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Glutathione and IGF-1 in bovine seminal cryopreservation: oxidative stress response and pregnancy rate

[*Glutationa e IGF-1 na criopreservação seminal bovina: resposta do estresse oxidativo e taxa degestação*]

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ABSTRACT

The objective of this study was to evaluate the rate of conception, metabolic, and structural conditions of cryopreserved bovine sperm cells, plus extender with IGF-1 and glutathione (GSH). 12 ejaculations of Nelore bulls were used, submitted to treatments: control, gSH (2mM/mL), IGF-1 (100ng/mL) and gSH (1mM/mL) + IGF-1 (50ng/mL). After cryopreservation and thawing the semen passed the fast thermo resistance test (TTR), plasma membrane and acrosomal integrity (PIAI), mitochondrial membrane potential (AP), oxidative stress, and conception rate. Tukey test was used for the statistical analysis of the parametric variables and the Friedman test for nonparametric. The gestation percentage was compared by the Chi-square test. There was no statistical difference (P<0.05) between treatments for the TTRr variable. Otherwise in the oxidative stress evaluated with the CellROX probe was noted that the IGF-1 showed the highest number of reactive cells (P<0.05). The PIAI, AP and gestation rate showed no difference among treatments (P>0.05), with an average of conceptions of 36.58%. It is concluded that IGF-1, gSH and their association did not cause changes in sperm motility, mitochondrial potential, plasma and acrosomal membrane integrity. IGF-1 increased oxidative stress, however, there was no difference in the gestation rate among the treatments.

Keywords: antioxidant, growth factor, gSH, zebu

RESUMO

Objetivou-se avaliar a taxa de concepção, as condições metabólicas e estrutural das células espermáticas bovinas criopreservadas, acrescidas de diluidores com fator de crescimento semelhante à insulina do tipo 1(IGF-1) e glutationa (GSH). Foram utilizados 12 ejaculados de touros da raça Nelore, submetidos aos tratamentos: controle, gSH (2mM/mL), IGF-1 (100ng/mL) e gSH (1mM/mL) + IGF-1 (50ng/mL). Após a criopreservação e descongelação, o sêmen passou pelos testes de termorresistência rápida (TTRr), integridade de membrana plasmática e acrossomal (PIAI), alto potencial mitocondrial (AP), estresse oxidativo e taxa de concepção. Utilizou-se o teste de Tukey para as análises estatísticas das variáveis paramétricas e o teste de Friedman para as não paramétricas, com significância de 5%. A percentagem de gestação foi comparada pelo teste do qui-quadrado. Não hove diferença estatística (P<0,05) entre os tratamentos para a variável TTRr. Já no estresse oxidativo avaliado com a sonda CellROX, observou-se que o IGF-1 apresentou maior quantidade de células reativas (P<0,05), 36,38± 24,10. A PIAI, o AP e a taxa de gestação não apresentaram diferença entre tratamentos (P>0,05), com média de concepções de 36,58%. Conclui-se que o IGF-1, a gSH e a sua associação não causaram mudanças na motilidade espermática, no potencial mitocondrial, na integridade da membrana plasmática e acrossomal. O IGF-1 aumentou o estresse oxidativo, porém sem diferença na taxa degestação entre os tratamentos.

Palavras-chave: antioxidante, fator de crescimento, gSH, zebu

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INTRODUCTION

Seminal plasma is a complex mixture of secretions originated in the testicles, epididymides, and accessory sexual glands (Manjunath *et al*., 1993), serving as a vehicle for ejaculated sperm, in addition to containing factors that influence their fertilization capacity (Calvete *et al*., 1996). Because of the interaction that exists between the seminal fluid and the sperm cells, the most recent research points to certain growth factors as determinants in the seminal quality. Among these, the insulin-like growth factor 1 (IGF-I) is considered one of the most important, as it favors the development of germ cells and the maturation of sperm (Skinner, 1991).

According to the review by Kumar *et al*. (2019), IGF-I supplementation improves semen quality in several species, such as dogs, stallions, and bulls, and their protective function may be due to antioxidant activities (Selvaraju *et al*., 2016), which is diminished or lost in the process of handling seminal plasma that routinely require dilution, reducing the concentration of these, which results in cell stress (Bilodeau *et al*., 2000). Another antioxidant that is lost in this seminal dilution is glutathione (GSH) which is responsible for the reconstruction of thiol groups (-SH) in proteins, which can be eliminated during oxidative stress, as well as protecting cell membranes from lipid oxidation preventing the formation of free oxygen (Lenzi *et al*., 1994). A great number of studies propose that the production of reactive oxygen species (ROS) and the semen's oxidative capacity enhance the harmful effects of seminal manipulation (Bustamante Filho *et al*., 2006).

Oxidative stress resulting from the imbalance between the concentration of antioxidants about the concentration of free radicals, generally (Nichi *et al*., 2006) imposes conditions that are extremely unfavorable to the maintenance of their viability. In spermatozoa, specifically, ROS causes structural damage to the acrosome, the head, and the intermediate part, as well as initiate apoptosis and induce DNA fragmentation (Castro, 2010). Knowledge about the oxidative damage caused to sperm cells resulting from the imbalance between the physiological concentrations of oxidants and antioxidants, resulting from increased production of ROS or reduced total antioxidant capacity of semen, can provide important information to improve sperm viability, mainly in post cryopreserved samples (Guerra *et al*., 2004). Thus, to improve the preservation of cell integrity, the association of antioxidants and hormones has been used in the semen freezing extenders of several species, including bovine (Beconi *et al*., 1993). Therefore, the objective of this study was to evaluate the conception rate, the metabolic and structural conditions of bovine sperm after freezing, using diluents supplemented with IGF-1, glutathione, and its association.

MATERIAL AND METHODS

The present study was approved by the Ethics committee of the Federal University of Acre, under the number 049/2015. The steps of cryopreservation of semen and artificial inseminations were performed in the municipality of Grajaú/MA, located 05°49'08" S and 46°08'20" O, with a tropical climate (Aw). The experiment was conducted at the State University of Maranhão, College São Luís/MA, and at the School of Veterinary Medicine and Animal Science (FMVZ) of University of São Paulo (USP), College Pirassununga/SP. Six Nellore bulls were used with average weight and age of 500kg and 36 months, respectively, classified as apt for reproduction, according to the Brazilian College of Animal Reproduction (Henry and Neves, 2003). All of them were collected by electroejaculation, going through repetition with an interval of one month, totalizing 12 ejaculation, which were divided among the treatments, evaluating volume, concentration, motility, vigor, and spermatic morphology.

The TRIS extender was used as described by Allen and Almquist (1981) [composition: Tris 2.42g (T1503, Sigma-Aldrich), citric acid 1.36g (C1909, Sigma-Aldrich), fructose 1.0g (F0127, Sigma-Aldrich), egg yolk 20mL,glycerol 7mL (G9012, Sigma-Aldrich), and penicillin 0.028g (P3032, Sigma-Aldrich), dd.H2O - q.s.p 100mL], being divided into four treatments: Control – only with TRIS, gSH – medium with glutathione reduced (2mM/mL -g4251, Sigma-Aldrich), IGF-1 – medium with insulin-like growth factor type 1 (100ng/mL - I3769, Sigma-Aldrich), gSH + IGF-1 – medium with gSH (1mM/mL) and IGF-1 (50ng/ml). After collection and dilution, cryopreservation was performed with the aid of the programmable cryopreservation system of portable semen (TK-3000®), with the curve P2S2 (0.5°c/min, positive curve e -20ºC/min, negative curve).

For the post-thawing analysis, two semen straw per treatment was used, thawed in a water bath at 37°c for 30 seconds, evaluating motility and vigor. For the rapid thermoresistance Test (TTRr), a seminal aliquot was maintained at 45°c for 30 minutes, spermatic motility and vigor were evaluated every 10 minutes, from the moment 0. The morphology of the spermatic cells was performed according to Henry and Neves (2003), with the count of 200 cells through the technique of moist preparation under differential interference contrast microscopy (DIC), being classified into major and minor defects, according to Blom (1972).

The analysis of the mitochondrial potential, the integrity of the plasmatic and acrosomal membrane was performed at the concentration of 20 x 10^6 spermatozoids/mL, adding 3µL of propidium iodide (PI: 0.5mg/mL in DPBS), 50µL of Pisum Sativum Agglutinin conjugated with fluorescein isothiocyanate (FITC-PSA: 100 µg/mL in DPBS) and 6µL of iodide 5,5 ', 6,6 ' – Tetracyloro-1, 3,3,3 '- Tetraethylbenzimidazolilcarbocyanin (JC – 1:153 µM), reading was performed under epifluorescence microscopy (microscope of epifluorescence brand Nikon, model Eclipse 80i) with a magnification of 1.000x. The cells were classified into eight categories according to the fluorescence emitted by each probe, as described by Celeghini *et al*. (2008).

The reactive oxygen species (ROS) were evaluated using the CellROX Fluorescent Probe™ Deep Red reagent, using 1µL de H3342 e 2µL de CellROX ™, incubated for 30 minutes at 37°c and the reading performed under epifluorescence microscopy (microscope of epifluorescence mark Nikon, model Eclipse 80i), classifying them with or without oxidative stress (Alves *et al*., 2015). Semen fertility was performed with 60 heifers per treatment, totalizing 240 animals inseminated at the fixed time (TAI) with the three-pass protocol (D0 – P4 implant and 2mg of estradiol benzoate; D8 – withdrawal of P4, 0.52mg of PGF2α, 1mg of estradiol cypionate and 300 IU of eCG; D10 – 0.01mggnRH). After 45 days of insemination, the pregnancy diagnosis was performed using ultrasound evaluation using a transrectal linear probe with a frequency of 5 MHz (Mindray DP 2200Vet). For statistical analysis, the variables went through the normality tests of Shapiro-Wilk and Lilliefors, and the parametric parameters (PI - intact plasma membrane; AI - intact acrosome; PIAI - spermatozoa with intact plasma and acrosomal membranes; AP - high mitochondrial potential and oxidative stress) were evaluated by ANOVA, comparing the averages through the Tukey test and the non-parametric (Motility, vigor, and TTRr) by Friedman's test, with a significance of 5%. The percentage of pregnancy was compared utilizing the chi-square test.

RESULTS

After cryopreservation, when motility, vigor, major and minor defects were evaluated, no significant difference was observed (P>0.05) between treatments (Table 1).

Table 1. The mean and standard deviation (X ± S.D.) of motility, vigor, major and minor defects spermatic of semen after cryopreservation of Nellore bulls

	Control	GSH	IGF-1	GSH + IGF-1
Motility (%)	37.9±7.21	43.33±8.61	42.08±9.15	40.0±9.77
Vigor (1-5)	2.33±0.49	2.58±0.51	2.58±0.51	2.5±0.52
Major defects (%)	7.29±1.81	6.15±1.87	6.75±1.98	6.83±1.51
Minor defects (%)	3.45±1.68	2.95±0.75	3.0±0.97	2.87±0.52

There was no difference between treatments (P> 0.05) by the Friedman test. gSH:glutathione; IGF-1: insulin-like growth factor 1; gSH + IGF-1: association of glutathione with insulin-like growth factor 1.

These values were adequate for insemination since they had at least 37% motility (control group) after thawing, being above the minimum required, 30%, by the Brazilian College of Animal Reproduction (Henry and Neves, 2003). The major (%) and minor (%) defects found in fresh semen and the control group were 5.5 ± 1.16, 3.25 ± 1.28, 7.29 ± 1.81, and 3.45 ± 1.68, respectively, with no significant difference between the evaluations before and after the seminal freezing. By the evaluation through the rapid Thermoresistance Test (TTR) there was no significant difference between the treatments (P>0.05). There is a difference (P<0.05) only between the initial and final values (TTR) within each treatment, but not between them (Table 2).

When evaluating the percentages of cells with mitochondrial potential (AP), plasma membrane (PI), and acrosomal membrane (AI) demonstrated by the association of fluorescent probes, it was not possible to verify a significant difference (P>0.05) between the treatments (Table 3). When analyzing the oxidative stress with the aid of the CellRox™ Deep Red probe, responsible for evaluating the amount of ROS, such as hydroxide and hydrogen peroxide, present in the sperm cells, with and without oxidative stress, it was observed that IGF-1 presented higher (P<0.05) number of cells with oxidative stress when compared with the other treatments (Table 4). It was observed that the pregnancy rate per treatment did not differ (P>0.05), with the mean and standard deviation between treatments of 36.58 ± 2.51% (Table 5).

Table 2. Mean and standard deviation (X ± S.D.) of motility at time 0 ', 10 ', 20 ', and 30' of thermoresistance test in different treatments of cryopreserved semen of Nellore bulls

Time (min)	Control	GSH	IGF-1	GSH+IGF-1
0'	40.80 ± 8.48[a]	40.80 ± 10.64[a]	41.25 ± 11.10[a]	39.16 ± 7.92[a]
10’	39.50 ± 7.21	42.00 ± 7.21	42.5 ± 10.11	40.41 ± 10.10
20’	30.40 ± 15.44	29.16 ± 12.76	35.00 ± 13.48	32.08 ± 13.72
30'	12.90 ± 16.84[b]	18.33 ± 18.50[b]	20.41 ± 15.29[b]	18.33 ± 18.38[b]
Δ	27.90	22.47	20.84	20.83

Different letters in the same column differ (P <0.05) by the Friedman test. gSH:glutathione; IGF-1: insulin-like growth factor 1; gSH + IGF-1: association of glutathione with insulin-like growth factor 1. Δ: Difference between initial and final values.

Table 3. Mean and standard deviation (X ± S.D.) of spermatozoa with acrosomal e plasma membrane intact and mitochondrial potential in different treatments of cryopreserved semen of Nellore bulls

Characteristics (%)	Control	GSH	IGF-1	GSH+IGF-1
PI	28.92 ± 20.28	34.33 ± 12.03	31.67 ± 20.31	36.58 ± 20.88
AI	44.08 ± 20.18	50.71 ± 14.11	47.63 ± 21.47	50.21 ± 21.58
PIAI	28.33 ± 20.18	34.29 ± 12.10	31.54 ± 20.11	36.50 ± 20.82
AP	93.38 ± 5.26	90.63 ± 6.92	87.11 ± 19.22	89.42 ± 14.40

There was no difference between treatments (P> 0.05) using the Tukey test. PI - intact plasma membrane; AI - intact acrosome; PIAI - spermatozoa with intact plasma and acrosomal membranes; AP - high mitochondrial potential.

Table 4. Mean and standard deviation (X ± S.D.) of spermatozoa with oxidative stress in different treatments of cryopreserved semen of Nellore bulls

	Control	GSH	IGF-1	GSH+IGF-1
oxidative stress (%)	14.54 ± 10.08b	17.21 ± 19.21b	36.38 ± 24.10a	24.04 ± 20.26b

Different letters on the same line differ by Fisher's exact test (P <0.05). gSH:glutathione; IGF-1: insulin-like growth factor 1;gSH + IGF-1: association of glutathione with insulin-like growth factor 1.

Table 5. Percentage of pregnant Nellore heifers after insemination with different treatments of cryopreserved semen of Nellore bulls

	Control	GSH	IGF-1	GSH+IGF-1
Pregnancy rate (%)	38.33 (23/60)	33.00 (20/60)	36.66 (22/60)	38.33 (23/60)

GSH:glutathione; IGF-1: insulin-like growth factor 1;gSH + IGF-1: association of glutathione with insulin-like growth factor 1.

DISCUSSION

The addition of gSH was expected to provide greater spermatic motility and vigor than the other treatments since antioxidants can maintain or decrease free radicals released by cellular metabolisms. They would avoid greater wear of sperm cells, which would endure the maintenance of speed and vigor, as observed in the results presented by Sariozkan *et al*. (2009) that showed improvement in sperm motility when added 2mM of gSH to the semen of Holstein cattle. However, the results of the present study were similar to those obtained by Tuncer *et al*. (2010), who, when performing evaluations of the semen of cattle with gSH at concentrations of 0,5 and 2,0mM, did not have a difference in sperm motility, post-cryopreservation, as well as in morphology.

However, this variation of results may be related to the types of diluters used, in addition to the antioxidant concentration.

Treatment with IGF-1 at the concentration of 100ng/mL did not present a significant difference (P>0.05) when compared with the other treatments for the analyses performed. IGF-1 is a growth factor that is associated with the increased sperm motility, as demonstrated by the results of Selvaraju *et al*. (2009), who evaluated the effect of the addition of 100ng/mL of IGF-1 to the semen of buffaloes, observed an increase in linear velocity and spermatic motility. Souza (2011) also obtained a positive response between the IGF-1 concentrations of the seminal plasma of gir-dairy bulls and spermatic motility, the same found by Padilha *et al*. (2012) when assessing the effect of the growth factor in the concentration of 100ng/mL in sheep semen. This response is partly due to the dilution of semen for the realization of cryopreservation because it promotes the dilution of the factors that compose the seminal plasma where it is contained, decreasing the concentration of this hormone. Therefore, it is expected that by recomposing its physiological values in the pre-dilution, we obtain a higher response of this variable after the freezing process.

The concentration used in the present study is per the physiological conditions pointed out by Henricks *et al*. (1998). However, it did not present higher spermatic motility after cryopreservation, and these results may be justified because of the existing function of this growth factor in capturing the nutrients present in the diluter, such as amino acids and sugars, which can facilitate the passage to the inner region of the cells, making the use of energy faster, leading, however, to release a greater number of free radicals, which consequently can promote cell death (Silva *et al*., 2013).

As for the sperm morphology of cryopreserved semen, there was no difference between treatments (P>0.05), by the Friedman test, for the major and minor defects. The use of gSH in the extender was intended to reduce the lesions that ROS can cause in sperm. One of the consequences of increased oxidative stress is the high number of spermatic defects. Thus, the addition of gSH could preserve the spermatic morphology after cryopreservation. Ansari *et al*. (2011), also observed that the use of antioxidants in Buffalo semen was able to maintain sperm morphology under control, as well as motility and spermatic viability. As for the addition of IGF-1 to semen, there are reports that it promotes improvement in the spermatic quality of horses (MacPherson *et al*., 2002), Buffaloes (Selvaraju *et al*., 2009), and pigs (Silva *et al*., 2011). In these cases, the spermatic morphology is preserved, suggesting that IGF-1 has a crucial function in preserving the integrity of these cells, which is corroborated by the findings of Souza (2011), who found a correlation between the seminal concentrations of IGF-1 and total defects during peripuberty in gir Bulls.

The motility at time 0', 10', 20' and 30' of TTRr, in different treatments, did not differ statistically (P>0.05). These data are like those found by Peixoto *et al*. (2013) who, when working with semen from dogs, observed that the diluter with gSH and Trolox did not interfere in motility when compared to the control during the slow TTR test for 60 minutes. However, when evaluating the maintenance of sperm viability within the treatments comparing the initial and final TTRr times, it was observed that the control group, gSH, IGF-1, and the association had significant differences (P < 0.05) within the time, but not between treatments, decreasing motility. This answer is part of the characteristics of this variable. It was expected that the treatments with gHS and IGF-1 had less variation in the values, initial and final, of the motility, something that had not been proven.

The basic principle of antioxidants is the reduction of free radicals in the face of the challenge of increasing metabolism (Bilodeau *et al*., 2000). Thus, what, in theory, could have favored the maintenance of spermatic motility in the face of its addition. It would be expected greater vigor and motility at the zero moments of thawing, with the consequent decrease in motility, within the time since it would have an inverse role to the antioxidants with increased velocity, increased metabolism, consequently greater production of ROS and, finally, lower motility at the end of the evaluation. Thus, greater vigor with greater motility was sought in the joint addition of gSH and IGF, since it would have control of ROS produced. A point that was not confirmed in the present study, like Tuncer *et al*. (2010), when performing evaluations with cryopreserved semen

of bovine with increased diluter of gSH (0.5 and 2.0mM), observed progressive motility when compared to the control group. Bilodeau *et al*., (2001), used cryopreserved semen of bovine plus 0.5mM of gSH and incubated for 6 hours at 38.5°c observed the maintenance of spermatozoa motility without an external source of oxidative stress (H_2O_2) added to the diluter.

In the present study, it is noted that the percentage of cells with the intact plasma membrane in all treatments was low, as well as that of the undamaged acrosome, but this response may be associated with increased fluorescence of the probes used by the TRIS diluter. However, due to the low response of the sperm membranes, it should be noted that the spermatozoids with intact plasmatic and acrosomal membranes are more apt to perform fertilization (Soares *et al*. 2011).

Thus, it was found that gSH and IGF-1, in the concentrations used, failed to improve the quality of the acrosomal and plasmatic membrane. These results may indicate that the gSH in the 2mM concentration has no positive effect, compared to the methods used in this study. This probably occurred due to the concentration used did not supplant the decrease occurring during the seminal dilution for the cryopreservation process. According to Bilodeau *et al*. (2000), after cryopreservation of bovine semen, there is a reduction of 78% of the concentration of gSH and, depending on the concentration, can cause damage to bovine spermatozoa, by altering the cell osmolarity, weakening the membrane and causing rupture in this structure (Bilodeua *et al*. 2001). The same was noted by Stradaioli *et al*. (2007), who observed that in the extenders for bovine egg yolks there is a decrease in the concentration of gSH when compared with those without products of animal origin. As was found by Soares *et al*. (2011), noting that the different concentrations were not able to preserve membrane integrity.

Sariozkan *et al*. (2009), when working with Holstein cattle, added to the 2mM diluter of gSH and observed the maintenance of the integrity of the spermatic membranes when compared with the control group. However, when assessing the rate of gestation in the used concentration of gSH, there was no significant difference. When referring to IGF-1, it was observed that it did not improve the maintenance of the integrity of the spermatic membranes. These data are contrary to those found by Brito *et al*. (2007) who observed a positive correlation between the IGF-1 concentration and the maintenance of membrane integrity. However, Selvaraju *et al*. (2010), when working with Buffalo semen and adding 100ng/ML of IGF-1, observed that the hormone did not affect the integrity of the acrosome despite having promoted a reduction of lipid lipoperoxidation. The same was found by Padilha *et al*. (2012) when evaluating IGF-1 action in sheep semen at concentrations of 100 and 250ng/mL.

It was noted that there was no difference between treatments when evaluating the percentage of cells with high mitochondrial potential. The values of this variable are important, since they are responsible for the ATP production, being indispensable to the flagellar beat for the motility of spermatozoa (Flesch and Gadella 2000). Studies have shown that human spermatozoids have a higher rate of *in vitro* fertilization, and those with high mitochondrial potential have a positive correlation between motility, hyperactivation of spermatozoa, the average velocity of the route, and mitochondrial membrane potential (Kasai *et al*., 2002). Ahmed *et al*. (2016), evaluated the reproduction of buffaloes outside the reproductive period by associating the semen quality parameters with fertility after 528 inseminations and observed that the integrity of the plasmatic, acrosomal membrane and potential of the mitochondrial membrane are directly related to fertility.

As for oxidative stress, the data of the present study corroborate the reports of Selvaraju *et al*. (2009), where they reported that IGF-1 is responsible for being an activator of the sperm metabolism, promoting an increase in sperm motility and, consequently, increased carbohydrate metabolism. O'Flaherty *et al*. (1997) affirmed that the increase in the energy metabolism of sperm cells is related to the generation of a greater number of free radicals, which may affect the sperm quality and its fecundating capacity. The reactions produced naturally in the organism through the metabolism itself can generate the ROS, who's most found free radicals are: the anion superoxide (O2-), hydroxyl radical (OH-), and the metabolite peroxide of Hydrogen (H_2O_2) (Chan *et al*., 1999, Borges *et al*., 2011). However, Selvaraju *et al.*

(2009) stated that when IGF-1 was added to Buffalo semen and subjected to malondialdehyde test (MDA), they observed a direct correlation with the reduction of lipid peroxidation levels.

The IGF-1 system may be involved in the transduction signal, leading to increased motility, spermatic capacitation and acrosome exocytosis (Gupta, 2005). Souza (2011) found a correlation between the concentrations of IGF-1 in the seminal plasma and the concentrations of protein peaks with affinity to heparin, which corroborates the idea of linking this hormone with the process of sperm capacitation. Thus, at the moment when the processes of sperm capacitation with alteration in sperm metabolism and motility are carried out, ROS production will also act in these processes (Soares and Guerra 2009, Borges *et al*., 2011, Walczak-Jedrzejowska *et al*., 2013).

The hydrogen hydroxide and peroxide present in the sample are the main subproducts of the increase in sperm metabolism induced by the growth factor (Alves *et al*., 2015). Thus, when analyzing the gSH data, it was observed that 82.79% of the sperm cells did not present oxidative stress, this demonstrates that although there was no significant difference when compared with the control, the gSH was more effective than IGF-1 in maintaining the quantity of ROS. Gadea *et al*. (2005), when working with gSH at the concentration of 1mM and 5mM, observed that the exogenous addition of this antioxidant only takes effect when the levels of endogenous gSH are below normal levels. However, Tuncer *et al*. (2010) observed that when assessing oxidative stress in bovine semen plus gSH (0.5 and 2.0mM), it decreased when compared to the control group.

The pregnancy rate is following the pattern of responses presented by the other variables analyzed, which did not present the statistical difference between the treatments, framing the recommendations adopted by the Brazilian College of Animal Reproduction (Henry and Neves, 2003), which indicate the use of reeds with at least 30% spermatic motility, after thawing, so that they are classified as satisfactory. Similar results were presented by Sariozkan *et al*. (2009), who use the Bioxcell diluter with 2mM of gSH to freezing, maintained the seminal characteristics, however, without increasing the gestation rate (P>0.05). As well as Tuncer *et al*. (2010), who performed evaluations in cryopreserved semen of bovine with diluters plus gSH (0,5 and 2,0mM) observed that the pregnancy rate of 233 cows inseminated with the frozen samples did not differ between the groups (GSH with 0.5 and 2.0mM/cysteine with 5 and 10mM). In the present study, although the gSH preserved 82% of the sperm cells against oxidative stress, it was not possible to obtain a significant difference between the rates of pregnancies, because other factors may have influenced at the time of fertilization.

CONCLUSIONS

IGF-1, gSH and its association did not cause changes in spermatic motility, mitochondrial potential, plasma and acrosomal membrane integrity. However, IGF-1 increased oxidative stress. There was no increase in the pregnancy rate resulting from any treatment.

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