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Antioxidant effect on viability of boar semen cooled to 15°C

[*Influência de antioxidantes na viabilidade do sêmen suíno resfriado a 15°C*]

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ABSTRACT

This study aimed to evaluate the addition of Vitamin C, reduced Glutathione and trolox on sperm characteristics of pork refrigerated semen. Six pigs were collected through the technique of gloved hand (10 ejaculates/animals). The semen was diluted in MR-A®. After the previous evaluations, the treatments were added: Control group: diluent only; Vitamin C Group: 200μM/mL Vitamin C; Trolox Group: 200μM/mL Trolox; Glutathione group: 2.5mM/ml Reduced glutathione. The semen was stored in thermal boxes and placed inside the refrigerator at 15°C and evaluated at D0, 12, 48, 72 hours. After 30 hours of incubation, each treatment was divided into two equal fractions and the same concentration of antioxidants was added in one of the parts. The results show that reduced glutathione supplementation preserves sperm motility after 24 hours but also has a higher percentage of acrosome intact in the presence of this antioxidant. There was no effect of adding a second dose of the antioxidants. In conclusion, the addition of reduced Glutathione to the swine semen diluent is a promising alternative for better preservation of sperm characteristics and the addition of the second dose of antioxidants during storage is detrimental to semen.

Keywords: glutathione, sperm, trolox, vitamin C

RESUMO

Este estudo tem como objetivo avaliar a adição da vitamina C, da glutationa reduzida e do trolox sobre características espermáticas do sêmen refrigerado de suínos. Seis cachaços foram coletados pela técnica de mão enluvada (10 coletas/animal). O sêmen foi diluído em MR-A®. Após as avaliações prévias, os tratamentos foram adicionados: grupo controle: apenas diluidor; grupo vitamina C: 200μM/mL de vitamina C; grupo trolox: 200μM/mL de trolox; grupo glutationa: 2.5mM/mL de glutationa reduzida. O sêmen foi armazenado em caixas térmicas e alocado dentro do refrigerador a 15oC e avaliado nos tempos zero, 12, 48 e 72 horas . Após 30 horas de incubação, cada tratamento foi dividido em duas frações iguais e adicionou-se a mesma concentração de antioxidantes em uma das partes. Os resultados demonstram que a suplementação de glutationa reduzida preserva a motilidade espermática após 24 horas, bem como tem maior percentual de acrossoma intacto na presença desse antioxidante. Não houve efeito ao se adicionar uma segunda dose dos antioxidantes. Em conclusão, o acréscimo da glutationa reduzida ao diluidor de sêmen suíno é uma alternativa promissora para melhor preservação das características espermáticas, e a adição da segunda dose dos antioxidantes durante o armazenamento é prejudicial ao sêmen.

Palavras-chave: espermatozoides, glutationa, trolox, vitamina C

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INTRODUCTION

Artificial insemination (AI) is the most frequently used reproduction technique in modern pig farming because it has several advantages. One of them is that there are rapid gains in genetic improvement (Bortolozzo *et al.*, 2005). The intense use of AI has increased the interest to develop proper conditions to store semen better for longer periods of time without affecting its fertility (Bordan *et al.*, 2016). However, the sperm characteristics of the semen stored for prolonged periods deteriorate gradually, probably related to the oxidative stress formed during its storage (Bordan *et al.*, 2016; Vongpralub *et al.*, 2016).

In addition, the plasma membrane of the swine spermatozoa has a large amount of polyunsaturated fatty acids and cholesterol and there is limited antioxidant capacity of spermatozoa to generate reactive oxygen species (ROS), which makes it susceptible to lipid peroxidation during refrigeration (Kumaresan *et al*., 2009, Severo *et al.*, 2011). The oxidative stress has been associated with male infertility. The ROS is responsible for several cellular alterations, also, productions of ROS decrease sperm motility, integrity and decrease fluidity of the plasma membrane, because of lipid peroxidation (El-Tohamy, 2012).

Thus, antioxidant supplementation can reduce the impact of oxidative stress during the sperm storage process and thus improve the quality of refrigerated semen (Rigo *et al.*, 2017). Antioxidants are compounds that regulate, remove and minimize the formation of ROS or block their actions, avoiding the onset or the spread of chain reactions of oxidation (Maneesh and Jayalekshmi, 2006).

Glutathione is characterized biochemically as the most abundant intracellular thiol tripeptide, with concentrations ranging from 0.1 to 10mM, having in its molecular structure, sulfur (Jedrzejowska *et al*., 2012). Vitamin C is in the form of ascorbate in the body, is a soluble micromolcule, present in aqueous compartments of organic tissues (Halliwell and Gutteridge, 1999), and the concentration in seminal plasma is about 10 times higher when compared with the blood serum (Jedrzejowska *et al*., 2012). Trolox, an analog of vitamin E, acts on lipids and low-density proteins present in the plasma membrane, conferring structural protection to the membrane of the oxidant action (Maia *et al.*, 2010).

Spermatozoa naturally have an intra and extracellular defense system, consisting of enzymatic and non-enzymatic antioxidants responsible for combating oxidative stress and lipid peroxidation (Aitken, 1995). However, during the processing of semen for storage, there is a large reduction of these antioxidants present in the ejaculate as a result of the dilution or removal of seminal plasma, thus compromising their protective capacity (Sarlós *et al.*, 2002). In this way, the objective of this study was to increase the viability of boar semen cooled for more than 72h at 15ºC by adding the antioxidants vitamin C, Trolox and glutathione to the semen extender.

MATERIAL AND METHODS

Semen samples were collected in the pig farm, AGROLUSA®, located in the municipality of São Luís (MA) (Latitude: 02º 31' 47" S and Longitude: 44º 18' 10" W). Six crossbred boars (Landrace x Large White, 2-4 years old), were used in this study. Semen collection was performed using a gloved hand technique by the same technician (10 ejaculates/animal). After separation of the gelatinous fraction of the ejaculate and for effective control of semen in use, an evaluation of physical aspects (volume - mL and concentration in a sperm densimeter) and microscopic aspects (sperm motility - %, and vigor - 0 to 5) was done, according to CBRA (Manual…, 2013).

Sperm morphology was analyzed through the diluted sample, preserved in a formal saline solution. The percentage of normal and pathological cells was estimated through phase-contrast microscopy (Nikon Eclipse 50i Microscope), with an increase of 1000x magnification, using 100 cells, and the results expressed as percentage, classified as major and minor defects, according to Blom (1973).

After the previous evaluations, the semen was diluted with MR-A® (30 x 10^6 sptzs/mL; Kubus, S.A., Spain), prepared according to the manufacturer. A sample of 40mL diluted semen was obtained, being divided into 4 equal fractions for subsequent addition of the

treatments. The semen was transported in isothermal packaging to the Laboratory of Animal Breeding, State University of Maranhão.

In the laboratory, the treatments were added, corresponding to the groups to be tested: Control group: only extender; Vitamin C group: adding 200µM/mL vitamin C; Trolox group: adding 200µM/mL Trolox; Glutathione group: 2.5mM/mL reduced glutathione, as such four initial treatments in total. The samples were stored in a thermal box kept in a refrigerator, calibrated to 15ºC. The samples were evaluated on days D0 (0h), D1 (24h), D2 (48h) and D3 (72h) after the antioxidants were added. The temperature was measured at those times with a digital thermometer, previously fitted to the refrigerator. After 30 hours of incubation, each treatment was divided into two equal fractions and the same concentration of antioxidants added in one of the parts. (Table 1).

Table 1. Experimental groups. Addition of Vitamin C, Trolox and Reduced glutathione at different times in the refrigerated semen of swine

D0 – first addition of antioxidants			
T1	T2	T3	T4
Control	200µM/mL Vitamin C	200µM/mL Trolox	2,5mM/mL Glutathione
D2 – second addition of antioxidants			
T1	T5	T6	T7
Control	400µM/mL Vitamin C	400µM/mL Trolox	5mM/mL Glutathione

D0: day zero of the addition of antioxidants to the seminal extender; D2: third day, 48 hours after the first addition of antioxidants, second addition of antioxidants.

All samples underwent the following tests: spermatic motility and vigor, slow thermosensitive test, eosin-nigrosin, acrosomal membrane integrity (Trypan blue/Giemsa) and hypoosmotic test (HOST). Sperm motility and vigor were measured at the time of dilution and after the period of stabilization at time zero for each day of evaluation (D0 to D3). For this purpose, the slow thermosensitive test was used; the samples were incubated for 60 minutes at 37°C and evaluated every 20 minutes.

For the evaluation of the integrity of the plasma membrane the proportion of live and dead sperm was determined through a combination of the eosin and nigrosin dyes in semen smears (10µL) in a 1:1 ratio (Arruda *et al.*, 2010). 100 cells were counted through light microscopy, under 100x magnification, in order to distinguish live cells (unstained) from dead cells (stained).

The acrosomal membrane integrity test and differentiation of viable spermatozoa were performed by means of double staining (Giemsa and Trypan blue), adapted from Feliciano Silva (1998). The present study used a DPBS (Dulbecco's Phosphate-Buffered Saline) solution to wash the cells by means of centrifugation at 600g for 6 minutes. After these initial procedures, the semen smears were incubated for 6 hours in a 4% Giemsa solution for subsequent reading. Phase-contrast microscopy (Nikon Eclipse Microscope 50i) was used, with 100x magnification; 100 cells were counted and classified into: live with acrosome (LW); dead with acrosome (DW); live without acrosome (LWo) and dead without acrosome (DWo), according to Feliciano Silva (1998).

The hypoosmotic test consisted in adding 100µL diluted semen in 2mL of hypo-osmotic solution (sodium citrate + fructose at 150mOsm) and kept in a water bath at 37°C for 15 minutes (Vazquez *et al.*, 1997). After the incubation period, 1mL of saline formalin was added to 1mL of the solution. Then, 100 cells were read in phase-contrast microscopy with 1000x magnification. The result was determined as percentage, after calculating the difference between the number of HOST-reactive spermatozoa and the number of spermatozoa which showed folded tails, according to Melo and Henry (1999).

The study used a randomized block, split-plot design, where each animal represented a block and each day of analysis, a plot. The SAS (2002) software was used for comparison of averages. Parametric variables were evaluated through ANOVA; the means were compared through Student's t-test. The non-parametric variables were analyzed using Friedman's test with a significance level of 5%. All the variables

underwent the Shapiro-Wilk and Lilliefors tests for normality. The variable HOST underwent logarithmic transformation for assessment of normality. The slow thermosensitive test was treated as non-parametric, because its response came from subjective observations. Samples were analyzed by the MIXED procedure of the SAS statistical package, according to Littell *et al.* (1998), to determine whether there was an interaction between time and treatment. This experiment was approved by the Animal Testing Ethics Committee of the State University of Maranhão, under protocol No. 037/2014.

RESULTS AND DISCUSSION

Mean concentration per mL and volume of the ejaculate without the gel fraction for the boars was 485 x 10^6sptz and 304mL, respectively. Fresh semen showed mean motility of 88.00±4.47% and vigor of 4.1±0.22. As for sperm morphology, the main defects found were strongly folded, abnormal middle piece and proximal cytoplasmic droplets. The overall mean of major, minor and total defects has not exceeded the normal values for pigs as advocated by CBRA (Manual…, 2013), hence they were classified as suitable for reproduction.

The evaluation of sperm motility and vigor showed no difference in treatment for time (0, 20, 40 and 60min) of the TRT (P> 0.05). Thus, the data were grouped by treatment for comparison between the evaluation days (D0, D1, D2 and D3) and between each of the treatments (Control, Vitamin C, Trolox and Glutathione). There was a difference (P< 0.05) among days within treatments. However, this response was expected, since storage time has a direct effect on this variable.

There was a statistical difference for sperm motility between treatments among the days, but not within a single day (P> 0.05; Table 2). After the first 24 hours of observation, glutathione stood out; it showed a difference (P< 0.05) compared with the control group.

Table 2. Mean (±standard deviation) of the sperm motility of the refrigerated semen of swine at the different evaluation times (D0, D1, D2 and D3) after addition of the antioxidants

Days	Treatments			
	Control	Vitamin C	Trolox	Glutathione
D0	60.00±13.35	61.66±12.99	57.92±17.06	63.45±16.69
D1	23.08±17.66^{b}	23.87±17.07^{ab}	28.16±19.71^{ab}	32.41±17.5^{a}
D2	17.16±13.14^{b}	13.70±14.01^{ab}	15.79±14.88^{ab}	25.75±16.61^{a}
D3	11.70±11.19^{b}	8.16±8.92^{ab}	10.62±10.83^{ab}	18.75±12.99^{a}

Different letters on the same line differ by the Friedman test (P< 0.05).

The sperm motility and vigor reduced throughout the days of observation. However, the addition of vitamin C and Trolox did not differ for progressive motility and vigor compared with the control group in the present study, unlike the results found by Zanella *et al.* (2016), whose study used semen diluted and cooled to 15^{o}C, and Trolox added at a concentration of 50µM. They found higher values of motility after the first 24 h of storage in samples that contained this antioxidant. A similar response was found by Peña *et al.* (2003), who suggested that the addition of Trolox (100 to 200µM), while freezing boar semen, showed a significant increase in sperm motility and speed, after thawing. Thus, in the study by Peña *et al.* (2003), the addition of vitamin C Trolox may have been able to offer greater protection to spermatozoa, as there is higher oxidative stress of these cells within the cryopreservation process - a different situation from the one reported in the present study. Similarly, Grossfeld (2007) reported that the addition of antioxidants to the boar semen extender has significantly improved sperm motility.

The addition of glutathione showed no improvement in sperm vigor, either. However, after 24 hours, it showed a better response for sperm motility in comparison to the control group. Because it is the main compound of non-protein thiol, glutathione assists in several cell functions, including transport of amino acids, DNA and protein synthesis, reduction of disulfide bonds and protection against oxidative stress, in which the sulfhydryl groups of glutathione offer protection against cell damage by oxidants and free radicals (Irvine, 1996).

According to the studies by Gadea *et al.* (2004), cryopreservation of boar semen is associated with a reduction in sperm motility and viability. By contrast, the author noted that the addition of 5mM of glutathione in the preservation of boar semen at 15°C did not reduce sperm viability. The results of Whitaker *et al.* (2008) were different; they stated that adding 5.0mM of glutathione to the extenders has significantly decreased the progressive motility of boar spermatozoa.

The integrity plasmatic membrane was quantified by a percentual of live and dead sperm cells determined by the vital dye eosin-nigrosin. There was no difference (P> 0.05) between treatments within time. In the control group, there was a difference (P< 0.05) between times D0 and D3. Similar results were found for the treatment with vitamin C and glutathione. However, there was a difference for Trolox between days D0, D2 and D3. This result showed that this reagent was not able to maintain viability until the last day without significant loss (Table 3).

Table 3. Mean (±standard deviation) of the integrity plasmatic membrane by eosin-nigrosin test of the refrigerated semen of swine at the different evaluation times (D0, D1, D2 and D3) after addition of the antioxidants

Days	Treatments			
	Control	Vitamin C	Trolox	Glutathione
D0	84.00±13.92^{a}	84.83±11.06^{a}	84.00±10.73^{a}	84.83±9.86^{a}
D1	63.16±16.90^{ab}	67.66±15.06^{ab}	62.83±20.36^{abc}	65.83±20.70^{ab}
D2	54.83±31.33^{ab}	59.83±31.95^{ab}	56.5±21.65^{b}	57.16±30.82^{ab}
D3	46.16±14.95^{b}	49.66±11.41^{b}	49.33±7.25^{c}	51.66±19.49^{b}

Different letters in the same column differ (P> 0.05) by the Student's t test.

The data presented above differ from those found by Peña *et al.* (2003), who concluded that Trolox has a protective effect on spermatozoa and that it influences the fraction of the ejaculate. These authors reported that this effect may be related to the different compositions of seminal plasma between the fractions, which may determine lower sensitivity to oxidative damage in the content of the semen in the first fraction of the ejaculate.

As for HOST, the addition of antioxidants to the extender did not affect the integrity of the plasma membrane, with no difference within time or between treatments (P> 0.05); only between animals. These results are restated by statements made by Boe-Hansen *et al.* (2005), indicating that some boars may have better characteristics in their seminal plasma than others, even with variation between races.

These results are very important for the present study, because the functionality of the membrane is correlated with sperm fertilization capacity, when the acrosome reaction occurs, a phenomenon of great biological importance, with relevant consequences in accordance to the stripping of the oocyte and the penetration of the zona pellucida (Esteves *et al.*, 2000).

As for the Giemsa/trypan blue test, there was an effect within treatment between the days of assessment in which the treatment with glutathione presented a greater number of live spermatozoa with preservation of the acrosome in D0, D2 and D3, compared to the control group (Table 4).

Table 4. Mean (±standard deviation) of the live sperm cells with acrosome of the refrigerated semen of swine at the different evaluation times (D0, D1, D2 and D3) after addition of the antioxidants

Days	Treatments			
	Control	Vitamin C	Trolox	Glutathione
D0	45.5±17.54^{c}	58±17.81^{b}	51.5±23.27^{b}	67.5±19.27^{a}
D1	42.16±18.01	39.16±24.59	42.33±17.13	48.66±25.03
D2	22.5±14.12^{b}	36±23.13^{a}	36.16±21.93^{a}	39.33±30.34^{a}
D3	19±12.83^{b}	34.33±18.88^{a}	26.66±14.14^{b}	34.33±19.07^{a}

Different letters on the same row show a statistical difference (P< 0.05) by the Student's t test.

In addition to glutathione, the treatments with vitamin C and Trolox showed significant differences when compared to the control group. They showed a higher percentage of sperm unstained by the vital dye. Unlike the results presented here, Araújo (2012) found that the addition of Trolox (1, 2 and 3mM) to a skimmed milk powder extender produced no effect on the percentage of sperm with intact acrosome; the percentage was similar among treatments during five days of preservation of boar semen. The same occurred with the addition of vitamin C (2.5, 5 and 10mM) to a Beltsville Thawing Solution (BTS) extender, which showed no significant difference in the viability and the parameters of acrosome integrity of boar semen (Breininger and Beconi, 2014).

Based on the variables analyzed, live and dead spermatozoa, with or without acrosome, only live spermatozoa with acrosome stood out, because these spermatozoa are the only potentially suitable for fertilization (Parrish *et al.*, 1988). The acrosome reaction follows sperm capacitation, a complex process that modifies the composition of the plasma membrane, sensitizing sperm cells to physiologically induce the acrosome reaction, an event required for ovum fertilization (Siciliano *et al.*, 2008). In this way, in an attempt to increase the number of non-reactive cells, a second dose of antioxidant was added to each treatment with the intention to superimpose the period of oxidation that such reagents could be exposed to.

Thus, the treatments containing Vitamin C, Trolox and glutathione, after receiving the second dose of antioxidant on the third day (D2), were observed for over 48 hours and were compared with the samples that did not receive the additional dose. These treatments showed no statistical difference within time, as for motility and vigor.

The addition of antioxidants after 30 hours of storage was shown to be unfeasible in this study, because it resulted in low or almost null values in the parameters of sperm motility and vigor. Since there was no difference between the treatments that received the second addition of antioxidants, a comparison was made between the treatments that did not receive such addition, i.e., positive controls. There was a difference ($P< 0.05$) in motility on the third day (D2); non-addition had a better response. On the fourth day (D3), there was a difference ($P< 0.05$) for Vitamin C; non-addition also showed a better response. There was no difference ($P> 0.05$) among those which received and those which did not receive the complementation of Trolox and glutathione (Table 5).

Table 5. Mean (±standard deviation) of the test of motility of the antioxidants evaluated between D2 and D3 with and without supplementation of antioxidants of the refrigerated semen of swine

Days	Treatments without the addition of antioxidants in D2		
	Vitamin C	Trolox	Glutathione
D2	13.70±14.01a	15.79±14.88a	25.75±16.6a
D3	8.16±8.92b	10.62±10.83b	18.75±12.99b
	Treatment with the addition of antioxidants in D2		
Days	Vitamin C	Trolox	Glutathione
D2	4.45±4.10c	8.08±6.49b	16.29±9.07b
D3	5.33±8.74c	8.79±11.47b	18.5±12.3b

Different letters in the same column show a statistical difference ($P< 0.05$) by the Friedman test. D2: third day of evaluation.

As for the eosin-nigrosin test, the treatments were compared between the days of evaluation, which showed no effect of treatment after the additional dose on days D2 and D3. The results of integrity of sperm and acrosome membranes, assessed by HOST and Giemsa/Trypan blue test, respectively, showed no effect of adding or not adding the antioxidant on the third and fourth days of evaluation.

The addition of antioxidant substances after 30 hours of storage was expected to improve the quality of some seminal parameters of the cooled samples, taking into account incubation time, because during the process of maturation, spermatozoa lose most of their cytoplasm, hence they limit the defense against oxidative stress and become dependent on the antioxidants present in seminal plasma (Baumber *et al.*,

2005), which would indicate the importance of using these substances in the extender within time. However, this response has not been found in this study.

CONCLUSION

It is concluded that the addition of reduced glutathione preserves sperm motility and plasma membrane integrity during storage and the addition of the second dose during storage is noisome to semen.

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