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Impact of adding different concentrations of IGF-I and insulin to the semen extender on bull sperm quality post-cryopreservation

[*Impacto da adição de diferentes concentrações de IGF-I e insulina ao diluidor de sêmen sobre a qualidade espermática bovina após a criopreservação*]

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ABSTRACT

This study aimed to evaluate the addition of different concentrations of IGF-I and insulin to egg yolk-based extender to improve bovine semen cryopreservation. Two experiments were developed to evaluate the effects of the additives in two commercial extenders, Botubov® (Experiment 1) and Triladyl® (Experiment 2), both with the same design. Three ejaculates from four bulls (n = 12) were used. Each ejaculate was divided into seven equal fractions for dilution ($60x10^6$ spermatozoa/mL) in the following treatments: CON: extender only; IGF100: IGF-I 100ng/mL; IGF200: IGF-I 200ng/mL; INS150: insulin 150µUI/mL; INS200: insulin 200µUI/mL; ASS1: IGF-I 100ng/mL + insulin 150µUI/mL; ASS2: IGF-I 200ng/mL + insulin 200µUI/mL. Semen was cryopreserved by an automated system. Post-thawed sperm were evaluated regarding motility by CASA (Computer-assisted sperm analysis), and membranes by fluorescent probes (H342, PI, FITC-PSA and JC-1). For Botubov® extender, INS150 was more efficient in preserving total and progressive motility, VCL, BCF, plasma and mitochondrial membranes. A similar response was seen when insulin was added to the Triladyl® extender, INS150 was more efficient in preserving sperm motility, plasma membrane integrity and mitochondrial potential. Thus, the addition of insulin 150µUI/mL, regardless of the composition of the extender, contributes to better preserving bovine sperm from the cryopreservation effects.

Keywords: Bull, Semen, Freezing, Botubov®, Triladyl®

RESUMO

Este estudo teve o objetivo de avaliar a adição de diferentes concentrações de IGF-I e insulina a diluidores, à base de gema de ovo, para melhorar a criopreservação do sêmen bovino. Dois experimentos foram desenvolvidos para avaliar os efeitos dos aditivos em dois diluidores comerciais: Botubov® (Experimento 1) e Triladyl® (Experimento 2), ambos com o mesmo delineamento. Foram utilizados três ejaculados de quatro touros (n=12). Cada ejaculado foi dividido em sete frações para diluição ($60x10^6$espermatozoides/mL), nos seguintes tratamentos: CON: somente diluidor; IGF100: 100ng/mL de IGF-I; IGF200: 200ng/mL de IGF-I; INS150: 150µUI/mL de insulina; INS200: 200µUI/mL de insulina; ASS1: 100ng/mL de IGF-I + 150µUI/mL de insulina; ASS2: 200ng/mL de IGF-I + 200µUI/mL de insulina. O sêmen foi criopreservado por sistema automatizado. Após a criopreservação, o sêmen foi avaliado quanto à motilidade espermática por CASA e quanto às membranas espermáticas (plasmática, acrossomal e mitocondrial) por sondas fluorescentes (H342, PI, FITC-PSA e JC-1). Para o diluidor Botubov®, INS150 foi mais eficiente em preservar motilidades total e progressiva, VCL, BCF, integridade da membrana plasmática e potencial mitocondrial. Resposta semelhante foi observada quando a insulina foi adicionada ao diluidor Triladyl®, INS150 foi mais eficiente na preservação da motilidade, integridade das membranas e potencial mitocondrial quando comparado aos demais grupos. Assim, a adição de 150µUI/mL de insulina aos diluidores, independentemente da composição, contribui para melhor criopreservação dos espermatozoides bovinos.

Palavras-chave: bovino, sêmen, congelação, Botubov®, Triladyl®

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INTRODUCTION

Semen cryopreservation keeps sperm cells at cryogenic temperatures for a long term (Treulen *et al*., 2018) and allows the widespread use of artificial insemination in cattle (Grotter *et al*., 2019). However, the process of freezing and thawing the semen results in irreversible changes in sperm kinetics, membranes, and DNA integrity (Celeghini *et al*., 2008; Amidi *et al*., 2016). These changes directly reflect on the fertility of cryopreserved semen (Oliveira *et al*., 2014). Moreover, damage to cells during freezing may be related to osmotic stress, the formation of intracellular ice crystals, the production of reactive oxygen species or, simultaneously, the combination of all these factors (Grotter *et al*., 2019).

The fertilization capacity of cryopreserved sperm depends on the preservation of the integrity and functionality of the sperm structures, which are acquired by components of the extenders as ionic and non-ionic substances, responsible for maintaining osmolarity and buffering. Some components of the extenders are high molecular weight lipoproteins, present in egg yolk, crucial to avoid cold shock; intracellular cryoprotectants, such as glycerol; energy sources, represented by sugars like glucose and fructose; and other additives (Oliveira *et al*., 2018).

To minimize some negative effects of semen cryopreservation, several additives are studied to guarantee better preservation of sperm cells, as reported by Pinto *et al*. (2020) in bovine, by Dalmazzo *et al*. (2018) in dogs, by Banday *et al*. (2017) in ovine and by Berkovitz *et al*. (2018) in humans. However, to the best of our knowledge, no additive was shown as minimizing the cryopreservation process by turning the post-thawed semen similar to *in natura* semen.

Bovine semen extenders supplemented with hormones such as IGF-I and insulin can improve post-thaw sperm metabolism and maximize the preservation of post-thaw sperm structures. Insulin is a protein composed of two chains (α and β, with 21 and 30 amino acids, respectively), which provides energy to sperm. This component is released into the seminal plasma, with the production of NADPH, influencing sperm capacitation and autocrine metabolism (Andó and Aquila, 2005). Autocrine metabolism is characterized by the releasing of insulin by sperm, which self-regulates glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that limits the pentose phosphate pathway (PPP), essential for sperm to acquire motility and sperm capacitation, in addition to promoting fusion between gametes (Andó and Aquila, 2005; Aquila *et al*., 2005). Previous studies adding insulin in the cryopreservation medium have shown better preservation of the plasma and acrosome membranes integrity, as well as the cellular metabolism (Van Tilburg *et al*., 2008), decreased sperm DNA fragmentation (Shokri *et al*., 2019) and increased sperm fertilizing capacity (Lampião and Plessis, 2008).

The insulin-like growth factor I (IGF-I) is a peptide similar to insulin and is a component of seminal plasma secreted by Leydig and Sertoli cells. IGF-I has a key role during spermatogenesis and steroidogenesis (Lee *et al*., 2016). Also, it preserves sperm motility, as well as plasma and acrosome membranes integrity, as demonstrated for bovine (Henricks *et al*., 1998), swine (Silva *et al*., 2011), buffalo (Selvaraju *et al*., 2016) and equine species with an increase in the pregnancy rate (Macpherson *et al*., 2002).

Although insulin and IGF-I already have beneficial effects on sperm quality during cryopreservation, the best concentration of these additives and the impact of their association have not yet to be elucidated in cryopreserved bull sperm. Based on that, this study aimed to evaluate the addition of different concentrations of insulin and IGF-I in egg yolk-based extenders (Botubov® and Triladyl®) for bovine semen cryopreservation on sperm kinetics and plasma, acrosome and mitochondrial membranes, the benefit of the addition being expected even when the composition of the extenders is unknown.

MATERIAL AND METHODS

Four Nellore bulls with an average age of 55 ± 0.95 months and body weight of 921.25 ± 78.46 kg were used. The animals were kept in paddocks with pasture of *Brachiaria sp.* and mineral salt and water *ad libitum*. Concentrate was provided according to the nutritional requirements indicated for the category and age following the National

Research Council (NRC, 2016). The animals were weighed every 15 days. Before the beginning of the experiment, endo and ectoparasite control was performed. This study was conducted into two experiments, according to commercial egg-yolk-based extender used: Experiment 1: Botubov® and Experiment 2: Triladyl®.

The experimental procedures were approved by the Ethics Committee on the Use of Animals (CEUA) of the School of Veterinary Medicine and Animal Science, the University of São Paulo-USP, Brazil (protocol CEUA number 2861160816).

In Experiment 1 (*Addition of different concentrations of IGF-I and insulin to the Botubov® extender for cryopreservation of semen*), three ejaculates from each four Nellore bulls were used (n = 12). The semen was collected by electroejaculation (Autojac, Neovet®, Uberaba/SP/Brazil), in a 15-mL graduated tube. After collection, *in natura* semen was kept in a water bath at 37°C during evaluation. Each ejaculate was evaluated for volume, concentration, motility, vigor, and morphology.

Semen volume was determined by the graduated tube used during collection. The sperm concentration was determined in a Neubauer chamber, under optical microscopy at 400x magnification, after the previous dilution (1:100) in a buffered formaldehyde solution (4%). Sperm motility and vigor were evaluated with a semen drop between a slide and a coverslip, under optical microscopy (Nikon, Eclipse E200) at 100x magnification. Motility was estimated as a percentage of moving sperm and sperm vigor as a score from 1 to 5, according to the recommendations for bovine (Neves *et al*., 2013). Sperm morphology was performed under differential interference contrast (DIC) microscopy (Nikon, Eclipse 80i), between slide and coverslip at 1000x magnification. A total of 200 cells was counted and the results were expressed as a percentage, classified into major and minor defects according to Blom (1973).

The results of the analysis of the semen *in natura* were used for the choice of the ejaculate for the cryopreservation and for the dilution and preparation for the cryopreservation procedure.

Each ejaculate was divided into seven equal fractions, all diluted to a final concentration of $60x10^6$ spermatozoa/mL in Botubov® (Botupharma®, Botucatu-SP) extender. Different concentrations of IGF-I (Sigma-Aldrich, I3760) and insulin (Sigma-Aldrich, I2643) were added. A total of seven treatment groups were performed as follows: BCON: only Botubov® extender (Control); BIGF100: Botubov® + 100 ng/mL of IGF-I; BIGF200: Botubov® + 200 ng/mL of IGF-I; BINS150: Botubov +150 µUI/mL of insulin; BINS200: Botubov + 200 µUI/mL of insulin; BASS1: Botubov +100 ng/mL of IGF-I + 150 µUI/mL of Insulin; BASS2: Botubov + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

The choice of IGF-I and insulin concentrations used in the experimental groups was supported by the basal concentrations of these hormones in the seminal plasma, as previously described (Henricks *et al*., 1998; Andó and Aquila, 2005).

The semen diluted and supplemented with different hormonal concentrations were incubated for 10 minutes at 37ºC for interaction between semen and treatment. Then, semen was packaged in French straws (0.25mL) labeled with the animal number, date, and treatment. Semen cryopreservation was performed in an automatic system (TK 3000 Plus®, Uberaba, MG, Brazil). For freezing, the semen was cooled at a rate of 0.25°C/minute until it reaches 5°C. Afterward, the straw holder was transferred to the thermal box containing liquid nitrogen until the temperature of -120°C, at a rate of -20°C/minute. Upon reaching this temperature, the straws were submerged in liquid nitrogen at -196°C. In the end, the straws were organized in racks and stored in cryogenic tanks according to each treatment and animal.

After thawing, two straws from each batch, bull and treatment were thawed in a water bath for 30 seconds at 37°C placed in microtubes, homogenized, and evaluated for motility and sperm membranes.

The characteristics of sperm motility were evaluated by Computer-Assisted Sperm Analysis – CASA (SCA®, Sperm Class Analyzer; MICROPTIC, Barcelona, Spain). The setup was previously adjusted to evaluate bovine sperm.

For the analysis, the samples were diluted in a concentration of $10x10^6$ spermatozoa/mL in the TALP sperm medium. The diluted semen sample was placed in the Makler® chamber (Selfi-Medical Instruments, Haifa, Israel) for evaluation. The investigated characteristics were total motility (TM%), progressive motility (PM%), velocity average path (VAP, µm/s), velocity straight line (VSL, µm/s), velocity curved line (VCL, µm/s), wobble (WOB%), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR%) and linearity (LIN, %).

The evaluation of the plasma, acrosome and mitochondrial membranes followed the protocol described by Celeghini *et al*. (2008). An 150 µL aliquot of the semen diluted in TALP sperm medium (Bavister and Lorraine, 1983) at $10x10^6$ concentration spermatozoa/mL was placed in a microtube and added 2 µL of Hoechst 33342 (H342, 0.5 mg/mL, Molecular Probes, H1399), 2 µL of propidium iodide (PI, 0.5 mg/mL in DPBS, Molecular Probes, P3566), 6 µL of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1, 153 µM in DMSO, Molecular Probes, T3168) and 20 µL of fluorescein isothiocyanate-*Pisum sativum* agglutinin (FITC-PSA, 100 µg/mL in DPBS, Sigma, L0770). The samples were incubated for 8 minutes at 37°C. After incubation, a drop (4 µL) of sample was placed between the slide and coverslip (preheated at 37°C) and the reading was performed under epifluorescence microscopy (Nikon Epifluorescence Microscope, Model Eclipse 80i) in a triple filter (D/F/R, C58420) presenting the UV-2E/C (340-380nm excitation and 435-485nm emission), B-2E/C (465-495nm excitation and 515-555nm emission) and G-2E/C (540-525nm excitation and 605-655nm emission) at 1,000x magnification.

A total of 200 sperm cells were counted and classified into eight categories according to the fluorescence emitted by each probe, according to Celeghini *et al*. (2008). After counting, the percentages of the following cell populations were considered for this study: plasma membrane integrity (PI), acrosome integrity (AI), high mitochondrial membrane potential (HMP), and spermatozoa showing plasma and acrosome membranes integrity and high mitochondrial membrane potential (PIAIHP).

In Experiment 2 (*Addition of different concentrations of IGF-I and insulin to the Triladyl® extender for cryopreservation of semen*), three ejaculates from each four Nellore bulls were also used (n = 12). Semen collection was performed by electroejaculation (Autojac, Neovet®, Uberaba/SP/ Brazil). After collection, *in natura* semen was kept in a water bath at 37°C during evaluation. Each ejaculate was evaluated for semen volume, sperm concentration, motility, vigor, and morphology, as described in Experiment 1, for the screening of the ejaculate to be used and for the cryopreservation procedure.

Each ejaculate was separated into seven equal fractions, all diluted to a final concentration of $60x10^6$ spermatozoa/mL in Triladyl® (prepared according to the manufacturer; Minitube®). Different concentrations of IGF-I (Sigma-Aldrich, I3760) and insulin (Sigma-Aldrich, I2643) were added with the same concentrations used in Experiment 1. A total of seven groups was performed as follows: TCON: only Triladyl® extender (Control); TIGF100: Triladyl® + 100ng/mL of IGF-I; TIGF200: Triladyl® + 200ng/mL of IGF-I; TINS150: Triladyl® + 150µUI/mL of insulin; TINS200: Triladyl® + 200µUI/mL of insulin; TASS1: Triladyl® + 100ng/mL IGF-I + 150µUI/mL insulin; TASS2: Triladyl® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

The semen diluted and supplemented with different hormonal concentrations were incubated for 10 minutes at 37ºC and packaged in French straws (0.25 mL) labeled with the animal number, date, and treatment. The semen cryopreservation process was the same as described in Experiment 1.

Two straws from each batch, bull, and treatment were thawed in a water bath (30 sec/37°C) placed in microtubes, homogenized, and evaluated for motility and sperm membranes.

For sperm motility, a total of 4 µL of semen were dropped between slide and coverslip (pre-heated) for reading by phase-contrast microscopy (Nikon, Model Eclipse 200) at 100x magnification. The percentage of sperm motility was estimated according to the recommendation for bovine (Neves *et al*., 2013) by an experienced technician.

To evaluate plasma and acrosome membranes integrity and mitochondrial membrane potential was used the same protocol described in Experiment 1. A total of 200 cells were counted and classified into eight categories according to the fluorescence emitted by each probe, according to Celeghini *et al*. (2008) and after counting, the percentages of the followed cell populations were considered: plasma membrane integrity (PI), acrosome integrity (AI), high mitochondrial membrane potential (HMP), and spermatozoa showing plasma and acrosome membranes integrity and high mitochondrial membrane potential (PIAIHP).

Both experiments were performed in randomized blocks. Parametric variables were tested by ANOVA and the means were compared by the Tukey test. All variables were tested for normality by Lilliefors. Variables that had subjective responses or did not show normal distribution after linear transformation were analyzed as nonparametric test, comparing the rankings using the Friedman test. The data were analyzed using the BioEstat 5.0 program. Statistical significance was defined as $P \leq 0.05$.

RESULTS

The *in natura* sperm characteristics from each bull are presented in Table 1. In this table, are shown the mean and standard deviation from three ejaculates before semen cryopreservation.

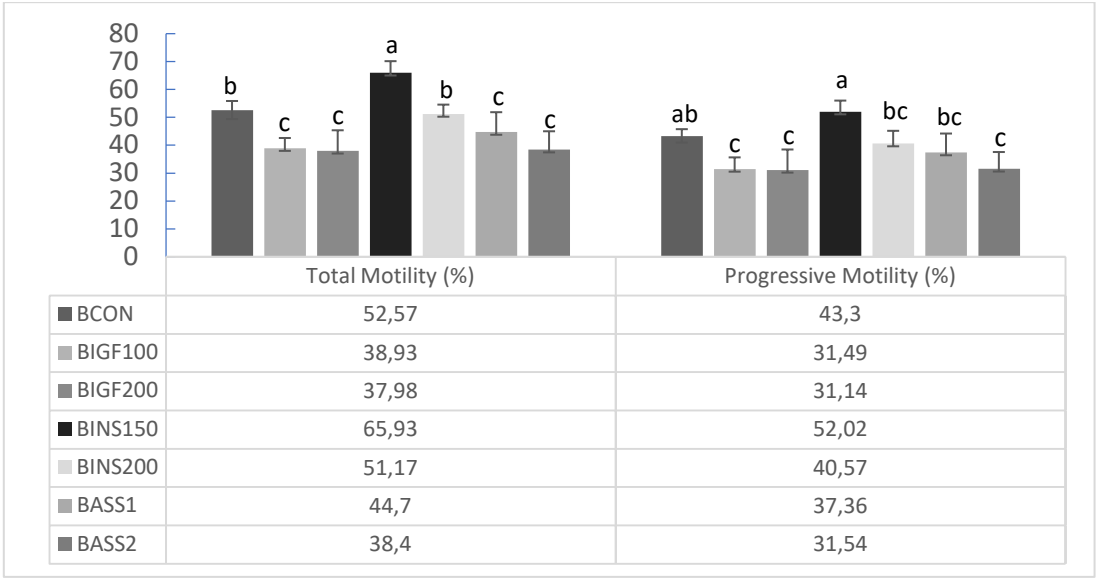
Table 1. Mean and standard deviation of *in natura* sperm characteristics considering the three ejaculates of each bull before semen dilution to cryopreservation

Sperm characteristics	Bulls			
	1	2	3	4
Motility (%)	75.0±5.0	78.33±2.88	71.66±2.88	73.33±2.88
Vigor (1-5)	2.83±0.76	2.66±0.28	2.83±0.38	3±0
Concentration ($x10^6$ sptz/mL)	215±80.46	470±268.37	169±56.712	570±247.84
Major defects (%)	7.66±0.57	6.66±2.08	6.33±2.08	4±2.64
Minor defects (%)	5.33±3.05	8.33±0.57	6.33±2.88	6±1.73
PIAIHP (%)	79.66±5.03	85.33±2.08	77.66±9.71	78±8.66

PIAIH= spermatozoa showing plasma and acrosome membranes integrity and high mitochondrial membrane potential

In experiment 1 using Botubov® extender, it was found that total motility was better preserved when 150µUI/mL of insulin (BINS150) was added to the Botubov® extender compared to the control group (BCON) and the other concentrations the IGF-I and insulin (Fig. 1). On the other hand, the addition of 200µUI/mL of insulin (BINS200) resulted in preservation of total motility similar to the control group (BCON), while all other treatments showed a decrease in total motility in relation to the BCON. The addition of 150 µUI/mL of insulin (BINS150) also preserved progressive motility better than the other additives (BIGF100, BIGF200, BINS200, BASS1and BASS2), but it was statistically similar to the BCON (Fig. 1).

In table 2, it is possible to observe that BINS150 group presented higher VCL and ALH than other groups. BCF was higher for BINS150 than for BIGF100, BIGF200 and BASS2, but it was similar to BCON. However, for VSL, VAP, LIN, STR and WOB characteristics, no effect of different concentrations of IGF-I and insulin using the Botubov® extender was observed.



	Total Motility (%)	Progressive Motility (%)
BCON	52,57	43,3
BIGF100	38,93	31,49
BIGF200	37,98	31,14
BINS150	65,93	52,02
BINS200	51,17	40,57
BASS1	44,7	37,36
BASS2	38,4	31,54

Figure 1. Means and standard error of total and progressive motility of cryopreserved bovine semen using Botubov® extender added with different concentrations of IGF-1, insulin, and combinations.
[a,bc] Different letters on the bars indicate statistically significant differences (P < 0.0001).
BCON (control): semen added only Botubov® extender; BIGF100: Botubov® + 100 ng/mL of IGF-I; BIGF200: Botubov® + 200 ng/mL of IGF-I; BINS150: Botubov® +150 µUI/mL of insulin; BINS200: Botubov® + 200 µUI/mL of insulin; BASS1: Botubov® +100 ng/mL of IGF-I + 150 µUI /mL of Insulin; BASS2: Botubov® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

Table 2. Means (± standard error) of sperm motility characteristics of cryopreserved bovine semen using Botubov® extender added with different concentrations of IGF-1, insulin, and combinations

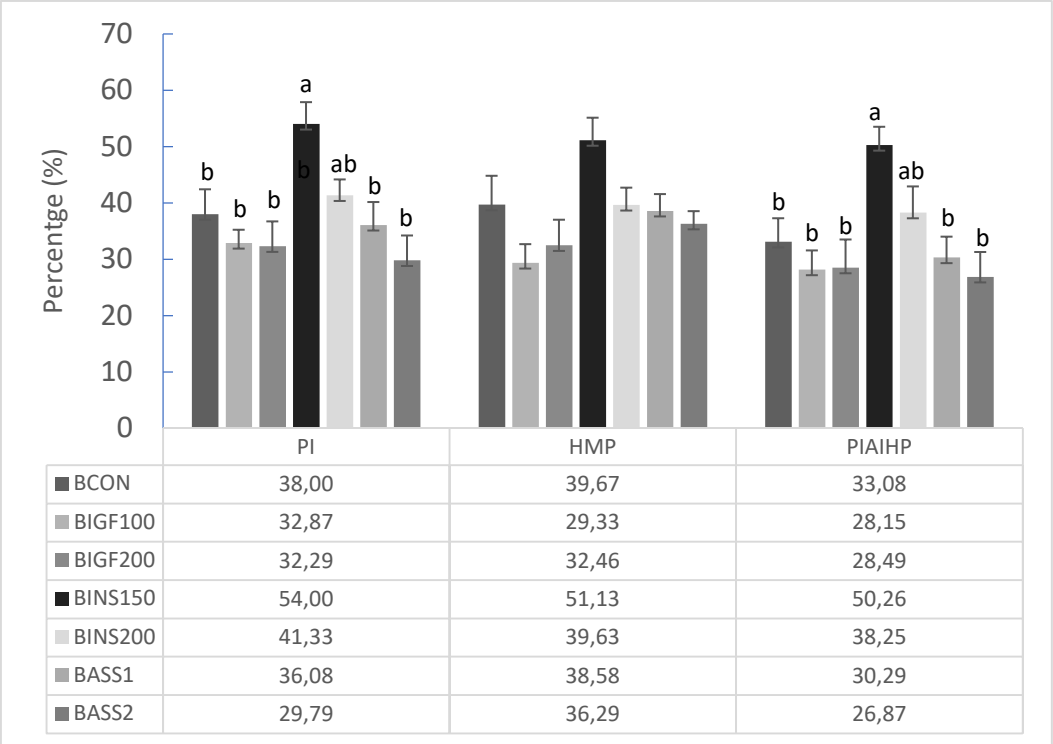
Parâmetros	BCON	BIGF100	BIGF200	BINS150	BINS200	BASS1	BASS2	p
VCL (µm/s)	127.75±7.7^{b}	134.18±12.6^{b}	124.51±10.0^{b}	152.16±10.2^{a}	132.39±11.2^{b}	135.42±13.3^{b}	129±10.5^{b}	≤0.05
VSL (µm/s)	86.43±8.0	82.79±5.9	77.74±7.3	89.72±8.1	81.07±7.2	84.74±7.7	80.77±5.9	≥0.05
VAP (µm/s)	99.68±9.2	95.94±7.5	89.98±8.2	98.23±9.8	94.88±8.6	97.25±8.6	93.18±6.5	≥0.05
LIN (%)	62.42±2.07	62.51±3.26	62.39±3.26	62.73±2.83	61.22±2.48	63.10±2.49	63.50±1.92	≥0.05
STR (%)	84.49±2.96	85.83±1.26	83.21±4.20	85.74±3.42	82.71±2.61	84.92±3.15	84.37±3.19	≥0.05
WOB (%)	71.95±1.67	72.28±2.54	72.14±2.70	72.47±1.92	71.54±1.70	72.18±2.08	73.08±1.76	≥0.05
ALH (µm)	2.44±0.10^{b}	2.43±0.20^{b}	2.21±0.16^{b}	2.81±0.16^{a}	2.40±0.14^{b}	2.42±0.18^{b}	2.36±0.18^{b}	≤0.05
BCF (Hz)	22.89±0.97^{ab}	21.64±1.14^{bc}	21.01±1.17^{c}	23.60±1.14^{a}	22.46±0.87^{abc}	22.85±1.17^{ab}	22.0±1.15^{bc}	<0.0001

[a,bc] Different letters in the same line indicate statistically significant differences (P ≤ 0.05).
BCON (control): semen added only Botubov® extender; BIGF100: Botubov® + 100 ng/mL of IGF-I; BIGF200: Botubov® + 200 ng/mL of IGF-I; BINS150: Botubov® +150 µUI/mL of insulin; BINS200: Botubov® + 200 µUI/mL of insulin; BASS1: Botubov® +100 ng/mL of IGF-I + 150 µUI /mL of Insulin; BASS2: Botubov® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin. VCL = curvilinear velocity; VSL = progressive velocity; VAP = path velocity; LIN = linearity; STR = straightness; WOB = oscillation of the real path; ALH = amplitude of the lateral head; BCF = beat cross frequency

As shown in Figure 2, the addition of insulin (BINS150 and BINS200) was beneficial in preserving the integrity of the plasma membrane after cryopreservation; however, only the concentration of 150 µUI/mL of insulin statistically better than the BCON and the groups with other additives. On the other hand, for the potential of mitochondrial membrane (Fig. 2)

and acrosomal integrity (Table 3), no effect was noted of supplementation of IGF-I and insulin with the Botubov® extender. For the sperm population with the intact plasma membrane, intact acrosome, and high mitochondrial membrane potential (PIAIHP), the BINS150 group demonstrated a positive effect on the preservation of sperm membranes when compared to the other groups.

Tables 4 and 5 show the individual responses of each bull to different semen additives. Table 4 presents the means and standard deviations of the sperm kinetics characteristics of three ejaculates from each bull, while in table 5 are the sperm membranes.



	PI	HMP	PIAIHP
BCON	38,00	39,67	33,08
BIGF100	32,87	29,33	28,15
BIGF200	32,29	32,46	28,49
BINS150	54,00	51,13	50,26
BINS200	41,33	39,63	38,25
BASS1	36,08	38,58	30,29
BASS2	29,79	36,29	26,87

Figure 2. Means and standard error of plasma membrane integrity (PI), high mitochondrial membrane potential (HMP) and spermatozoa showing plasma and acrosome membranes integrity and high mitochondrial membrane potential (PIAIHP) of cryopreserved bovine semen using Botubov® extender added with different concentrations of IGF-1, insulin, and combinations

[a,bc] Different letters on the bars indicate statistically significant differences ($P \leq 0.05$). BCON (control): semen added only Botubov® extender; BIGF100: Botubov® + 100 ng/mL of IGF-I; BIGF200: Botubov® + 200 ng/mL of IGF-I; BINS150: Botubov® +150 µUI/mL of insulin; BINS200: Botubov® + 200 µUI/mL of insulin; BASS1: Botubov® +100 ng/mL of IGF-I + 150 µUI /mL of Insulin; BASS2: Botubov® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

Table 3. Means (± standard error) of acrosome integrity (AI) of cryopreserved bovine semen using Botubov® extender added with different concentrations of IGF-1, insulin, and combinations

Parameters	BCON	BIGF100	BIGF200	BINS150	BINS200	BASS1	BASS2
AI (%)	78.04±1.57	70.13±1.82	78.42±3.23	81.13±1.38	79.96±2.28	76.71±3.93	75.92±3.52

No statistically significant differences ($P > 0.05$).

BCON (control): semen added only Botubov® extender; BIGF100: Botubov® + 100 ng/mL of IGF-I; BIGF200: Botubov® + 200 ng/mL of IGF-I; BINS150: Botubov +150 µUI/mL of insulin; BINS200: Botubov® + 200 µUI/mL of insulin; BASS1: Botubov +100 ng/mL of IGF-I + 150 µUI/mL of Insulin; BASS2: Botubov + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

Table 4. Means and standard deviation from three ejaculates of each bull of sperm motility characteristics of cryopreserved bovine semen using Botubov® extender added with different concentrations of IGF-1, insulin, and combinations

Bull	Treatment	SM (%)	TM (%)	PM (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
1		36.67±	47.77±	39.10±	128.43±	101.47±	114.20±	67.16±	88.77±	75.57±	2.47±	24.67±
	BCON	5.77	14.72	13.19	7.45	12.57	13.27	3.59	1.32	3.09	0.06	1.69
		33.33±	31.23±	22.93±	124.67±	82.57±	95.57±	65.60±	85.87±	76.23±	2.30±	21.93±
	BIGF100	5.77	8.27	9.40	13.77	19.31	17.27	8.33	4.78	5.47	0.10	1.50
		30.00±	34.87±	26.27±	127.67±	87.27±	98.53±	68.47±	88.63±	77.27±	2.20±	22.53±
	BIGF200	10.00	14.43	8.73	8.09	1.56	3.75	3.12	1.76	2.15	0.17	2.17
		56.67±	64.37±	47.50±	157.57±	114.77±	112.70±	70.40±	91.53±	75.97±	2.67±	25.43±
	BINS150	5.77	9.99	3.66	10.06	6.65	8.88	0.60	6.81	5.46	0.12	1.24
		46.67±	47.67±	33.50±	136.53±	85.47±	106.43±	62.57±	85.60±	73.07±	2.47±	23.27±
	BINS200	11.55	20.75	11.52	6.39	7.06	15.88	3.07	2.86	1.96	0.12	0.23
		35.00±	40.73±	34.17±	140.73±	95.33±	107.30±	67.53±	88.73±	76.03±	2.43±	23.27±
	BASS1	8.66	14.07	10.73	7.79	14.53	14.96	6.85	1.19	6.88	0.23	2.51
		31.67±	27.10±	21.60±	132.97±	88.97±	101.43±	66.73±	87.40±	76.20±	2.40±	22.17±
	BASS2	2.89	4.06	5.36	4.81	12.44	9.34	7.06	4.29	4.50	0.00	1.19
2		45.00±	57.23±	45.07±	148.73±	99.13±	116.97±	61.77±	84.87±	72.77±	2.67±	24.47±
	BCON	5.00	7.48	6.10	13.54	8.76	11.57	2.16	1.01	3.03	0.23	1.42
		40.00±	37.53±	30.50±	168.47±	99.63±	117.30±	59.33±	84.87±	69.80±	2.83±	24.70±
	BIGF100	17.32	23.83	21.06	11.42	6.18	3.87	5.37	2.72	4.69	0.25	1.75
		33.33±	37.57±	29.00±	151.63±	92.13±	108.20±	60.77±	85.17±	71.40±	2.67±	22.97±
	BIGF200	5.77	11.64	8.23	4.49	3.19	3.21	3.21	2.33	2.08	0.21	1.10
		58.33±	69.77±	51.83±	175.40±	103.80±	119.87±	61.30±	85.43±	71.63±	3.10±	25.63±
	BINS150	7.64	17.15	10.45	8.26	3.48	4.57	6.35	2.80	5.02	0.17	0.81
		48.33±	56.53±	44.93±	162.10±	99.33±	117.20±	61.43±	84.73±	72.40±	2.70±	24.53±
	BINS200	2.89	6.79	9.32	6.48	6.31	2.76	5.97	3.35	4.12	0.20	0.55
		38.33±	44.67±	36.10±	169.77±	100.73±	116.27±	59.67±	86.43±	68.83±	2.83±	25.97±
	BASS1	7.64	17.38	13.67	17.28	12.14	8.98	8.37	4.49	6.63	0.38	1.50
		31.67±	39.10±	31.43±	152.73±	92.77±	106.13±	61.30±	87.43±	70.10±	2.77±	25.03±
	BASS2	12.58	19.05	16.98	19.55	6.37	6.11	7.43	1.24	7.94	0.49	3.86
3		40.00±	59.00±	49.33±	111.93±	73.23±	82.90±	63.60±	88.33±	72.00±	2.17±	21.50±
	BCON	10.00	19.63	18.92	10.72	2.51	2.60	1.51	0.31	1.67	0.12	0.72
		41.67±	48.73±	42.73±	108.77±	74.57±	83.47±	68.57±	89.27±	76.80±	1.93±	20.50±
	BIGF100	2.89	5.65	5.89	11.52	7.67	7.92	2.30	1.18	1.85	0.15	2.00
		48.33±	57.50±	51.77±	108.67±	71.77±	81.60±	66.30±	88.33±	75.03±	1.97±	20.77±
	BIGF200	10.41	3.42	6.86	7.17	5.92	6.51	1.51	0.59	1.55	0.06	0.21
		56.67±	73.67±	63.13±	126.27±	77.93±	84.60±	67.23±	89.80±	74.87±	2.43±	22.27±
	BINS150	7.64	13.32	5.25	3.70	1.18	2.72	1.75	0.44	1.56	0.06	0.12
		51.67±	57.00±	51.33±	110.50±	73.47±	82.07±	66.40±	85.60±	74.17±	2.00±	21.27±
	BINS200	2.89	7.40	5.49	3.44	6.37	5.19	3.70	2.86	2.46	0.00	0.25
		50.00±	63.73±	55.87±	107.30±	72.03±	81.00±	67.20±	88.90±	75.53±	1.93±	21.53±
	BASS1	10.00	3.03	2.42	9.44	5.59	6.64	0.82	0.66	0.93	0.15	0.50
		46.67±	56.60±	48.43±	101.77±	67.93±	77.20±	66.77±	87.83±	76.07±	1.87±	21.30±
	BASS2	7.64	5.64	5.42	16.18	10.24	10.39	2.11	1.89	2.00	0.21	1.47
4		41.67±	46.30±	39.73±	121.93±	71.92±	81.33±	57.17±	84.23±	67.47±	2.47±	20.93±
	BCON	18.93	32.54	29.44	7.58	16.62	10.40	10.00	7.22	6.31	0.21	1.21
		41.67±	38.23±	29.83±	134.83±	74.40±	87.43±	56.53±	85.20±	66.30±	2.67±	19.43±
	BIGF100	22.55	27.45	21..32	33.76	6.66	9.42	8.42	1.81	9.04	0.81	2.72
		23.33±	22.00±	17.53±	110.10±	59.80±	71.60±	54.03±	82.00±	64.87±	2.00±	17.80±
	BIGF200	5.77	24.39	18.28	3.12	19.84	16.91	16.95	9.89	13.91	0.26	3.12
		50.00±	54.93±	45.63±	149.43±	84.93±	82.07±	55.30±	81.90±	67.40±	3.07±	21.07±
	BINS150	17.32	25.28	21.08	20.27	5.16	15.77	6.63	5.38	3.91	0.55	1.22
		41.67±	43.50±	32.53±	120.43±	66.03±	80.50±	54.47±	81.30±	66.53±	2.43±	20.77±
	BINS200	18.93	29.70	24.16	6.15	16.11	13.91	10.86	6.12	8.41	0.21	0.92
		26.67±	29.67±	23.30±	123.90±	70.90±	84.43±	58.00±	83.53±	68.30±	2.50±	20.63±
	BASS1	5.77	20.13	15.68	4.01	12.68	8.62	12.58	6.91	8.84	0.35	0.95
		26.67±	30.80±	24.73±	128.53±	73.43±	87.97±	59.20±	83.90±	69.93±	2.43±	19.50±
	BASS2	5.77	13.47	13.15	30.20	1.60	7.08	13.21	7.88	9.70	0.67	0.72

SM = subjective motility; TM = total motility; PM = progressive motility; VCL = curvilinear velocity; VSL = progressive velocity; VAP = path velocity; LIN = linearity; STR = straightness; WOB = oscillation of the real path; ALH = amplitude lateral of the head; BCF = beat cross frequency. BCON (control): semen added only Botubov® extender; BIGF100: Botubov® + 100ng/mL of IGF-I; BIGF200: Botubov® + 200ng/mL of IGF-I; BINS150: Botubov® +150µUI/mL of insulin; BINS200: Botubov® + 200µUI/mL of insulin; BASS1: Botubov® +100ng/mL of IGF-I + 150µUI /mL of Insulin; BASS2: Botubov® + 200ng/mL of IGF-I + 200µUI/mL of insulin.

Table 5. Means and standard deviation from three ejaculates of each bull of plasma membrane integrity (PI), intact acrosome (IA), high mitochondrial membrane potential (HMP) and spermatozoa showing plasma and acrosome membranes integrity and high mitochondrial membrane potential (PIAIHP) of cryopreserved bovine semen using Botubov® extender added with different concentrations of IGF-1, insulin, and combinations

Bull	Treatment	PI (%)	IA (%)	HMP (%)	PIAIHP (%)
1	BCON	45.00±23.94	78.33±18.99	44.17±22.75	32.83±13.32
	BIGF100	31.83±14.22	66.00±16.04	22.33±16.65	21.67±17.18
	BIGF200	29.33±10.42	76.00±19.98	25.83±9.78	20.50±3.77
	BINS150	59.00±11.00	80.17±13.53	52.33±13.16	56.33±12.50
	BINS200	41.33±18.66	73.67±21.33	39.83±17.96	39.83±17.96
	BASS1	27.50±6.08	65.83±15.00	41.17±24.57	26.17±5.20
	BASS2	17.83±5.80	74.17±28.29	41.17±40.90	15.83±3.25
2	BCON	45.67±12.66	80.33±6.21	52.00±19.92	45.00±13.48
	BIGF100	39.50±17.39	74.83±9.22	38.50±17.76	37.83±16.60
	BIGF200	30.83±12.39	75.83±8.08	31.17±12.34	29.33±11.24
	BINS150	65.50±12.13	82.17±7.52	55.50±17.30	61.50±15.88
	BINS200	50.00±10.58	84.00±6.14	47.83±11.41	54.00±17.50
	BASS1	32.50±15.80	78.50±19.22	41.83±6.01	31.17±15.63
	BASS2	32.83±8.69	71.17±3.01	33.67±12.35	31.67±9.83
3	BCON	32.67±7.37	80.00±4.36	32.33±15.12	30.83±7.08
	BIGF100	31.83±3.18	70.33±24.95	28.00±4.09	26.50±4.58
	BIGF200	41.50±6.50	88.00±2.60	45.67±4.65	39.17±7.94
	BINS150	48.33±9.45	84.33±5.03	41.00±11.27	46.33±8.50
	BINS200	40.33±5.35	79.67±3.25	37.67±5.11	35.67±0.29
	BASS1	43.83±16.43	84.67±2.57	41.67±15.82	40.67±17.47
	BASS2	38.83±3.62	86.33±4.19	38.83±4.25	35.83±1.89
4	BCON	27.67±14.98	73.50±5.27	30.17±18.82	27.00±16.09
	BIGF100	22.83±12.09	63.33±14.05	28.50±4.77	21.00±11.32
	BIGF200	24.17±17.62	73.83±9.45	27.17±16.65	21.83±17.82
	BINS150	46.50±15.88	77.83±5.20	39.67±17.06	45.00±16.53
	BINS200	34.17±15.73	82.50±12.32	33.17±16.44	29.50±17.76
	BASS1	30.83±17.25	77.83±11.62	29.67±17.92	23.50±14.41
	BASS2	24.33±8.02	71.17±8.25	30.17±13.48	21.50±9.26

BCON (control): semen added only Botubov® extender; BIGF100: Botubov® + 100ng/mL of IGF-I; BIGF200: Botubov® + 200 ng/mL of IGF-I; BINS150: Botubov® +150µUI/mL of insulin; BINS200: Botubov® + 200µUI/mL of insulin; BASS1: Botubov® +100 ng/mL of IGF-I + 150 µUI /mL of Insulin; BASS2: Botubov® + 200ng/mL of IGF-I + 200µUI/mL of insulin.

In experiment 2 using Tryladyl® extender, the addition of insulin regardless of concentration (150 and 200µUI/mL), to the Triladyl® extender for cryopreservation of semen improved the preservation of sperm motility when compared to the other groups (Fig. 3).

According to the results, the highest percentages of plasma membrane integrity (Fig. 4) and high

mitochondrial membrane potential (Fig. 5) for post-cryopreservation semen were obtained when 150 µUI/mL of insulin (TINS150) was added to the Triladyl® extender; however, these values did not differ from the TCON group. The same result was observed for the percentage of PIAIHP cells (Fig. 6). On the other hand, acrosome integrity was not affected by the addition of hormones (Table 6).

In the table 7 are shown the means and standard deviations of the sperm characteristics of three ejaculates from each bull after cryopreservation with the Triladyl® extender with the different additives, allowing the individual response of each bull to be evaluated.

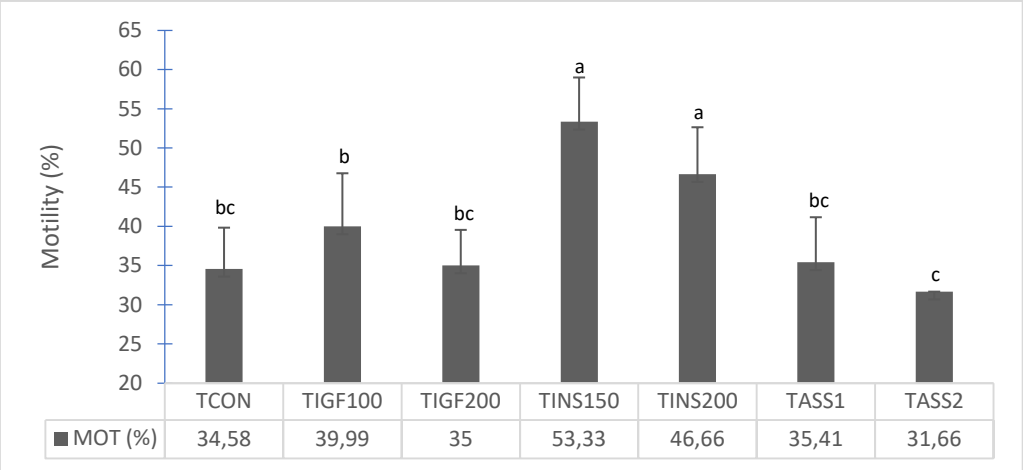


Figure 3. Means and standard error of sperm motility of cryopreserved bovine semen using Triladyl® extender added with different concentrations of IGF-1, insulin, and combinations.
[a,bc] Different letters on the bars indicate statistically significant differences (P ≤ 0.0001). TCON (Control): semen added only Triladyl®; TIGF100: Triladyl® + 100 ng/mL of IGF-I; TIGF200: Triladyl® + 200 ng/mL of IGF-I; TINS150: Triladyl® + 150 µUI/mL of insulin; TINS200: Triladyl® + 200 µUI/mL of insulin; TASS1: Triladyl® + 100 ng/mL IGF-I + 150 µUI/mL insulin; TASS2: Triladyl® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

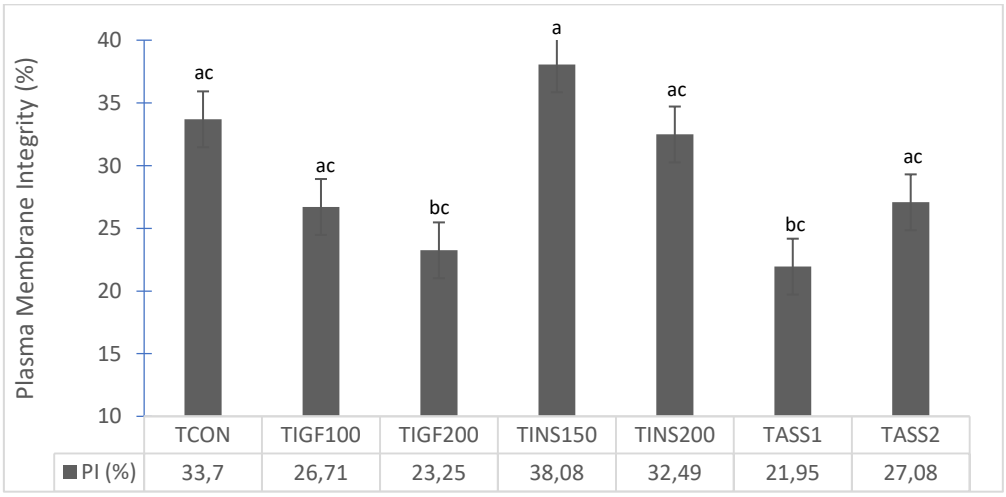


Figure 4. Means and standard error of plasma membrane integrity (PI) of cryopreserved bovine semen using Triladyl® extender added with different concentrations of IGF-1, insulin, and combinations.
[a,bc] Different letters on the bars indicate statistically significant differences (P ≤ 0.05). TCON (Control): semen added only Triladyl®; TIGF100: Triladyl® + 100 ng/mL of IGF-I; TIGF200: Triladyl® + 200 ng/mL of IGF-I; TINS150: Triladyl® + 150 µUI/mL of insulin; TINS200: Triladyl® + 200 µUI/mL of insulin; TASS1: Triladyl® + 100 ng/mL IGF-I + 150 µUI/mL insulin; TASS2: Triladyl® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

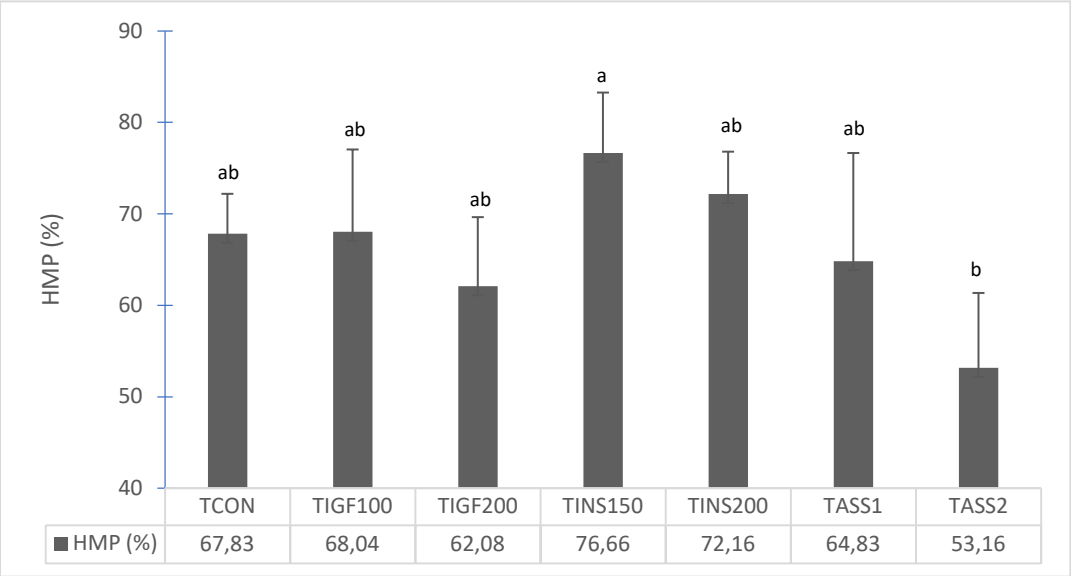


Figure 5. Means and standard error of high mitochondrial membrane potential (HMP) of cryopreserved bovine semen using Triladyl® extender added with different concentrations of IGF-1, insulin, and combinations.
[a,bc] Different letters on the bars indicate statistically significant differences (P ≤ 0.05). TCON (Control): semen added only Triladyl®; TIGF100: Triladyl® + 100 ng/mL of IGF-I; TIGF200: Triladyl® + 200 ng/mL of IGF-I; TINS150: Triladyl® + 150 µUI/mL of insulin; TINS200: Triladyl® + 200 µUI/mL of insulin; TASS1: Triladyl® + 100 ng/mL IGF-I + 150 µUI/mL insulin; TASS2: Triladyl® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

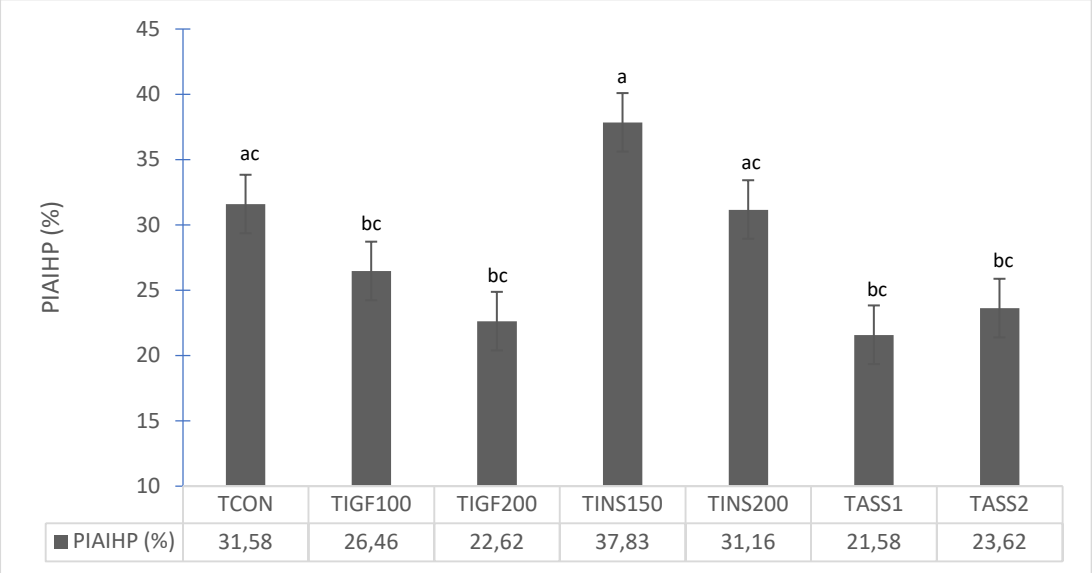


Figure 6. Means and standard error of percentage of spermatozoa showing plasma and acrosome membranes integrity and high mitochondrial membrane potential (PIAIHP) of cryopreserved bovine semen using Triladyl® extender added with different concentrations of IGF-1, insulin, and combinations[a,bc] Different letters on the bars indicate statistically significant differences (P ≤ 0.0001).
TCON (Control): semen added only Triladyl®; TIGF100: Triladyl® + 100 ng/mL of IGF-I; TIGF200: Triladyl® + 200 ng/mL of IGF-I; TINS150: Triladyl® + 150 µUI/mL of insulin; TINS200: Triladyl® + 200 µUI/mL of insulin; TASS1: Triladyl® + 100 ng/mL IGF-I + 150 µUI/mL insulin; TASS2: Triladyl® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

Table 6. Means (± standard error) of acrosome integrity (AI) of cryopreserved bovine semen using Triladyl® extender added with different concentrations of IGF-1, insulin, and combination

Parameters	TCON	TIGF100	TIGF200	TINS150	TINS200	TASS1	TASS2
AI (%)	81.70±3.21	81.58±1.82	80.45±3.50	87.47±3.89	82.29±2.07	83.91±3.71	83.62±2.33

No statistically significant differences (P > 0.05). TCON (Control): semen added only Triladyl®; TIGF100: Triladyl® + 100 ng/mL of IGF-I; TIGF200: Triladyl® + 200 ng/mL of IGF-I; TINS150: Triladyl® + 150 µUI/mL of insulin; TINS200: Triladyl® + 200 µUI/mL of insulin; TASS1: Triladyl® + 100 ng/mL IGF-I + 150 µUI/mL insulin; TASS2: Triladyl® + 200 ng/mL of IGF-I + 200 µUI/mL of insulin.

Table 7. Means and standard deviation from three ejaculates of each bull of subjective motility (SM), plasma membrane integrity (PI), intact acrosome (IA), high mitochondrial membrane potential (HMP), and spermatozoa showing plasma and acrosome membranes integrity and high mitochondrial membrane potential (PIAIHP) of cryopreserved bovine semen using Triladyl® extender added with different concentrations of IGF-1, insulin, and combinations

Bull	Treatment	SM (%)	PI (%)	IA (%)	HMP (%)	PIAIHP (%)
1	TCON	46.67±5.77	42.50±7.47	76.33±14.75	80.17±9.67	40.83±9.00
	TIGF100	48.33±5.77	37.67±9.57	77.17±13.14	81.67±9.50	37.67±9.57
	TIGF200	40.00±5.00	23.50±10.00	78.50±18.03	67.33±14.07	23.17±9.75
	TINS150	65.00±0.00	48.17±12.85	87.00±9.84	85.17±5.80	48.17±12.85
	TINS200	58.33±7.64	50.83±6.83	78.83±5.25	80.17±4.19	48.33±7.11
	TASS1	45.00±0.00	31.50±4.27	84.33±14.01	75.17±10.30	31.33±4.25
	TASS2	38.33±7.64	34.00±15.52	84.50±12.62	62.83±14.92	33.50±15.31
2	TCON	40.00±0.00	54.50±10.40	85.67±9.07	66.83±19.21	50.00±10.04
	TIGF100	53.33±5.77	40.83±4.91	80.83±11.45	70.50±16.50	40.50±5.22
	TIGF200	45.00±0.00	34.00±9.99	78.00±15.22	67.83±13.33	33.00±9.73
	TINS150	60.00±5.00	55.00±5.77	95.83±4.37	86.33±0.58	54.50±4.92
	TINS200	55.00±5.00	46.33±6.66	85.00±8.67	80.17±8.28	45.67±7.29
	TASS1	43.33±14.43	32.33±10.56	85.33±6.90	79.00±4.44	32.00±9.99
	TASS2	36.67±11.55	49.50±20.66	77.67±20.89	62.67±13.58	36.67±8.22
3	TCON	26.67±5.77	18.83±10.25	88.67±9.29	64.50±43.75	16.50±12.58
	TIGF100	35.00±8.66	21.67±17.16	86.00±3.12	78.00±18.21	21.00±16.09
	TIGF200	30.00±5.00	23.83±15.25	90.67±5.48	73.33±27.43	23.50±15.26
	TINS150	48.33±2.89	27.17±2.02	89.83±6.79	77.33±26.84	27.00±2.00
	TINS200	40.00±0.00	19.33±7.25	86.67±9.39	64.83±40.18	17.50±6.06
	TASS1	33.33±2.89	15.50±10.04	92.00±8.05	75.67±18.76	14.83±9.65
	TASS2	26.67±7.64	15.83±7.65	83.33±13.32	58.33±24.10	15.50±7.37
4	TCON	25.00±5.00	19.00±9.34	76.17±14.77	59.83±36.30	19.00±9.34
	TIGF100	23.33±15.28	6.67±4.25	82.33±13.29	42.00±35.38	6.67±4.25
	TIGF200	25.00±13.23	11.67±10.75	74.67±21.68	39.83±33.14	10.8310.05±
	TINS150	40.00±15.00	22.00±8.72	77.17±14.34	57.83±22.68	21.67±8.50
	TINS200	33.33±20.21	13.50±10.64	78.67±14.22	63.50±38.67	13.17±10.91
	TASS1	20.00±0.00	8.50±8.79	74.00±16.82	29.50±30.63	8.17±8.69
	TASS2	25.00±8.66	9.00±0.87	89.00±19.05	28.83±23.96	8.83±1.15

TCON (Control): semen added only Triladyl®; TIGF100: Triladyl® + 100ng/mL of IGF-I; TIGF200: Triladyl® + 200ng/mL of IGF-I; TINS150: Triladyl® + 150µUI/mL of insulin; TINS200: Triladyl® + 200µUI/mL of insulin; TASS1: Triladyl® + 100ng/mL IGF-I + 150µUI/mL insulin; TASS2: Triladyl® + 200ng/mL of IGF-I + 200µUI/mL of insulin.

DISCUSSION

Several factors during sperm cryopreservation process affect the sperm performance after thawing, which includes the *in natura* sperm quality, the freeze/thaw method, and the extenders and cryoprotectants used (Grotter *et al*., 2019). The process of semen freezing induces physical and chemical stress, which causes damage to sperm plasma membrane, mitochondria, acrosome, motility, and morphology. All of these result in a decrease in the sperm fertilization ability (Shokri *et al*., 2019). In this study, IGF-I and insulin, at

different concentrations, were added to two extenders egg yolk-based, to improve the preservation of function and structure of bovine sperm from cryopreservation damage, and this benefit is expected even when the definition of extenders composition is unknown.

The first experiment was carried out with a commercial extender (Botubov®), which has in its composition sugars, antioxidants, amino acids, egg yolk, glycerol, and water (Botupharma®, Botucatu-SP). In the second experiment, the interaction of the additives in another commercial semen extender (Triladyl®) was tested; this one with sugars, TRIS (Hydroxymethyl), citric acid, buffers, antibiotics, egg yolk, glycerol, and water (Minitube®) in its composition. Although both extenders are based on egg yolk and have similar components, the exact composition of each one is unknown, protected by trade secrets. Thus, different interactions could occur that could show different benefits of the additives in favor of one of them. However, similar results were observed regarding the characteristics that were evaluated. The groups that received insulin at a concentration of 150 µUI/mL, in both experiments, presented better preservation of the sperm cells during freezing, reflecting in a higher percentage of sperm motility and intact cells components after thawing.

The improvement in the preservation of sperm motility with the addition of insulin, found in both experiments, can be explained by its performance, already well defined in the previous literature. Briefly, sperm motility is a process dependent on the production of ATP and the glycolytic enzymes present in the fibrous sheath of the flagella are one of the main energy suppliers. In addition, the mitochondria located in the intermediate piece are also energy suppliers by oxidative phosphorylation (Takei *et al*., 2014). Energy is important for motility maintenance, sperm capacitation and subsequently oocyte fertilization, as the energy is produced by the metabolism of external substrates (Ding *et al*., 2015). Small molecules such as monosaccharides easily cross the sperm plasma membrane bilayer, the other molecules receive support from membrane proteins that can actively or passively transport glucose (Ding *et al*., 2015). The presence of insulin is crucial for the glucose movement across the plasma membrane; however, when semen is diluted for cryopreservation, there is a decrease in the concentration of insulin present in the seminal plasma, so it is believed that its addition to the extender in the present study enabled the glucose to move across membranes, subsequently producing more ATP, which is essential for sperm motility.

Other studies corroborate the present, such as in humans, in which the cryopreserved semen with different concentrations of insulin (10, 100, 500 and 1000 ng/mL) increased the sperm motility percentage (Shokri *et al*., 2019). In other study, human semen supplemented with insulin (10 µmol) and leptin has shown a prolonged preservation of sperm motility and acrosomal reaction, thus increasing the oocyte's fertilization capacity (Lampião and Plessis, 2008). Men with insulin deficiency production had lower percentages of sperm motility and increased sperm defects, mainly from the acrosome region (Baccetti *et al*., 2002). The action of insulin on sperm kinetics is due to this affinity of 100 to 1,000 times for the heterologous receptors present in the sperm membranes, in this case, those of IGF-I, thus favoring sperm motility for greater glucose uptake (Clemmons *et al*., 1992).

In the present study, it was observed a higher percentage of cells with the intact plasma membrane, intact acrosome, and high mitochondrial membrane potential (PIAIHP) when insulin (150 µUI/mL) was added to both extenders. Such responses to the insulin action can be assured, since the plasma membrane, acrosomal and metabolism are cytological targets (Souza *et al*., 2012). During semen cryopreservation, between 15º to 5º C, mostly membranes alterations occur, such as rupture and loss in the phospholipid arrangements. In this interval, the plasma membrane changes its state from liquid to gel, decreasing the motility and increasing its permeability. Integral proteins, present in the plasma membrane, also suffer modulation during cryopreservation (Watson, 2000). Thus, it is believed that insulin binds to the sperm membrane receptors, which allows more protection during the cryopreservation process.

Reinforcing the results presented in this study, according to Aquila *et al*. (2005), the sperm mobilizes insulin from its granules at the time of

hyperactivation, and this process activates a better source of energy to the sperm cell, which, in turn, destabilizes the components that maintain the integrity of the plasma and acrosomal membranes before acrosomal reaction. This statement was verified in the present study, as the treatments that used insulin, especially in the concentration of 150 µUI/mL, had the highest percentage of intact cells (PIAIHP). The same was observed when membranes integrity was separately evaluated for both extenders tested.

Van Tilburg *et al*. (2008) found a significant effect of insulin on acrosome integrity. They reported that the higher the concentration of insulin was added to the freezing extender the greater is the sperm with intact acrosome, still, insulin does not induce the acrosome reaction but preserves the integrity of the acrosome, attributing the action of this by inhibiting the binding of cAMP to cAMP-dependent protein kinase and activating the phosphatidylinositol 3-, 4-bisphosphate and phosphatidyl -inositol 3,4,5-triphosphate, mechanisms responsible for capacitation and acrosome reaction. However, this effect was not found in the present study.

In relation to IGF-I, no significant effect was reported of its action on the different extenders, the same was observed by Costa *et al*. (2020) in a study with bovine cryopreserved semen. They added 150 ng/mL of IGF-I to the extender and no effects were also observed. Differently, Henricks *et al*. (1998), Pan *et al*. (2015), Sang-Min *et al*. (2014), Serveraju *et al*. (2009), Maskarevich *et al*. (2014) and Padilha *et al*. (2012), in cattle, dogs, buffalo ram, goats and ovine, respectively, found an improvement in the sperm quality when extenders were supplemented with different concentrations of IGF-I. According to Kumar *et al*. (2019), in buffaloes, IGF-I increased the concentration of intracellular calcium, resulting in better progressive sperm motility. However, this was not noticed in the current study and could be explained by the difference in membranes composition, by the sensitivity to cryopreservation between species and by the concentration, IGF-I added in the cryopreservation extenders.

The addition of IGF-I and insulin combined did not improve sperm quality after thawing in both Triladyl® and Botubov® extenders. It was expected that the association of these hormones would guarantee better cell preservation, as IGF-I acted on sperm motility and linear speed, through the connection with receptors present in sperm, and that insulin acted on metabolism (Souza *et al*., 2012). The associated groups showed a lower percentage of sperm motility and a lower number of intact cells. The concentration used in these associations likely provided a cytotoxic effect to sperm, decreasing sperm motility, membrane integrity and providing greater damage to cells.

CONCLUSION

In conclusion, the addition of 150 µUI/mL of insulin to egg yolk-based extenders (Botubov® and Triladyl®) improves the preservation of sperm motility, plasma membrane integrity and high mitochondrial membrane potential in cryopreserved bovine semen, regardless of the extender composition.

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