.6 ± 14.1	38.9 ± 14.0	0.36	14.1
Sperm with intact acrosome, %	65.7 ± 7.7 a	66.4 ± 5.8 a	64.8 ± 5.6 a	61.4 ± 7.6 b	59.9 ± 5.1 b	56.1 ± 7.3 b	0.02	7.0
Sperm with high MA, %	27.0 ± 27.0	31.5 ± 16.3	26.1 ± 12.2	29.9 ± 15.7	25.9 ± 10.1	25.6 ± 12.7	0.77	14.7
Intensity of cells with high MA	15.0 ± 0.8	15.7 ± 0.9	15.0 ± 0.7	15.3 ± 1.4	15.3 ± 1.3	15.1 ± 1.5	0.14	–
Viable sperm with high SO[1], %	26.1 ± 8.2 a	28.5 ± 10.8 a	27.6 ± 8.3 a	27.5 ± 9.6 a	24.7 ± 11.3 b	22.9 ± 11.5 b	0.05	15.1
Intensity of cells with high SO[1]	167 ± 13 a	162 ± 13 a	153 ± 22 b	151 ± 18 b	142 ± 18 c	137 ± 24 c	0.01	–
Viable sperm with high PO[1], %	24.6 ± 5.3 a	23.2 ± 6.6 a	23.6 ± 4.2 a	22.6 ± 5.3 b	18.4 ± 4.1 c	16.7 ± 2.5 c	< 0.01	–
Intensity of cells with high PO[1]	147 ± 69	145 ± 61	148 ± 61	144 ± 65	140 ± 73	149 ± 77	0.57	–
Permeability of intact membrane, %	63.2 ± 7.8	64.7 ± 7.8	63.5 ± 6.8	61.6 ± 6.4	57.9 ± 8.6	59.0 ± 7.3	0.06	8.0

MA: mitochondrial activity; SO: superoxide; PO: peroxide.
[a,b]Means followed by different letters in the column differ according to the SNK test ($P < 0.05$).
[1] No significance by Friedman´s test ($P > 0.05$).

($P < 0.05$) of the evaluated substances on the remaining semen characteristics evaluated.

4. Discussion

Reports evaluating the effects of adding GSH to the freezing extenders of boar semen alone or in combination with IGF-I or its antibody (anti-IGF-I) have not been published. The results of this study provide evidence that the addition of GSH could be beneficial from an oxidative perspective, which corroborates other authors (Yeste et al., 2014b; Betarelli et al., 2018), but only in samples in which IGF-I or its antibody were added before cooling. Using IGF-I or its antibody, however, appears to be beneficial for maintaining

the quality of both fresh and liquid-stored semen.

The advantage of adding GSH to freezing extenders is the increased antioxidant capacity of the frozen-thawed semen (Yeste et al., 2014b; Giaretta et al., 2015). The boar sperm compared to sperm of many other species are very susceptible to lipid peroxidation due to the relatively greater concentrations of polyunsaturated fatty acids in the membrane (Cerolini et al., 2000). Furthermore, the concentration of GSH present in the sperm is relatively less in pigs compared with that in other species (Li, 1975). The mean GSH content is 0.03 nmol/10^8 sperm. In humans, GSH is 5.3 nmol/10^9 sperm and in cattle is 2.93 nmol/10^8 sperm (Agrawal and Vanha-Perttula, 1988).

In the present study, evidence of the effect of GSH was observed only when this substance was combined with IGF-I or anti-IGF-I, suggesting a possible interaction between these factors. This result partially explains the discrepancies in results of previous studies where there was use of GSH in semen cryopreservation protocols (Gadea et al., 2004). The IGF-I hormone is a natural substance that is present in boar semen in varying concentrations (Zangeronimo et al., 2013). The purpose of using the anti-IGF-I in the present study was to neutralize any endogenous IGF-I and to evaluate the effects of GSH on boar semen in the absence of this hormone.

The IGF-I receptors have been identified in different cells of the spermatogenic lineage, including sperm (Vannelli et al., 1988; Henricks et al., 1998). It, therefore, has been suggested that IGF-I has an important function in the metabolic control of the sperm cell (Glander et al., 1996; Vickers et al., 1999). In most published studies there has been evaluation of the addition of IGF-I to semen as a metabolic activator (Silva et al., 2011), and possible antioxidant (Mendez et al., 2013). Although the combination of GSH with IGF-I or anti-IGF-I enhanced the antioxidant capacity of frozen-thawed boar semen, the integrity of the acrosome appeared to be impaired. Acrosome integrity is important to prevent premature loss of acrosomal enzymes and thus maintain the fertilization capacity of the sperm (Lucio et al., 2016). Because metabolic substances derived from polyunsaturated fatty acids (i.e., reactive oxygen species) have an important function in the acrosome reaction of sperm (Tripodi et al., 2003), the use of antioxidants usually reduces the frequency of acrosomal defects in semen (Sarlos et al., 2002; Betarelli et al., 2018). In the present study, the observed decrease in the number of sperm with an intact acrosome in insemination samples with GSH supplementation was not expected.

The addition of IGF-I alone during the dilution of fresh semen did not lead to enhanced sperm quality for frozen boar semen. Benefits were observed only when IGF-I was combined with GSH. For fresh semen (precooling), adding either IGF-I or its antibody resulted in an increase in osmotic resistance and the percentage of sperm with an intact acrosome. These results indicate IGF-I may be involved in the antioxidant protection pathways of sperm cells (Selvaraju et al., 2009; Silva et al., 2011).

Mendez et al. (2013) reported that there were lesser concentrations of malondialdehyde when adding 30 ng/mL IGF-I to boar semen. In the present study, however, the effects of adding IGF-I or its antibody to cells with greater concentrations of superoxides or peroxides were not statistically significant in fresh semen. A lesser fluorescence intensity of cells with greater concentrations of superoxides and a greater percentage of cells with greater mitochondrial activity, however, were observed in liquid-stored semen that had been stored for 24 h, which is consistent with findings from a previous study (Mendez et al., 2013). Additionally, the increase in the percentage of sperm with a relatively greater mitochondrial activity may be related to the capacity of IGF-I to facilitate energy use (the acceleration of metabolism) by sperm cells (Henricks et al., 1998).

Interestingly, the results obtained in the present study for IGF-I were similar to those obtained with the addition of its antibody. This suggests that instead of activating IGF-I-mediated hormonal mechanisms, the addition of this hormone at 30 ng/mL could have inactivated such mechanisms because excessive amounts of IGF-I may have activated the downregulation mechanisms of the receptors, thereby inactivating the signaling pathways involved with this hormone (Hadley and Levine, 2000). The concentration used (30 ng/mL) was ˜10 times greater than the physiological concentration of the free hormone (3.46 ± 0.83 ng/mL) in the seminal plasma of the boars tested. Under these conditions, instead of activating the signaling cascade initiated by this hormone, the addition of IGF-I may have inhibited this signaling cascade. This is the first study in which there was comparison of the effects of adding either IGF-I or its antibody on boar semen. Further studies should be conducted to investigate the specific effects on molecular signaling, extent of semen dilution, and number of IGF-I-binding proteins (IGFBPs) present in the ejaculate.

In practice, the standardization of an efficient cryopreservation protocol for boar semen remains challenging due to the biochemical characteristics of the ejaculates. Semen characteristics change depending on the breed, age and nutritional status, season, and even between ejaculates from the same animal (Holt, 2000; Sonderman and Luebbe, 2008). It, therefore, is necessary to gain and enhanced understanding of the cellular responses related to cold shock damage because these responses represent the first of the cellular mechanisms that protect against the cold shock damage caused by cryopreservation.

5. Conclusion

Adding GSH to the freezing extender improved the quality of the frozen-thawed boar semen, particularly when IGF-I or anti-IGF-I was added to the insemination sample. The present results suggest a possible association between these factors, although the molecular mechanisms involved in the cellular response to cryodamage require elucidation. The addition of IGF-I or its antibody improved the quality of fresh and liquid-stored semen. There, however, was no advantage for use of IGF-I or anti-IGF-I for frozen-thawed semen processing, except when GSH was also added to the freezing extender.

Declaration of Competing Interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the study submitted.

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