Original Research Paper

Oxidative Status of Boar Semen during Storage

[2]Carlos Bondan, [2]Eraldo Zanella, [2]Ricardo Zanella,
[1]Márcia Rósula Poetini, [3]Mariana Groke Marques and [1]Julio C.M. Soares

[1]*Universidade Federal do Pampa-UNIPAMPA-Campus de Caçapava do Sul, RS, Brazil*
[2]*Faculdade de Agronomia e Medicina Veterinária (FAMV), Universidade de Passo Fundo, Passo Fundo, RS, Brazil*
[3]*Embrapa Suínos e Aves, Concórdia, SC, Brazil*

Corresponding Author:
Julio C.M. Soares
Universidade Federal do Pampa-
UNIPAMPA-Campus de
Caçapava do Sul, RS, Brazila
Email: jcmsoares@yahoo.com.br

Abstract: The intensification of the use of artificial insemination in the swine industry has increased the interest to develop proper conditions to store semen better for longer periods of time without affecting its fertility. Trolox is a vitamin E analog with antioxidant property to prevent oxidative stress. Therefore, the objective of this study was to evaluate the Trolox antioxidant capacity on boar semen during storage period (86 h) at 15°C. For that, semen samples from 5 different boars were collected and diluted with a Long-Term (LT) extender with and without the addition of Trolox in a concentration of 50 µM. The sperm motility, oxidative stress and antioxidant protection were measured in those samples during an 84 h storage time. Our results indicated that Trolox was able to minimize the loss of sperm motility after 24 h of storage. The storage time was positively correlated with TBARS production. However in the presence of Trolox, after 12 h, TBARS content were significantly lower than those of sperm cells stored with LT diluent only. The concentrations of Non-Protein SH groups (NPSH), after 36 h of storage, were lower in sperm cells stored only with LT diluent when compared to the group supplemented with Trolox. Catalase and Superoxide Dismutase activities were decreased after 48 h storage at 15°C in both groups (with or without Trolox). Spontaneous spermatozoa ROS formation was high in viable swine sperm during the LT storage period in samples without Trolox. In conclusion, LT diluent with the addition of antioxidant as Trolox can reduce the oxidative stress in sperm cells, which could be used to protect spermatozoa against oxidative damage, preventing motility losses in swine semen.

Keywords: Trolox, ROS, Swine, Sperm Cells, Semen Storage

Introduction

The intense use of artificial insemination in the swine industry has increased the interest to develop proper conditions to store semen better for longer periods of time without affecting its fertility. In addition to that, dilution and storage of sperm facilitate its transportation to distant locations (Roca *et al*., 2006; Dimitrov *et al*., 2009). However, parameters associated with sperm viability are affected, depending on the duration and the condition that those cells remain (Bansal and Bilaspuri, 2010; El-Tohamy, 2012).

The formation of ROS in the sperm cells is a physiological condition. However, oxidative stress has been associated with male infertility (Bansal and Bilaspuri, 2010; El-Tohamy, 2012). The toxicity of ROS is responsible for several cellular alterations

(Soares *et al*., 2014) also, productions of ROS decrease sperm motility (Ogbuewu *et al*., 2010). Because of lipid peroxidation, the plasma membrane decrease fluidity and integrity (Bansal and Bilaspuri, 2010).

Physiologic antioxidant defenses neutralize ROS activity but, the high production of ROS can result in increased rate of cellular damage (Zanella *et al*., 2010; Soares *et al*., 2011). ROS can induce sperm cells damage, causing oxidative damage to the cell membrane, starting lipid peroxidation cascade, through of attack of polyunsaturated fatty acids (Ogbuewu *et al*., 2010).

The toxic effect of ROS results in lipid peroxidation, oxidation and modifications of protein synthesis and alteration gene expression. (El-Tohamy, 2012).

Vitamin E is the main component of the antioxidative system of sperm cells, one of the first protective membranes against lipid peroxidation (Hu *et al*., 2011).

The vitamin E breaks the covalent links between ROS and polyunsaturated fatty acids side chains in the membrane lipids, preventing the formation of ROS and lipid peroxidation (Jeong *et al.*, 2009).

Brezezińska-Slebodzińska *et al.* (1995) presented that over short periods of incubation, vitamin E and GSH protects against lipid peroxidation reducing losses in the semen quality. Additional studies have shown that Trolox protects cryopreserved sperm decreasing lipid peroxidation (Minaei *et al.*, 2012) and bovines (Hu *et al.*, 2011), reducing motility losses. The beneficial effect has also demonstrated in cryopreserved swine semen (Varo-Ghiuru *et al.*, 2015; Jeong *et al.*, 2009; Peña *et al.*, 2004; 2003). Despite its beneficial effect in the sperm cryopreservation, few studies have been conducted to evaluate its protection on cooling protocols and storage at 15°C of swine sperm cells. Therefore, the objective of this study was to evaluate the antioxidant potential of Trolox on boar semen during the long-term storage period (86 h) at 15°C.

Materials and Methods

Semen Sample Collection

Semen samples used in this study obtained from a commercial AI center that follows all the standard protocols for animal welfare. Semen was collected using a standard technique with polyvinyl gloves and just the sperm-rich fraction was used. Five semen samples from boars were divided into aliquots and transferred to sterile polypropylene tubes. Semen was centrifuged for 10 min at 1,500× g to separate the sperm cells from the seminal fluid. Following the centrifugation, the pellet was resuspended with diluent for LT semen storage. (66.25 g Glucose; 11.00 g Sodium Citrate; 13.50 g Sodium Bicarbonate; 6.50 g EDTA; 2.00 g Tris; 1.59 g Sodium Chloride; 0.75 g Gentamicin Sulfate; 1,00 L deionized water; 309 mOsm Osmolality and pH 7.20), in a dose of $1,5 \times 10^9$ sptz mL^{-1}. After dilution, samples from the treated group were supplemented with 50 µM of antioxidant Trolox-C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid). Samples stored at 15°C for 86 h. Samples then evaluated after 0, 12, 24, 36, 48, 60, 72 to 86 h of storage, for motility, thiobarbituric acid reactive species, sulfhydryl status, the antioxidant capacity of semen and concentrations of Catalase and Superoxide Dismutase (SOD).

Sperm Motility

Evaluated by microscopy under 200× magnification at 37°C. The sperm motility evaluation conducted for three times by the same observer and the average motility was then calculated and expressed as percentage.

Thiobarbituric Acid Reactive Species Assay (TBARS)

TBARS were quantified in accord with Ohkawa *et al.* (1979). Realized the semen precipitation with trichloroacetic acid 40% and kept on ice before centrifugation. After were added 1000 µL of the supernatant fractions to the color reaction medium. TBA-RS levels were measured at 532 nm, using Malondialdehyde (MDA) as standard curves.

Sulfhydryl Groups Assay in the Semen (NPSH)

Semen sulfhydryl groups assay were quantified using the modified Ellman's reagent. Semen was precipitated with trichloroacetic acid 40%. NPSH groups were quantified after centrifugation and neutralized with Tris-NaOH. The sulfhydryl levels were calculated through of standard curve of reduced glutathione

Antioxidant Capacity of Semen

The antioxidant activity of semen was measured, using the stable radical of 1,1-diphenyl-2-picryl hydrazyl (DPPH) (Gil-Villa *et al.*, 2010). Briefly, semen samples were lysed using a sperm-lysis buffer and centrifuged at 660 g for 10 min. After centrifugation, 200 µL supernatant was added to 4 mL DPPH radical solution (6.09×10^{-5} mol L^{-1} methanol). Assay it was left at room temperature for 10 min to stabilize the color reaction. The reaction was quantified in the spectrophotometer at 515 nm. Data was measured at 10 min intervals for 60 min. An ascorbic acid solution (0.25 mg ascorbic acid/mL methanol) was used as an antioxidant capacity control. We conducted the calculations using the formula:

$$Inhibition\ \% = \frac{\left(Absorbency\ of\ control - Absorbency\ of\ sample \right)}{Absorbency\ of\ control} \times 100$$

Catalase and SOD

Catalase activity was quantified according to Sinha (1972) and the SOD activity was quantified according to Sun and Zigman (1978).

Statistical Analysis

Spearman's correlation test was realized between the sperm motility and the production of antioxidant properties of the semen. The comparisons within and between the treated and the control groups were conducted every 12 h up to 84 h. The averages within treatment were compared between times using a student t-test and significance was considered if a $p < 0.05$.

Results

Trolox was able to maintain sperm motility up to 24 h of the storage. Although the percentage of sperm motility decreased with the time, motility was significantly

higher in spermatozoa stored in diluent for long-term storage with Trolox when compared to diluent only (Fig. 1). This fact indicates the possible capability of Trolox to prevent the reduction of sperm motility in swine semen diluted with a long-term extender. The percent motility of the spermatozoa exhibits a negative correlation between and time storage ($r = -0.8$; $p < 0.001$).

Also, was encountered a significant negative correlation between percent sperm motility and TBARS content in both groups ($r = -0.8$, $p < 0.0001$). The storage of spermatozoa increased TBARS levels, however in the presence of Trolox, after 12 h, this increase minimized and TBARS content was significantly lower than those of sperm cells stored with diluent only (Fig. 2).

Thiol groups, which are part of the NPSH, protect sperm cells against oxidative stress (Bansal and Bilaspuri, 2008). The concentrations of Non-Protein SH groups (NPSH), after 36 h of storage, were lower in sperm cells stored only with diluent when compared to the group supplemented with Trolox (Fig. 3).
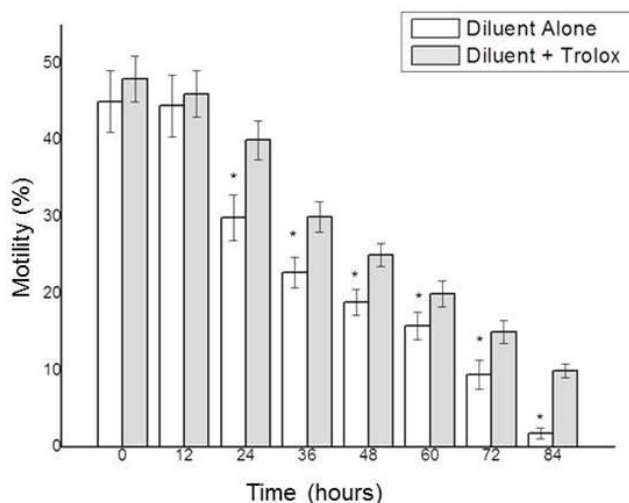

Fig. 1. Motility percentage in semen stored in diluent for long-term semen preservation at 15°C of 0 up to 84 h (doses of $1{,}5 \times 10^9$ sperm mL^{-1}) in the absence or presence of Trolox (50 μM). Data are expressed as mean ± SD (*indicates a difference between the groups in each storage period; $p < 0.001$)
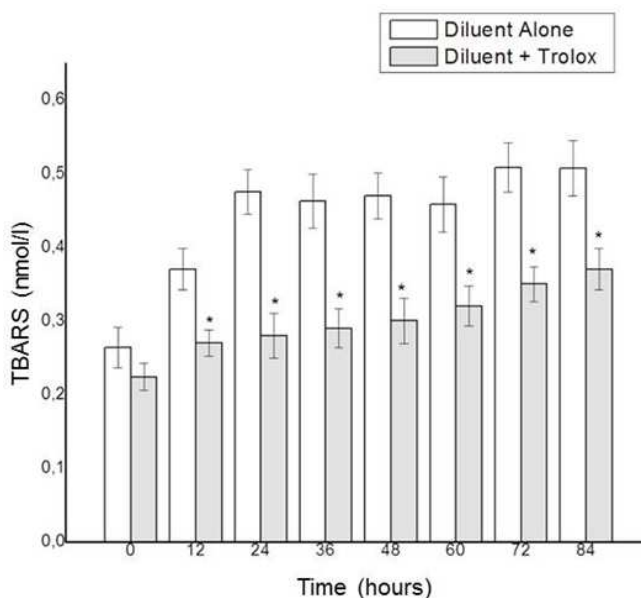

Fig. 2. TBARS content in semen stored in diluent for long-term semen preservation at 15°C of 0 up to 84 h (doses of $1{,}5 \times 10^9$ sperm mL^{-1}) in the absence or presence of Trolox (50 μM). Data are expressed as mean ± SD (*indicates a difference between the groups in each storage period; $p < 0.001$)
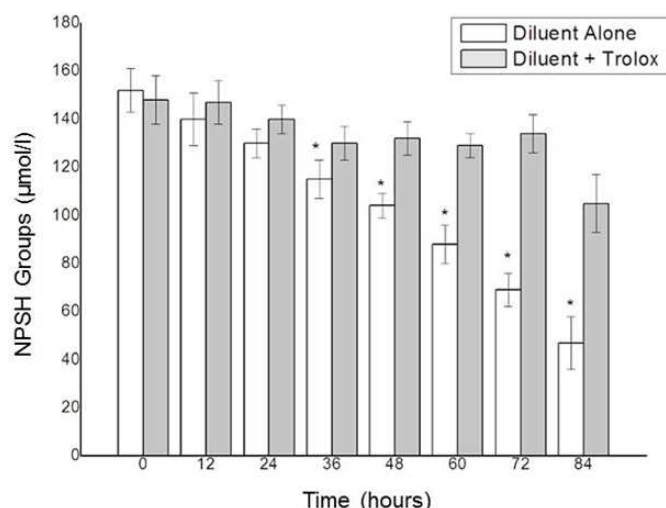
Fig. 3. NPSH in semen stored in diluent for long-term semen preservation at 15°C of 0 up to 84h (doses of $1{,}5 \times 10^9$ sperm mL^{-1}) in the absence or presence of Trolox (50 μM). Data are expressed as mean ± SD (*indicates a difference between the groups in each storage period; p<0.001)

Table 1. DPPH Inhibition, Catalase and SOD activities in semen stored in diluent for long-term preservation at 15°C for 84 h (doses of $1{,}5 \times 10^9$ sperm mL^{-1}) in the absence or presence of Trolox (50 μM). Data expressed as mean ± SD. Different superscript letters indicate a difference between the storage period in each group (p<0.001). There was no difference between groups (Diluent Alone or Diluent + Trolox)

		Time (h)							
		0	12	24	36	48	60	72	84
	DPPH Inhibtion	55±7.0[a]	52±3.0[a]	47±3.0[b]	35±6.0[b]	27±4.0[b]	18±2.0[b]	10±2.0[b]	5±2.0[b]
Catalase	Diluent Alone	14±1.2[a]	14±1.0[a]	15±1.1[a]	12±1.0[a]	7±0.9[b]	7±1.0[b]	6±0.8[b]	6±0.7[b]
	Diluent + Trolox	15±1.5[a]	14±1.3[a]	14±1.5[a]	11±1.2[a]	8±1.5[b]	7±1.5[b]	5±1.5[b]	6±1.0[b]
SOD	Diluent Alone	32±4.0[a]	30±3.5[a]	29±4.0[a]	28±4.5[a]	21±1,8[b]	15±1.5[b]	12±1.5[b]	5±1.6[b]
	Diluent + Trolox	34±4.6[a]	32±3.0[a]	31±4.7[a]	29±4.5[a]	18±2.5[b]	17±2.0[b]	11±1.5[b]	7±1.7[b]

After SOD convert superoxide anion in H_2O_2 and oxygen. The H_2O_2 is scavenged by catalase and peroxidase, result in water and oxygen (Bathgate, 2011). The activity of catalase and SOD decreased after 48 h storage at 15°C (p<0.001) in both groups (Table 1). Furthermore, the addition of diluent to the Trolox was unable to reverse this reduction. The scavenging ability of semen on DPPH free radical examined, spermatozoa storage with diluent for LT semen preservation resulted in a significant decrease time-dependent inhibition of DPPH radical after 24 h of storage (Table 1). A positive correlation was verified between the percent motility of the spermatozoa and percent inhibition of DPPH radical (r = 0.9, p<0.001).

Discussion

Changes in the spermatozoa viability and aging during storage time are a natural process and may be determined by the conditions and length of the process (Johnson *et al.*, 2000). Among these changes, the reduction of motility was the primary parameter used to judge the decrease of fertilizing ability of sperm cells (Johnson *et al.*, 2000). Sperm cells and seminal plasma have a complex antioxidant system to remove the oxygen radicals and prevent damage under normal physiological conditions. This process involves enzymatic and non-enzymatic defenses. Nevertheless, the antioxidant system of those cells is not sufficient to prevent the lipid peroxidation completely, during the period of storage, when the production of free radicals is significantly higher.

Spermatozoa are known to be susceptible to lipid peroxidation, resulting in motility losses in the presence of exogenous oxidant (Buffone *et al.*, 2012; Minaei *et al.*, 2012; Maia *et al.*, 2010; Guthrie and Welch, 2006). The increase in spermatozoa TBARS levels could be attributed to oxidative damage caused by long-term storage (Maia *et al.*, 2010). This result indicates that long-term storage can increase the oxidative stress, determined by TBA-RS content, which is in agreement with our data.

The progressive reduction on spermatozoa motility is negatively correlated with storage time, suggesting that oxidative damage generated by ROS is directly related to the exposure time. Similar results have indicated that loss of motility is correlated with lipid peroxidation (Minaei *et al.*, 2012; Maia *et al.*, 2010). In our study, we have identified that using diluent for LT storage with the addition of antioxidant resulted in better levels of sperm motility, indicating that anti-oxidative mechanisms contribute to a high percentage of motile to prevent losses in the sperm-membrane. Also, a positive correlation found between the percent motility of the spermatozoa and antioxidant activity time-dependent corroborate with this fact.

NPSH groups from spermatozoa in diluent for LT storage resulted in a significant time-dependent reduction and the addition of Trolox decreases ROS production and simultaneously was able to maintain levels of NPSH as initial levels. Membrane sulfhydryl groups serve as an important defense mechanism of the spermatozoa to fight against oxidative stress. Reduction in motility and losses of sperm functions can mask the activity of sulfhydryl groups and can be associated with male infertility (Bansal and Kaur, 2009; Bansal and Bilaspuri, 2008; Nivsarkar *et al.*, 1998). In addition to that, the nonprotein thiols play a significant role in eliminating toxic compounds (Seligman *et al.*, 2005). The scavenging action of NPSH acts counteracting the effect of oxidative stress in sperm cells, which could result in lipoperoxidation of the plasma membrane (Bansal and Kaur, 2009). As the increase of TBARS content remained constant 24 h after the beginning of the experiment up to 84 h of incubation. Probably this steady growth was due by the consumption of SH groups. Our results have indicated that LT storage can negatively influence the activity of important antioxidant enzymes. Storage of spermatozoa in long-term conservation caused a significant decrease catalase and sod activity, to the increase in peroxide formation. The enzymatic activity decreased should probably at increased free radicals produced as a consequence of long-term storage evidenced by TBARS content.

Furthermore, endogenous enzymes antioxidants as SOD and catalase activity and NPSH groups have no effect on controlling the oxidative stress during long-term boar semen storage. Also, all the samples presented a reduction in the scavenging activity being time dependent. A complex antioxidant system composed of different enzymatic activities, is present in spermatozoa and seminal plasma, to remove the oxygen radicals, under normal physiological conditions. Nevertheless, the antioxidant system of the cells is not potent enough to prevent the lipid peroxidation completely, especially during in vitro storage.

Conclusion

In conclusion, spontaneous spermatozoa ROS formation was high in viable swine sperm during the long-term storage period in samples without Trolox; consequently, sperm was quite susceptible to spontaneous lipid peroxidation and cell damage. Diluent with an antioxidant property that reduces oxidative stress and improves sperm motility could be useful for long-term storage of swine semen and other species. New studies are in development to understand the mechanisms involved with this antioxidant protection, including individual animal genetic variation.

Author Contribution

Carlos Bondan: Experimental design and Laboratory work.

Ricardo Zanella: Experimental design, Laboratory work and manuscript writing.

Eraldo Zanella: Experimental design and sample acquisition.

Márcia Rósula Poetini: Laboratory work.

Mariana Groke Marques: Manuscript writing, discussions and manuscript review.

Julio C.M. Soares: Experimental design, manuscript writing and coordination.

Conflict of Interest

None reported.

References

Bansal, A.K. and G.S. Bilaspuri, 2008. Oxidative stress alters membrane sulfhydryl status, lipid and phospholipid contents of crossbred cattle bull spermatozoa. Anim. Reprod. Sci., 104: 398-04. DOI: 10.1016/j.anireprosci.2007.06.017

Bansal, A.K. and A.R.J. Kaur, 2009. Cooperative functions of manganese and thiol redox system against oxidative stress in human spermatozoa. J. Hum. Reprod. Sci., 2: 76-80. DOI: 10.4103/0974-1208.57227

Bansal, A.K. and G.S. Bilaspuri, 2010. Impacts of oxidative stress and antioxidants on semen functions. Vet. Med. Int., 2011: 1-7. DOI: 10.4061/2011/686137

Bathgate, R., 2011. Antioxidant mechanisms and their benefit on post-thaw boar sperm quality. Reprod. Domest. Anim., 46: 23-5.
DOI: 10.1111/j.1439-0531.2011.01826.x

Buffone, M.G., J.C. Calamera, S. Brugo-Olmedo, S. Vincentiis and M.M. Calamera *et al.*, 2012. Superoxide dismutase content in sperm correlates with motility recovery after thawing of cryopreserved human spermatozoa. Fertil Steril., 97: 293-98. DOI: 10.1016/j.fertnstert.2011.11.012

Brezezińska-Slebodzińska, E., A.B. Slebodzinski, B. Prietas and G. Wieczorek, 1995. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. Biol. Trace. Element. Res., 47: 69-74.

Dimitrov, S., V. Atanasov, E. Dichlyanova and R. Petrova, 2009. Comparison of three commercial Diluents for short-term storage of boar Semen. Trakia J. Sci., 7: 58-62.

El-Tohamy, M.M., 2012. The mechanisms by which oxidative stress and free radical damage produces male infertility. Life Sci. J., 9: 674-78.

Gil-Villa, A.M., W. Cardona-Maya, A. Agarwal, R. Sharma and A. Cadavid, 2010. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. Fertil Steril., 94: 1465-72.
DOI: 10.1016/j.fertnstert.2009.05.042

Guthrie, H.D. and G.R. Welch, 2006. Determination of Intracellular reactive oxygen species and high mitochondrial flow cytometry membrane potential in Percoll-treated viable boar sperm using fluorescence-activated. J. Anim. Sci., 84: 2089-100. DOI: 10.2527/jas.2005-766

Hu, J.H., X.L. Zhao, W.Q. Tian, L.S. Zan and Q.W. Li, 2011. Effects of vitamin E supplementation in the extender on frozen-thawed bovine semen preservation. Animal, 5: 107-112.
DOI: 10.1017/S1751731110001679

Jeong, Y.J., M.K. Kim, H.J. Song, E.J. Kang and S.A. Ock *et al.*, 2009. Effect of alpha-tocopherol supplementation during boar semen cryopreservation on sperm characteristics and expression of apoptosis related genes. Cryobiology, 58: 181-9. DOI: 10.1016/j.cryobiol.2008.12.004

Johnson, L.A, K.F. Weitze, P. Fiser and W.M. Maxwell, 2000. Storage of boar semen. Anim. Reprod. Sci., 62: 143-72. DOI: 10.1016/S0378-4320(00)00157-3

Maia, M.S., S.D. Bicudo, C.C. Sicherle, L. Rodello and I.C.S. Gallego, 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. Anim. Reprod. Sci., 122: 118-23.
DOI: 10.1016/j.anireprosci.2010.08.004

Minaei, M.B., M. Barbarestani, S. Nekoonam, M.A. Abdolvahabi and N. Takzare *et al.*, 2012. Effect of Trolox addition to cryopreservation media on human sperm motility. Iran J. Reprod. Med., 10: 99-04.

Nivsarkar, M., B. Cherian and S. Patel, 1998. A regulatory role of sulfhydryl groups in modulation of sperm membrane conformation by heavy metals: Sulfhydryl groups as markers for infertility assessment. Biochem. Biophys. Res. Commun., 247: 718-19. DOI: 10.1006/bbrc.1998.8841

Ohkawa, H., N. Ohishi and K. Yagi, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., 95: 351-58.

Ogbuewu, I.P., N.O. Aladi, I.F. Etuk, M.N. Opara and M.C. Uchegbu *et al.*, 2010. Relevance of oxygen free radicals and antioxidants in sperm production and function. Res. J. Vet. Sci., 3: 138-64.
DOI: 10.3923/rjvs.2010.138.164

Peña, F.J., A. Johannisson, M. Wallgren and H. Rodriguez-Martinez, 2004. Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: Changes in sperm membrane lipid architecture. Zygote, 12: 117-24.
DOI: 10.1017/S096719940400262X

Peña, F.J., A. Johannisson, M. Wallgren and H. Rodriguez-Martinez, 2003. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim. Reprod. Sci., 78: 85-98.
DOI: 10.1016/S0378-4320(03)00049-6

Roca, J., J.M. Vázquez, M.A. Gil, C. Cuello and I. Parrilla *et al.*, 2006. Challenges in pig artificial insemination. Reprod. Domest. Anim., 41: 43-53.
DOI: 10.1111/j.1439-0531.2006.00768.x

Seligman, J., G.L. Newton, R.C. Fahey, R. Shalgi and N.S. Kosower, 2005. Nonproteinthiols and disulfides in rat epididymal spermatozoa and epididymalfluid: role of gamma-glutamyl-transpeptidase in sperm maturation. J. Androl., 26: 629-37. DOI: 10.2164/jandrol.05040

Sinha, K.A., 1972. Colorimetric assay of Catalase. Anal. Biochem., 47: 389-94.

Soares, J.C.M., V. Folmer, J.B.T. Rocha and C.W. Nogueira, 2014. Ebselen exhibits glycation-inhibiting properties and protects against osmotic fragility of human erythrocytes in vitro. Cel. Biol. Int., 38: 625-30. DOI: 10.1002/cbin.10253

Soares, J.C.M., R. Zanella, C. Bondan, L.P. Alves and M.R. Lima *et al.*, 2011. Biochemical and antioxidant changes in plasma, serum and erythrocytes of horses before and after a jumping competition. J. Eq. Vet. Sci., 31: 357-60. DOI: 10.1016/j.jevs.2011.03.017

Sun, M. and S. Zigman, 1978. An Improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. Anal. Biochem., 90: 81-89. DOI: 10.1016/0003-2697(78)90010-6.

Varo-Ghiuru, F., I. Miclea, A. Hettig, I. Ladoşi and V. Miclea *et al*., 2015. Lutein, Trolox, ascorbic acid and combination of Trolox with ascorbic acid can improve boar semen quality during cryopreservation. Cryo. Lett., 36: 1-7

Zanella, R., C. Bondan, J.C.M. Soares, E.L. Zanella and M.R. Lima, 2010. Use of antioxidants vitamins to improve the pregnancy rate in beef cattle submitted to a synchronization protocol with progesterone (P4). Ci. Anim. Bras., 11: 477-481.