

Review Article/Artículo de Revisión

## OXIDATIVE STRESS CHALLENGES DURING THE SPERM CRYOPRESERVATION IN DOGS DESAFIOS DEL ESTRÉS OXIDATIVO DURANTE LA CRIOPRESERVACION ESPERMATICA EN PERROS

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### ABSTRACT

Cryopreservation is one of the most important reproductive biotechnologies in dogs, which allows the preservation and propagation of genetic material even post mortem. However, sperm cryopreservation success depends on the adequacy of sperm cells to the extender, cryoprotectant, cooling curve and freezing/thawing processes. Moreover, during sperm cryopreservation, metabolites called Reactive Oxygen Species (ROS) are produced and play a key role on sperm oxidative stress. Thus, studies are required to improve sperm quality after thawing. Therefore, the aim of this review is to describe possible extenders for semen of dogs, techniques for cryopreservation (one and two steps) and thawing (fast and slow), the effect of oxidative stress in sperm quality of cryopreserved semen and the use of cryopreserved semen in artificial insemination protocols in bitches.

**Keywords:** Reactive Oxygen Species; Extenders; Artificial Insemination; One and Two steps; Dogs  
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### RESUMEN

La criopreservación es una de las biotecnologías más importantes en perro, la cual permite la preservación y propagación de material genético incluso post mortem. Sin embargo, el éxito del proceso de criopreservación depende de la adaptación de las células espermáticas al diluyente, al crioprotector, a la curva de enfriamiento y al proceso de congelado/descongelado. Además, durante la criopreservación espermática, se producen los metabolitos llamados Especies Oxígeno Reactivas (ROS) que juegan un rol clave en el estrés oxidativo. necesitándose estudios para mejorar la calidad espermática luego del descongelado. Por lo tanto, el propósito de esta revisión es describir posibles diluyentes para el semen de perros, técnicas para la criopreservación (una o dos etapas) y el descongelado (rápido o lento), el efecto del estrés oxidativo en la calidad espermática del semen criopreservado y el uso de este en los protocolos de inseminación artificial en perras.

**Palabras clave:** Especies Oxígeno Reactivas; Diluyentes; Inseminación Artificial; Una y Dos Etapas; Perros.  
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## INTRODUCTION

Nowadays, breeding dogs has a major importance. The high zootechnical, economic value and the affective character of some individuals increase the necessity of genetic material preservation for future generations. The advance of reproductive biotechnologies applied to small and farm animals, such as artificial insemination, semen cryopreservation, in vitro fertilization and cloning, promotes a higher reproductive performance (Choudhary et al. 2016). However, the development of assisted reproduction techniques in dogs is scarce compared to livestock animals (Goodrowe et al. 2000, Luvoni et al. 2005).

Among the reproductive biotechnologies applied to dogs, semen cryopreservation is one of the most important to allow the preservation and propagation of genetic material even post mortem, facilitating the semen transportation over to long distances, and reducing animal transportation costs. Moreover, cryopreservation can be used as a tool for increases the genetic diversity of the species, hence the dog is considered an alternative model for endangered species (Thomassen and Farstad 2009).

Despite the biological importance, semen cryopreservation depends on initial sperm viability and adequacy of sperm cells to the extender, cryoprotectant, cooling curve and freezing/thawing processes (Peña et al. 1998). Therefore, selection of the adequate extender for dogs is a critical point in cryopreservation (Holt 2000), as well as the freezing process (one or two steps) and thawing protocol (Silva et al. 2006). Therefore, further studies to improve these processes for the canine specie are required, since cryopreservation may promote loss of membrane integrity, decreased sperm motility and sperm DNA damage (Lucio et al. 2016a).

This reduction of sperm quality is closely related to the semen cryopreservation process, which leads to the production of Reactive Oxygen Species (ROS) by the spermatozoa. However, the ROS effect is related to physiological occurrence of oxidative stress of sperm cells, which is necessary for sperm capacitation and, therefore, fertilization (Lenzi et al. 2002). Nevertheless, oxidative stress can be premature during the cryopreservation process (Halliwell 1991, Sharma et al. 2012), altering proteins, carbohydrate moieties, sperm motility, plasma membrane functionality, and sperm DNA (Birben et al. 2012). Consequently, studies are required to attempt to improve sperm quality by preventing the ROS attack during cryopreservation (Bilodeau et al. 2001, Keskes-Ammar et al. 2003).

Thus, more scientific works are necessary to standardize protocols and techniques that may improve canine sperm cryopreservation. In this context, this review aims to explain the main topics of the sperm cryopreservation technique in dogs and their main critical points, such as the oxidative stress condition.

## EXTENDERS

On species in which artificial insemination with cryopreserved semen is a common practice, failures attributed to extender are easily detected and modified, favoring homogenization of the technique (Farstad 2009). However, there is no standard formula for the extenders commonly used for the canine specie, nor semen cryopreservation protocol. Fertility data with the use of cryopreserved semen are still scarce, preventing the homogenous evaluation and standardization of the technique for dogs (Farstad 2009).

Extender is a necessary substance for semen cryopreservation, as it has the function to maintain cell membrane stability due to its buffer property, controlling the pH (around 7), and to provide the electrolyte balance and osmolarity to the medium (Eilts 2005). The extender promotes sperm nutrition and cellular protection during refrigeration and cryopreservation, in addition to preventing bacterial growth (Farstad 2009).

Sperm extenders are constituted by: extracellular cryoprotectant (milk, egg yolk), intracellular cryoprotectant (glycerol, ethylene glycol or dimethyl sulfoxide), buffer agent (tris), sugar (glucose, sucrose, lactose, fructose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin, amikacin). There are several extenders recommended for semen cryopreservation in the canine specie, such as soy lecithin, low density lipoproteins (LDL), reduced glutathione, milk, liposomes and powder coconut water (Kmenta et al. 2011, Kasimanickam et al. 2012, Uchoa et al. 2012, Bencharif et al. 2013, Mota et al. 2014, Ogata et al. 2015, Lucio et al. 2016b, Gharajelar et al. 2016, Belala et al. 2016). However, tris-egg yolk- acid citric is the most worldwide employed due to its buffer activity and its reducing action on fructose metabolism by sperm cell, contributing to preservation of sperm energy (Farstad 1996). The egg yolk protects sperm from thermal shock during refrigeration and prevents losses of membrane phospholipids during cryopreservation process.

Along the sperm cryopreservation process, freezing of the intra and extracellular fluid medium leads to crystal ice formation. According to Elts 2005, intracellular crystal ice promotes loss of the semi permeability capacity of the plasma membrane, causing cellular death. Therefore, the exposure to the cryoprotectant is a fundamental stage to sperm cell survival during cryopreservation. The most frequently used cryoprotectant in dogs is glycerol, which preserves sperm fertility during the freezing and thawing processes, with variable final concentrations between 2 to 8% due to its potential toxic effect (Peña et al. 1998, Farstad 2009). According to Kim et al. 2007, in dogs, glycerol leads to better total and progressive motility after thawing in relation to other cryoprotectants. However, this agent has toxic effects on sperm cells, thus, its concentration must be balanced between cryoprotectant and toxic effects (Silva et al. 2002). Nevertheless, the moment of the cryoprotectant addition is variable, occurring whether the semen is at 37°C or after its refrigeration (5°C) (Peña et al. 1998, Silva et al. 2006, Farstad 2009).

## PROTOCOLS OF CRYOPRESERVATION AND THAWING PROCESSES

During semen cryopreservation, it is necessary to subject sperm to a decline of the temperature, dehydration and freezing. The drop of temperature during semen cryopreservation process is called refrigeration or cooling. In dogs, such stage occurs in a slow curve (0.5°C per minute) in which the sperm, initially at 37°C, reaches the temperature of 5°C (Lucio et al. 2016b). Along this period, changes occur on the lipid layer of the plasma membrane, with alterations of its functional state (Watson 1995, Watson 2000). The refrigeration curve is important, because the temperature decrease leads to changes on membrane permeability and cellular calcium regulation, influencing the process of sperm capacitation and acrosomal reaction (Barbas and Mascarenhas 2009).

During freezing process, intracellular crystal ices are formed, which may cause plasma membrane lesion. Therefore, it is necessary that the sperm loses its liquid content (Barbas and Mascarenhas 2009). Dehydration occurs during the glycerolization phase, which is the moment in which the cryoprotectant enters the sperm cell, in replacement to intracellular water (Barbas and Mascarenhas 2009). The cryoprotectant can be added both at 37°C or at 4°C according to Peña et al. 1998 and Silva et al. 2006. The difference between protocols of one or two steps is just the moment of cryoprotectant addition. The cryopreservation in one step consists on the addition of the cryoprotectant together with the entire extender on semen at the temperature of 37°C. On the other hand, the two steps protocol consists on the addition of half volume of extender (without cryoprotectant) at 37°C, the mixture goes to refrigerating curve and after it achieve 4°C the other half of extender (containing the cryoprotectant) is added (Brito et al. 2016).

The causes of cellular alterations during the cryopreservation process are mainly due to sperm temperature decrease, toxicity and osmotic stress caused by cryoprotectant exposure and the intra and extracellular crystal ice formation. Despite the advantages of cryopreservation, spermatozoa suffer various forms of stress, which cause damage to sperm integrity, membrane structure and sperm function (Yoon et al. 2015). In addition, sperm mitochondrial activity can serve as a generator of ROS, which can affect cellular composition and organelle functions (Starkov 2008).

Even though some authors refer that adding cryoprotector at 37°C may be toxic to sperm cell. Brito et al. 2016, showed that there is no difference between both protocols of cryoprotectant addition. Apart from the temperature of cryoprotectant addition, there are also different possibilities on packing methods and thawing temperatures. Canine semen on cryopreservation process is most often stored at 0,5 ml or at 0,25 ml plastic straws (Farstad 2010), however Strzezek et al. 2015, showed that there is also the possibility of storing dog semen in 5,0 ml aluminum tubes.

The process of semen thawing must be cautious, because the elevation of the temperature and activation of cellular metabolism must occur gradually, reducing the chances of sperm lesion (Barbas and Mascarenhas 2009). For the canine species, the thawing protocol mostly adopted is the exposure of the straws to a 37°C temperature during 30 seconds (Thomassen et al. 2006). Thawing process can be done at 37°C for 30 seconds (Brito et al. 2016) or also at 70°C for 8 seconds (Kim et al. 2007). Kim et al. (2007) reported that thawing semen at 70°C for 8 seconds provides better stabilization of sperm plasmatic membrane in dogs, however, Brito et al. 2016, exposed that the slow protocol of thawing (37°C for 30 seconds) increased the fertilizing ability of the cryopreserved canine spermatozoa. Moreover, the thawing process can be upgraded with the use of a thawing medium, such as described in goat and bull with reduced glutathione supplementation (Gadea et al. 2013, Gadea et al. 2008)

On canine specie, there is no standard protocol for semen cryopreservation or thawing. Consequently, there is no consensus as to the extender composition, the cryoprotectant to be used or the insemination dose of cryopreserved semen for dogs.

## OXIDATIVE STRESS AND SPERM CRYOPRESERVATION

The oxygen is an essential element for the maintenance of sperm function, because sperm cell performs their function on aerobic environments. However, high concentrations of oxygen can cause serious damage to cells, due to the formation of Reactive Oxygen Species (ROS) or free radicals (Ortega et al. 2003). The unbalance between formation and removal of free radicals on organic systems generate a pro-oxidant state promoting lesions and culminating on cellular death. This state is defined as oxidative stress (Rover Júnior et al. 2001).

ROS formation occurs because of oxygen ( $O_2$ ) reduction when receiving four electrons, with a subsequent formation of  $H_2O$ . On this reaction, the intermediary products formed are: superoxide anion ( $O_2^-$ ), hydroperoxyl ( $HO_2$ ) and hydroxyl ( $OH$ ) (Nordberg and Arner 2001). Besides these substances, there is the formation of hydrogen peroxide ( $H_2O_2$ ), which causes serious cellular damage, since it possesses high longevity, with the ability to go through biological membranes and produce free radicals (Nordberg and Arner 2001).

ROS are produced by sperm in physiological conditions and play an important role on mammal sperm function (Saleh and Agarwal 2002, Bennetts and Aitken 2005). In low concentrations, ROS are necessary to achieve sperm fertilizing capacity, since they are involved on kinetic function, as well as in sperm capacitation and hyperactivation and fusion of the oocyte with the spermatozoa (Aitken et al. 1993, Saleh and Agarwal 2002). The control of the free radical concentration is done by the antioxidants of the semen fluid and inside the sperm cell.

Semen has enzymatic (superoxide dismutase — SOD, catalase and glutathione peroxidase — GPx) and no enzymatic antioxidants (reduced glutathione — GSH, vitamin E and ascorbate) (Griveau and Le Lannou 1997, Michael et al. 2008). The contribution of each antioxidant system is different between species; however, it is believed that there is a synergistic action between them (Michael et al. 2008). According to Ferreira and Matsubara 1997, the antioxidants can act by inactivating free radicals (GSH, SOD, catalase, GPx and vitamin E) or repairing the oxidative stress lesions (ascorbate, glutathione reductase — GR and GPx).

Excessive production of free radical is responsible for alterations on semen quality, caused by decrease in sperm motility, as well as protein and DNA damage, resulting in cell apoptosis (Griveau and Le Lannou 1997, Lucio et al. 2016a). Spermatozoa are particularly sensitive to oxidative stress, because its plasma membrane has a high content of polyunsaturated lipid acids and they have low concentrations of protective enzymes on their cytoplasm (Sharma and Agarwal 1996). Polyunsaturated acids are susceptible to free radical action because of their double bonds of carbon, which is a more fragile target. In addition, intracellular antioxidant enzymes do not protect acrosomal and plasma membrane, so this must be protected by semen fluid (Iwasaki and Gagnon 1992, Zini et al. 1993).

Cryopreservation process induces free radical formation on semen samples (Watson 2000). According to Lucio et al. 2016a, the production of free radicals by sperm cells begins during glycerolization, and intensifies after thawing. Thermal shock and exposure to atmospheric oxygen and removal of semen fluid favor the lipid peroxidation by higher ROS production (Bucak et al. 2007). Physiological antioxidant concentration at sperm cytoplasm is insufficient to protect sperm cell during cryopreservation process (Bansal and Bilaspuri 2011). As a consequence of lipid peroxidation, destruction of the lipid matrix structure has been observed, leading to a loss of membrane permeability, decrease of sperm motility, sperm DNA lesion and reduction of sperm fertilizing capacity (Aitken and Elton 1984, Alvarez and Storey 1989, Aitken et al. 1993, Bucak et al. 2010). DNA compaction gives some protection to free radical action, however Bennetts and Aitken 2005, verified that mitochondrial DNA is in general more vulnerable to oxidative lesions than nuclear DNA (Aitken et al. 2003).

To minimize ROS deleterious effects on sperm during cryopreservation, research has involved the addition of antioxidant agents to the extender, such as GSH, ascorbate and catalase. Antioxidants supplementation to the extender during cryopreservation allows for the improvement of sperm quality after thawing and higher sperm longevity, which is considered an important indicator to the successful of cryopreservation in dogs (Monteiro et al. 2009). In fact, Lucio et al. 2016b, observed a higher acrosome protection and higher pregnant bitches with sperm cryopreserved with 10 mM of reduced glutathione (GSH). However, there is no consensus about the substance that provides better results after thawing.

## ARTIFICIAL INSEMINATION WITH CRYOPRESERVED SEMEN

Artificial insemination with fresh semen in bitches presents pregnancy rates similar to natural mating. Nevertheless, the use of cryopreserved semen leads to the reduction of those indexes (Yu et al. 2002). In the bovine species, the use of cryopreserved semen for artificial insemination presents good results worldwide (Barbas and Mascarenhas 2009). Linde-Forsberg and Forsberg 1993, verified that artificial inseminations done with cryopreserved semen in dogs have a reduction of 30% on pregnancy rate and litters 31% lower, when compared to inseminations

with fresh semen. Cryopreserved sperm present lower longevity, which compromises the technique results, as well as a lower sperm concentration compared to fresh semen (Silva et al. 1996). The success of artificial insemination with cryopreserved semen depends mainly on the artificial insemination technique, the method of detection of ovulation, the moment of insemination, the number of inseminations and sperm concentration (Eilts 2005).

However, the most important aspect of artificial insemination with cryopreserved semen is that the frozen-thawed semen is deposited in the uterus (Manson and Rous 2014, Romagnoli and Lopate 2014). Linde-Forsberg et al. 1999, compared three techniques of artificial insemination with cryopreserved semen: intravaginal, intrauterine with the use of a Norwegian catheter and intrauterine with the use of fiber optic endoscope. As a conclusion, authors found that intrauterine insemination with the Norwegian catheter presents pregnancy rate and number of pups per litter similar to those obtained in natural mating, assuming good sperm quality after thawing and proper monitoring of the estrous cycle of the bitch.

More specifically, Thomassen and Farstad 2009, verified that artificial insemination with cryopreserved semen presents better results when realized by intrauterine technique, with the use of semen with proper quality after thawing and 2 to 3 day after ovulation, considering that the insemination was done with a rigid catheter after fixation of the cervix by abdominal palpation, without needing sedation of the bitch. Additionally, authors suggest that, when performing two inseminations at intervals of 24 hours, the accuracy of the procedure increases.

## CONCLUSION

Semen cryopreservation process seems to be crucial for dog reproduction. Thus, careful selection of extenders and protocols for freezing and thawing are necessary to reduce the deleterious effects of the oxidative stress. In summary, materials, protocols and processes discussed here should be considered in order to obtain high fertility rates.

## REFERENCES

- Aitken RJ., Baker MA., Sawyer D. 2003. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. **Reproductive Biomedicine Online** 7:65-70.
- Aitken RJ., Buckingham D., Harkiss D. 1993. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. **Journal of Reproduction and Fertility** 97:441-450.
- Aitken RJ., Elton RA. 1984. Significance of Poisson distribution theory in analysing the interaction between human spermatozoa and zona-free hamster oocytes. **Journal of Reproduction and Fertility** 72:311-321.
- Alvarez JG., Storey BT. 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. **Gamete Research** 23:77-90.
- Bansal AK., Bilaspuri GS. 2011. Impacts of oxidative stress and antioxidants on semen functions. **Veterinary Medicine International** 2011: doi.org/10.4061/2011/686137
- Barbas JP., Mascarenhas RD. 2009. Cryopreservation of domestic animal sperm cells. **Cell and Tissue Banking** 10:49-62.
- Belala R., Briand-Amirat L., Vinciguerra L., Tainturier D., Kaidi R., Thorin C., Michaud S., Anton M., Bencharif D. 2016. Effect of equilibration time on the motility and functional integrity of canine spermatozoa frozen in three different extenders. **Research in Veterinary Science** 106:66-73.
- Bencharif D., Amirat-Briand L., Le Guillou J., Garand A., Anton M., Schmitt E., Desherces S., Delhomme G., Langlois ML., Destrumelle S., Vera-Munoz O., Barriere P., Tainturier D. 2013. Canine-chilled sperm. study of a semen extender made with low-density lipoproteins from hen egg yolk supplemented with glutamine. **Reproduction in Domestic Animals** 48:258-266.
- Bennetts LE., Aitken RJ. 2005. A comparative study of oxidative DNA damage in mammalian spermatozoa. **Molecular Reproduction and Development** 71:77-87.
- Bilodeau JF., Blanchette S., Gagnon C., Sirard MA. 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. **Theriogenology** 56:275-286.
- Birben E., Sahiner UM., Sackesen C., Erzurum S., Kalayci O. 2012. Oxidative stress and antioxidant defense. **World Allergy Organization Journal** 5(1):9-19. doi:10.1097/WOX.0b013e3182439613.
- Brito MM., Lúcio CF., Angrimani DSR., Losano JDA., Dalmazzo A., Nichi M., Vannucchi CI. 2016. Comparison of Cryopreservation Protocols (Single and Two-steps) and Thawing (Fast and Slow) for Canine Sperm. **Animal Biotechnology** 1-7. doi.org/10.1080/10495398.2016.1203797
- Bucak MN., Atessahin A., Varisli O., Yuca A., Tekin N., Akcay A. 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process. **Theriogenology** 67:1060-1067.
- Bucak MN., Tuncer PB., Sariozkan S., Baspinar N., Taspinar M., Cayan K., Bilgili A., Akalin PP., Buyukleblebici S., Aydos S., Ilgaz S., Sunguroglu A., Oztuna D. 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters. antioxidants protect DNA integrity against cryodamage. **Cryobiology** 61:248-253.

- Choudhary KK., Kavya KM., Jerome A., Sharma RK. 2016. Advances in reproductive biotechnologies. **Veterinary World** 9:388-395.
- Eilts BE., 2005. Theoretical aspects of canine semen cryopreservation. **Theriogenology** 64:692-697.
- Farstad W. 1996. Semen cryopreservation in dogs and foxes. **Animal Reproduction Science** 42:251-260.
- Farstad W., 2009. Cryopreservation of canine semen - new challenges. **Reproduction in Domestic Animals** 44(Suppl 2):336-341.
- Farstad W. 2010. Artificial insemination in dogs. In: England G, von Heimendahl A (eds) **BSAVA Manual of Canine and Feline Reproduction and Neonatology**, 2nd ed., British Small Animal Veterinary Association, Quedgeley, Gloucester, pp 80-88
- Ferreira ALA., Matsubara LS. 1997. Radicais livres, conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo. **Revista da Associação Médica Brasileira** 43:61-68.
- Gharajelar SN., Sadrkhanloo RA., Onsoni M., Saberivand A. 2016. A comparative study on the effects of different cryoprotectants on the quality of canine sperm during vitrification process. **Veterinary Research Forum** 7:235-239.
- Gadea J., Gumbao D., Canovas S., Garcia-Vazquez FA., Grullon LA., Gardon JC. 2008. Supplementation of the dilution medium after thawing with reduced glutathione improves function and the in vitro fertilizing ability of frozen-thawed bull spermatozoa. **International Journal of Andrology** 31:40-49.
- Gadea J., Gumbao D., Gomez-Gimenez B., Gardon JC. 2013. Supplementation of the thawing medium with reduced glutathione improves function of frozen-thawed goat spermatozoa. **Reproductive Biology** 13:24-33.
- Goodrowe KL., Walker SL., Ryckman DP., Mastromonaco GF., Hay MA., Bateman HL., Waddell WT. 2000. Piecing together the puzzle of carnivore reproduction. **Animal Reproduction Science** 60-61:389-403.
- Griveau JF., Le Lannou D. 1997. Influence of oxygen tension on reactive oxygen species production and human sperm function. **International Journal of Andrology** 20:195-200.
- Halliwell B. 1991. Reactive oxygen species in living systems. source, biochemistry, and role in human disease. **The American Journal of Medicine** 91:S14-S22.
- Holt WV. 2000. Basic aspects of frozen storage of semen. **Animal Reproduction Science** 62:3-22.
- Iwasaki A., Gagnon C. 1992. Formation of reactive oxygen species in spermatozoa of infertile patients. **Fertility and Sterility** 57:409-416.
- Kasimanickam VR., Kasimanickam RK., Memon MA., Rogers HA. 2012. Effect of extenders on sperm mitochondrial membrane, plasma membrane and sperm kinetics during liquid storage of canine semen at 5 degrees C. **Animal Reproduction Science** 136:139-145.
- Keskes-Ammar L., Feki-Chakroun N., Rebai T., Sahnoun Z., Ghazzi H., Hammami S., Zghal K., Fki H., Damak J., Bahloul A. 2003. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. **Archives of Andrology** 49:83-94.
- Kim HJ., Oh HJ., Jang G., Kim MK., 2007. Birth of puppies after intrauterine and intratubal insemination with frozen-thawed canine semen. **Journal of Veterinary Science** 8:75-80.
- Kmenta I., Strohmayer C., Muller-Schlosser F., Schafer-Somi S. 2011. Effects of a lecithin and catalase containing semen extender and a second dilution with different enhancing buffers on the quality of cold-stored canine spermatozoa. **Theriogenology** 75:1095-1103.
- Lenzi A., Gandini L., Lombardo F., Picardo M., Maresca V., Panfili E., Tramer F., Boitani C., Dondero F. 2002. Polyunsaturated fatty acids of germ cell membranes, glutathione and blutathione-dependent enzyme-PHGPx. from basic to clinic. **Contraception** 65:301-304.
- Linde-Forsberg C., Forsberg M. 1993. Results of 527 controlled artificial inseminations in dogs. **Journal of Reproduction and Fertility**. (Supplement) 47:313-323.
- Linde-Forsberg C., Strom Holst B., Govette G. 1999. Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen. a retrospective study. **Theriogenology** 52:11-23.
- Lucio CF., Regazzi FM., Silva LC., Angrimani DS., Nichi M., Vannucchi CI. 2016a. Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. **Theriogenology** 85:1568-1575.
- Lucio CF., Silva LC., Regazzi FM., Angrimani DS., Nichi M., Assumpcao ME., Vannucchi CI. 2016b. Effect of reduced glutathione (GSH) in canine sperm cryopreservation. In vitro and in vivo evaluation. **Cryobiology** 72:135-140.
- Luvoni GC., Chigioni S., Allievi E., Macis D. 2005. Factors involved in vivo and in vitro maturation of canine oocytes. **Theriogenology** 63:41-59.
- Mason SJ., Rous NR. 2014. Comparison of endoscopic-assisted transcervical and laparotomy insemination with frozen-thawed dog semen. a retrospective clinical study. **Theriogenology** 82:844-850.
- Michael AJ., Alexopoulos C., Pontiki EA., Hadjipavlou-Litina DJ., Saratsis P., Ververidis HN., Boscos CM. 2008. Quality and reactive oxygen species of extended canine semen after vitamin C supplementation. **Theriogenology** 70:827-835.
- Monteiro JC., Goncalves JS., Rodrigues JA., Lucio CF., Silva LC., Assumpcao ME., Vannucchi CI. 2009. Influence of ascorbic acid and glutathione antioxidants on frozen-thawed canine semen. **Reproduction in Domestic Animals** 44(Suppl 2):359-362.
- Mota AC., Silva HVR., Nunes TGP., de Souza MB., de Freitas LA., de Araujo AA., da Silva LDM. 2014. Cryopreservation of canine epididymal sperm using ACP-106c and TRIS. **Cryobiology** 69:17-21.
- Nordberg J., Arner ES. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. **Free Radical Biology & Medicine** 31:1287-1312.
- Ogata K., Sasaki A., Kato Y., Takeda A., Wakabayashi M., Sarentonglaga B., Yamaguchi M., Hara A., Fukumori R., Nagao Y. 2015. Glutathione supplementation to semen extender improves the quality of frozen-thawed canine spermatozoa for transcervical insemination. **Journal of Reproduction and Development** 61:116-122.
- Ortega AM., Izquierdo AC., Gómez JH., Corichi IMO., Torres VMM., Méndez JVV. 2003. Peroxidación lipídica y antioxidantes en la preservación de semen. una revisión. **Interciencia** 28:699-704.
- Peña AI., Barrio F., Quintela LA., Herradon PG. 1998. Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. **Theriogenology** 50:163-174.

- Romagnoli S., Lopate C. 2014. Transcervical artificial insemination in dogs and cats. review of the technique and practical aspects. **Reproduction in Domestic Animals** 49(Suppl 4):56-63.
- Rover Júnior L., Hoehr NF., Vellasco AP., Kubota LT. 2001. Sistema antioxidante envolvendo o ciclo metabólico da glutatona associado a métodos eletroanalíticos na avaliação do estresse oxidativo. **Química Nova** 24:112-119.
- Saleh RA., Agarwal A. 2002. Oxidative stress and male infertility. from research bench to clinical practice. **Journal of Andrology** 23:737-752.
- Sharma P., Jha AB., Dubey RS., Pessarakli M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. **Journal of Botany** 2012: Article ID 217037. doi.org/10.1155/2012/217037
- Sharma RK., Agarwal A. 1996. Role of reactive oxygen species in male infertility. **Urology** 48:835-850.
- Silva AR., Cardoso RC., Silva LD. 2006. Influence of temperature during glycerol addition and post-thaw dilution on the quality of canine frozen semen. **Reproduction in Domestic Animals** 41:74-78.
- Silva AR., de Cassia Soares Cardoso R., Uchoa DC., Machado da Silva LD. 2002. Effect of tris-buffer, egg yolk and glycerol on canine semen freezing. **Veterinary Journal** 164:244-246.
- Silva LD., Onclin K., Lejeune B., Verstegen JP. 1996. Comparisons of intravaginal and intrauterine insemination of bitches with fresh or frozen semen. **The Veterinary Record** 138:154-157.
- Starkov AA., 2008. The role of mitochondria in reactive oxygen species metabolism and signaling. **Annals of the New York Academy of Sciences** 1147:37-52.
- Strzezek R., Polakiewicz P., Kordan W. 2015. The effect of two packaging systems on the post-thaw characteristics of canine sperm. **Polish Journal of Veterinary Sciences** 18:249-253.
- Thomassen R., Farstad W. 2009. Artificial insemination in canids. a useful tool in breeding and conservation. **Theriogenology** 71:190-199.
- Thomassen R., Sanson G., Krogenaes A., Fougner JA., Berg KA., Farstad W. 2006. Artificial insemination with frozen semen in dogs. a retrospective study of 10 years using a non-surgical approach. **Theriogenology** 66:1645-1650.
- Uchoa DC., Silva TFP., Mota AC., Silva LDM. 2012. Intravaginal Artificial Insemination in Bitches Using Frozen/Thawed Semen after Dilution in Powdered Coconut Water (ACP-106c). **Reproduction in Domestic Animals** 47:289-292.
- Watson PF. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. **Reproduction Fertility and Development** 7:871-891.
- Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. **Animal Reproduction Science** 60-61:481-492.
- Yoon SJ., Kwon WS., Rahman MS., Lee JS., Pang MG. 2015. A novel approach to identifying physical markers of cryo-damage in bull spermatozoa. **PloS One** 10(5):e0126232. doi: 10.1371/journal.pone.0126232
- Yu I., Songsasen N., Godke RA., Leibo SP. 2002. Differences among dogs in response of their spermatozoa to cryopreservation using various cooling and warming rates. **Cryobiology** 44:62-78.
- Zini A., de Lamirande E., Gagnon C. 1993. Reactive oxygen species in semen of infertile patients. levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. **International Journal of Andrology** 16:183-188.