B-4411
[1-6]

RESEARCH ARTICLE Indian Journal of Animal Research, Volume Issue : ()

Comparing Glycerol with Dimethylformamide and Combination for Cryopreservation of Pug Dog Semen in Egg Yolk Plasma Containing Extender

A. Sharma[1], Ranjna S. Cheema[2], A. Pathania[2]

10.18805/IJAR.B-4411

ABSTRACT

Background: In order to find an alternative cryoprotectant for canine semen, different cryoprotectants, as dimethyl sulphoxide and ethylene glycol, dimethyle formamide either alone or in combination with glycerol have been evaluated on pooled semen of different breeds with different opinions.

Methods: In the current study the effect of glycerol, DMF and their combination in Tris-citric acid-fructose egg yolk plasma extender on motility, viability, plasma membrane integrity (PMI), acrosome integrity (AI), inner mitochondrial membrane potential (IMMP), reactive oxygen species (ROS) and antioxidant enzymes in frozen-thaw semen of pug breed was compared.

Result: Values for motility, viability, (PMI), AI were significantly ($p<0.05$) high in TCFEYP-G compared to TCFEYP-DMF and TCFEYP-G+DMF at post thaw. However, HIMMP and MIMMP/MDA were non-significantly ($P>0.05$) high and low in TCFEYP-G compared to TCFEYP-DMF and TCFEYP- G + DMF at post thaw, respectively. MDA concentration was significantly ($p<0.05$) low in TCFEYP-G compared to TCFEYP-DMF and TCFEYP-G + TCFEYP-DMF extenders. However, H_2O_2, scavenging capacity of spermatozoa, cryopreserved in TCFEYP-G was significantly ($p<0.05$) higher than in TCFEYP-DMF and TCFEYP-G + TCFEYP-DMF. However, there was no significant ($p>0.05$) difference in superoxide free radical scavenging activity and nitrite concentration of spermatozoa among the three extenders. Activity of GPX, SOD and Catalase was also significantly ($p<0.05$) higher in TCFEYP-G compared to TCFEYP-DMF and TCFEYP-G+DMF. However, values were reverse for GRE activity. This study concludes that glycerol is a healthier cryoprotectant to protect canine spermatozoa from cryo-injury during freezing-thawing process.

Key words: Cryopreservation, DMF, Glycerol, Pug, Semen.

INTRODUCTION

There is an increasing demand for preservation of canine semen among the breeders breeding specific breeds. Cryopreservation facilitates a wide use of ejaculates of stud dogs, irrespective of time and location. The success of the freezing and thawing of semen is dependent upon a number of steps expected to achieve less damage to the sperm and securing acceptable longevity *in vitro* and *in vivo* (Fastad, 1996). Many protocols have been described for canine semen cryopreservation and differences are linked to the composition of the extenders (Peña *et al.* 2003) and nature of the cryoprotectants (Olar *et al.* 1989). Most of the researchers have used Tris-egg-yolk extender (Peña and Linde-Forsberg, 2000, Peña *et al.* 2003). In order to find an alternative cryoprotectant for canine semen, different cryoprotectants, as dimethylsulphoxide and ethylene glycol, had been used either alone or in combination with glycerol (Cavalcanti *et al.* 2002, Martins-Bessa *et al.* 2006, Rota *et al.* 2006). The observations of those studies were based only on the analysis of post-thaw sperm motility or a few functional tests such as the hypoosmotic swelling test (HOST). Some studies indicated that these cryoprotectants are similar (Martins-Bessa *et al.* 2006, Rota *et al.*, 2006) or sometimes worse (Cavalcanti *et al.* 2002) than glycerol. Other alternative cryoprotectants for semen freezing are the amides, which have been frequently used as an option for equine semen cryopreservation (Alvarenga *et al.* 2005, Carmo *et al.* 2005). Amides have been also tested in canine semen freezing but with dissimilar observations on pooled semen samples of different breeds (Lopes *et al.* 2009, Oliveira *et al.* 2006, Mota Filho *et al.* 2011).

Cryoprotective effects of amides are due to lower molecular weights (73.09) and viscosities in comparison with glycerol (92.05) and for higher membrane permeability.

[1]Department of Veterinary Gynecology and Obstetrics, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India.
[2]Department of Bioinformatics, College of Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India.

Corresponding Author: Ranjna S. Cheema, Department of Bioinformatics, College of Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India. Email: ranjna.cheema@gmail.com

How to cite this article: Sharma, A., Cheema, R.S. and Pathania, A. (2021). Comparing Glycerol with Dimethylformamide and Combination for Cryopreservation of Pug Dog Semen in Egg Yolk Plasma Containing Extender. Indian Journal of Animal Research. DOI:10.18805/IJAR.B-4411

Submitted: 25-01-2021 **Accepted:** 22-07-2021 **Online:**09-08-2021

Since, addition of the methyl (CH3) radical into the amide molecule increases its permeability through the sperm membrane, therefore, it may improve the efficiency of its cryoprotective action (Bianchi *et al.* 2008). Therefore, the aim of the current study was to compare the effects of glycerol, DMF and their combination on motility, viability, plasma membrane integrity (PMI), acrosome integrity (AI), inner mitochondrial membrane potential (IMMP), reactive oxygen species (ROS) and antioxidant enzymes in frozen - thaw semen of pug breed.

MATERIALS AND METHODS

Maintenance of dogs and semen collection

All the procedures were approved by the CPCSEA, New Delhi vide F. No 25-19-2018-CPCSEA, dated 22/11/2018. The experiments were conducted at Reproductive Biology Lab, Department of Veterinary Gynecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, Punjab, India from May, 2020 to October, 2020.

Analysis of sperm attributes in frozen-thaw semen

Semen was collected from four Pug dogs by digital method at an interval of 3 days. Motility was evaluated by wet mount and track method. Viability was assessed by eosin-nigrosin (Dhillon *et al.*, 2020). Pooled ejaculates of four dogs were divided into three parts and extended with 1) Tris citric acid-fructose-egg yolk plasma-glycerol (TCFEYP-G), 2) TCFEYP-Dimethylformamide (TCFEYP-DMF) and 3) TCFEYP-G+DMF extenders and processed for cryopreservation as per standardized protocol in our lab (Cheema *et al*., 2021). AI of spermatozoa was assessed using Coomassie brilliant blue stain (CBB, R-250, Feng *et al.*, 2007). Inner mitochondrial membrane potential was evaluated by staining the spermatozoa with JC-1 stain (JC-1 stain kit, Sigma – Aldrich, Cheema *et al.* 2021). About 200 spermatozoa were counted in different fields for all sperm attributes and percentage of motility, viability, plasma membrane integrity, acrosome integrity and IMMP [high (H), medium (M) and low (L)] was calculated.

Reactive oxygen species

Sperm extract was prepared in Tris buffer to evaluate following free radicles and antioxidant enzymes (Cheema *et al.* 2021)

Superoxide anion radical assay (Robak *et al.* 1988)

The reaction mixture was prepared by mixing 75 µl phosphate buffer (pH 7.4), 25 µl NBT (100 µM), 25 µl NADH (468 µM) and 25 µl sperm extract. The reaction was started by adding 25 µl phenazine methosulfate solution (60µM) in to the reaction mixture followed by incubation at 25°C for 5 min. A control was also run without sample. The absorbance was measured at 560 nm against blank. Percentage of SO_2 scavenging activity was calculated by using the following formula:

$$(\text{Abs sample} - \text{Abs control}) / \text{Abs sample} \times 100$$

H_2O_2 radical assay (Ruch *et al.* 1989)

A solution of hydrogen peroxide (H_2O_2, 40 mmol/l) was prepared in phosphate buffer (pH 7.4). Reaction mixture was prepared by mixing 150 µl of 40 mmol H_2O_2 and 25 µl sperm extract and incubated at room temperature for 10 min. Absorbance of 40 mM H_2O_2 (control) and reaction mixture was measured at 230 nm against PBS, pH 7.4. H_2O_2 activity (%) was calculated by the following formula:

$$(\text{Abs control} - \text{Abs sample}) / \text{Abs control} \times 100$$

Nitrite radical assay (Marcocci *et al.* 1994)

Griess reagent (50 µl) and sperm extract (25 µl) were mixed and incubated at room temperature for 10 mins. A blank consisting of 50 µl PBS, pH 7.4 and 25 µl sperm extract was also ran along with the samples. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with 1-naphthyl ethylene diamine dihydrochloride was immediately read at 540 nm. NO_2^- concentration in the samples was calculated from standard curve plotted for 1mM-10 mM concentrations of sodium nitrite.

Lipid peroxidation and antioxidant enzymes

Malondialdehyde concentration (end product of LPO), Glutathione reductase (GRE), Glutathione peroxidase (GPx) and Catalase (CAT) were estimated in sperm extracts as per modified and standardized methods in our earlier study (Cheema *et al.* 2021). MDA concentration and enzyme activity were calculated by using the following formulas:

MDA content (µM) =

$$\frac{\text{OD} \times \text{Volume of assay mixture}}{\text{Volume of sample taken} \times \text{Coefficient extinction}}$$

GRE (IU/10^9 spermatozoa/min) = $\Delta T - \Delta C$

GPX (IU/10^9 spermatozoa/min) = $\Delta T - \Delta C$

Where,

ΔT - Change in OD_{Test} at 60 sec interval and ΔC - Change in $OD_{Control}$ at 60 sec interval.

CAT (KU/10^9 spermatozoa) =

$$\frac{\text{A (Sample)} - \text{A (Blank 1)} \times 27.1}{\text{A (Blank 2)} - \text{A (Blank 3)}}$$

RESULTS AND DISCUSSION

Comparative evaluation of extenders based on sperm attributes

Values for motility, viability, PMI, AI were significantly ($p<0.05$) high in TCFEYP-G compared to TCFEYP-DMF and TCFEYP-G+DMF at post thaw. However, HIMMP and MIMMP/MDA were non-significantly ($P>0.05$) high and low in TCFEYP-G compared to TCFEYP-DMF and TCFEYP- G + DMF at post thaw, respectively (Fig 1). There was a difference of 23.4%, 21.2%, 19.4%, 14.7%, 15.0%, 8.7%, 40.1 µM and 21.7%, 24.1%, 7.7%, 24.7%, 12.5%, 21.2%,

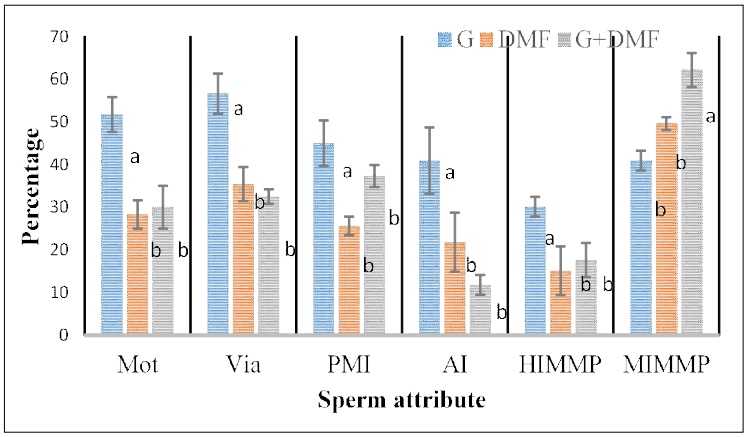

Fig 1: Sperm attributes in post thaw semen cryopreserved in different extenders. Superscripts a, b indicates significant (p<0.05) differences in sperm attributes among the extenders.

42.5 µM in motility, viability, PMI, AI, HIMMP, MIMMP and MDA concentration between TCFEYP-G x TCFEYP-DMF and TCFEYP-G x TCFEYP-G + DMF extenders, respectively. Higher motility, viability, PMI, AI, HIMMP and low MIIMP / Lipid per oxidation (MDA production) in TCFEYP-G extender indicated that glycerol gives more protection to the sperm against cryo-effect than DMF and combination of both. A study on canine semen indicated significant difference (P < 0.05) between glycerol and DMF with regard to subjective progressive motility, objective progressive motility, velocity average pathway and amplitude of lateral head, which also confirmed the efficiency of glycerol (Lopes *et al.*, 2009) for cryopreservation of canine semen. Overall, sperm motility and membrane intactness/function were higher when glycerol was used as a cryoprotectant, as compared to DMF (P < 0.05) (Hernandez-Aviles *et al.*, 2020).

Penetrating and non-penetrating Cryoprotectants (CPAs) reduce the physical and chemical stress exerted by the freezing process on spermatozoa. The most frequently used cryoprotectant for dog semen cryopreservation is glycerol which has a mixed intracellular and extracellular action. Glycerol reduces the salt concentration, thus lowering the freezing point of the solution and the risk of membrane perforation. It can interact by hydrogen bonding with water and can permeate across the plasma membrane of many different cell types at a relatively slow rate. Membrane permeability of DMF is higher in comparison to glycerol (molecular weight 92.05) due to its lower molecular weight (73.09) and viscosity. In general, permeability decreases as the molecular size of the substance increases. CPAs with low permeability can cause more osmotic stress than CPAs with high permeability. Although, glycerol has a high molecular weight compared to DMF, but there was low level of cryoinjury in TFCEYP-G compared to TFCEYP-DMF and combination of both extenders. It may be revealed from this study that there was no significant advantage using DMF to replace glycerol in cryopreservation of canine semen. Our observations are in agreement with that reported in canine (Futino *et al.* 2008, Lopes *et al.* 2009, Hernandez-Aviles *et al.* 2020). However, it is suggested that 5% DMF in lactose extender could efficiently preserve post thaw quality of canine semen, with motile sperm values of 45.0% (Oliveira *et al.* 2006). DMF at a concentration of 7% was added to the Tris base extender in the present study. In earlier studies, concentration of DMF and buffer were different (Oliveira *et al.* 2006, Futino *et al.* 2008, Lopes *et al.* 2009). It may be assumed that when DMF is used to cryopreserve canine semen, the concentration (Pena *et al.* 1998), method (Silva *et al.* 2003) and temperature (Silva *et al.* 2006) of its addition may affect the quality of frozen-thawed sperm.

Comparative evaluation of extenders based on lipid per oxidation, free radical scavenging and antioxidant enzyme activity

MDA concentration (µM/10^9 spermatozoa) was significantly (p<0.05) low in TCFEYP-G (47.7±1.3) compared to TCFEYP-DMF (96.6±17.5) and TCFEYP-G + TCFEYP-DMF (102.6 ± 3.2) extenders (Fig 2). However, H_2O_2, scavenging capacity of spermatozoa, cryopreserved in TCFEYP-G (90.5±0.4 %) was significantly (p<0.05) higher than in TCFEYP-DMF (70.9±6.0 %) and TCFEYP-G + TCFEYP-DMF (81.3±1.7 %) (Fig 2). However, there was no significant (p>0.05) difference in superoxide free radical scavenging activity and nitrite concentration of spermatozoa among the three extenders (Fig 3). Activity of GPX, SOD and Catalase was also significantly (p<0.05) higher in TCFEYP-G (0.34±0.06, 144.2±1.0, 471±5.5 IU/10^9 spermatozoa) compared to TCFEYP-DMF (0.27±0.08, 149±2.3, 451.3±3.2 IU/10^9 spermatozoa) and TCFEYP-G+DMF (0.07±0.02, 137.3±1.7, 384±4.5 IU/10^9 spermatozoa) (Fig 2, 3). However, values were reverse for GRE activity (Fig 3).

Studies conducted in other animals, also have demonstrated a reduction in the sperm membrane intactness following semen cryopreservation using DMF as a cryoprotectant (Bezerra *et al.* 2011 and Forero-Gonzalez *et al.* 2012). It may be assumed from poor results yielded using DMF as a cryoprotectant that the sperm plasma membrane from those species has a similar composition to the canine

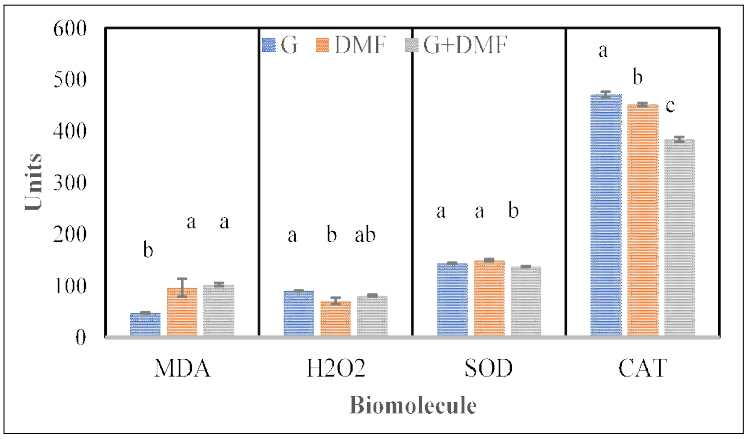

Fig 2: Malonaldehyde (MDA, µM/10^9 spermatozoa), hydrogen peroxide scavenging activity (H_2O_2, %)), superoxide dismutase (SOD, IU/10^9 spermatozoa) and catalase enzyme ativity (CAT, KU/10^9 spermatozoa) in post thaw semen cryopreserved in different extenders. Superscripts a, b indicates significant ($p<0.05$) differences in biomolecules among the extenders.

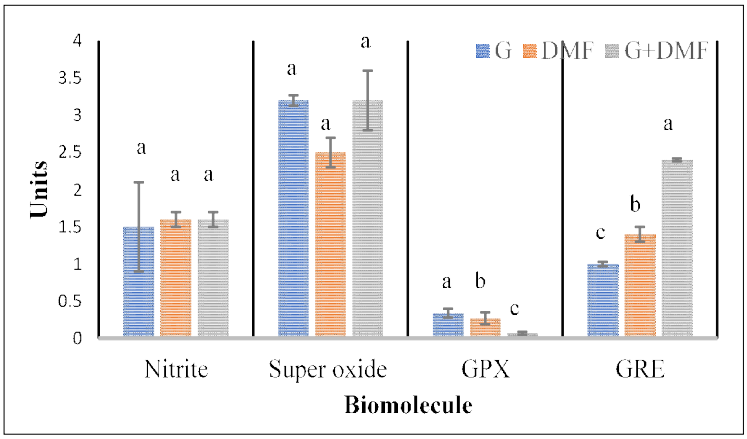

Fig 3: Nitrite (µM/10^9 spermatozoa), Super oxide scavenging activity (%)), glutathione peroxidase (GPX, IU/10^9 spermatozoa) and glutathione reductase enzyme activity (GRE, IU/10^9 spermatozoa) in post thaw semen cryopreserved in different extenders. Superscripts a, b, c indicates significant ($p<0.05$) differences in biomolecules among the extenders.

sperm membranes and hypothesized that a common susceptibility to low-molecular weight cryoprotectants could exist in these species (Hernandez-Aviles *et al.* 2020). The presence or absence of different transmembrane proteins with affinity for a specific cryoprotectant might account for the species-specific response to the effect of DMF or glycerol during freezing. Horse sperm cryopreserved in extender containing DMF and with better tolerance to DMF exhibits no difference in the relative expression of Aquaporin-7 (AQP-7). On the other hand, the use of this same extender was related to a differential expression of AQP-3 and AQP-11 (Bonilla-Correal *et al.* 2017)

In other species, where semen freezing relatively goes well with amide-containing extenders (boars and bulls), the differential expression of these water and glycerol transporting proteins has also been demonstrated (Prieto-Martínez *et al.* 2017a, Prieto-Martínez *et al.* 2017b). It can be assumed from these observations that the transport of amides could be differentially affected depending on the type of cryoprotectant used in the extender, which in turn may be differentially related to the presence of aquaporins in canine semen. Lower osmotic coefficient of DMF may be another reason that allowed its faster passive diffusion through the plasma membrane, which reduced the amount of cryoprotectant available within the sperm before freezing (Oldenhof *et al.* 2012).

Deterioration of PMI, AI and HIMMP in DMF containing extender compared to glycerol correspond to the motility and viability during the present study. It indicated that HOST and HMMP were the parameters which explained the variability in post-thaw motility observed after freezing (Hernandez-Aviles *et al.* 2018). The HOST predicts the function of the sperm membrane by evaluating the capacity of the sperm cell to react to an osmotic challenge when is incubated in a low-osmolality solution (Ramu and Jeyendran, 2013) and a higher mitochondrial membrane potential is associated with a more efficient energy production through the mitochondrial electron transport chain (Amaral *et al.* 2013). Further deviant mitochondrial function is associated to oxidative stress and apoptosis (Aitken *et al.* 2016). MDA

production indicating oxidative stress was high, while H_2O_2 scavenging activity and SOD, catalase, GPX enzyme activity of spermatozoa were low in DMF containing extender compared to glycerol. It may be predicted that sperm plasma membrane osmotic capacity is reduced when the plasma membrane intactness or the mitochondrial electron transport chain are compromised due to osmotic stress or oxidative stress (Gonzalez-Fernandez *et al.* 2012 and Ortega-Ferruso *et al.* 2017,). This compromise was more in spermatozoa cryopreserved in DMF containing extender. Higher percentage of free radical scavenging activity and higher activity of SOD, GPX and catalase in semen, cryopreserved in TCFEYP-G revealed that balance of antioxidants and free radical formation was better maintained by glycerol than DMF or their combination. Therefore, higher percentage of motile, viable spermatozoa, higher intact plasma membrane, intact acrosome and high potential of mitochondrial membrane were a result of reduced oxidative stress in TCFEYP-G extender. 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 (Lucio *et al.* 2016).

To our knowledge, this is the first study in which the effect of DMF on the membranes of dog spermatozoa is evaluated on a combination of sperm function test including oxidative stress, ROS and antioxidant enzymes.

CONCLUSION

This study concludes that glycerol is a healthier cryoprotectant than dimethyl formamide to protect canine spermatozoa from cryo-injury during freezing-thawing process of canine semen.

REFERENCES

Aitken, R.J., Gibb, Z., Baker, M.A., Drevet, J. and Gharagozloo, P. (2016). Causes and consequences of oxidative stress in spermatozoa. Reproduction Fertility Development. 28: 1-10.

Alvarenga, M.A., Papa, F.O., Landim-Alvarenga, F.C. and Medeiros, A.S.L. (2005). Amides as cryoprotectants for freezing stallion semen: a review. Animal Reproduction Science. 89: 105-13.

Amaral, A., Lourenco, B., Marques, M. and Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. Reproduction. 146: 163-74.

Bezerra, F.S.B., Castelo, T.S., Alves, H.M., Oliveira, I.R.S. and Lima, G.L., *et al.* (2011). Objective assessment of the cryoprotective effects of dimethylformamide for freezing goat semen. Cryobiology. 63: 262-66.

Bianchi, I., Calderam, K., Maschio, E.F., Madeira, E.M., Ulguim, R.R., Corcini, C.D., Bongalhardo, D.C., Correˆa, E.K., Lucia, T. Jr, Deschamps, J.C. and Correˆa, M.N. (2008). Evaluation of amides and centrifugation temperature in boar semen cryopreservation. Theriogenology. 69: 632-38.

Bonilla-Correal, S., Noto, F., García-Bonavila, E., Rodríguez-Gil, J.E., Yeste, M. and Miro, J. (2017). First evidence for the presence of aquaporins in stallion sperm. Reproduction in Domestic Animals. 52: 61-64.

Carmo, M.T., Papa, F.O., Medeiros, A.S.L., Araujo, G.H.M. and Alvarenga, M.A. (2005). Improvement of stallion semen post-thaw motility with the association dimethyl formamide. Animal Reproduction Science. Abstr. 89: 286.

Cavalcanti, M.C.O., Moura, C.S., Guerra, M.M.P. and Silva, S.V. (2002). Cryoprotector action of the glycerol and ethylene glycol in the freezing of the dog semen. Revista Brasileira Reproduction Animals. 26:174-76.

Cheema, R.S., Kaur, S., Mavi, G.K., Singh, A.K., Honparkhe, M. and Gandotra, V.K. (2021). *In vitro* evaluation of Labrador dog spermatozoa cryopreserved in Tris-citric acid fructose buffer supplemented with different combinations of extracellular and intracellular cryoprotectants. Animal Biotechnology. 32: 352-365.

Dhillon, N.S., Cheema, Ranjna S., Kaswan, S. and Singhal, S. (2020). Seminal attributes, antioxidant defense system and fertility rate reveal an association in Beetal Goat bucks. International Journal of Livestock Research. 10: 85-96.

Fastad, W. (1996). Semen cryopreservation in dog and foxes. Animal Reproduction Science. 12: 145-50.

Feng, H.L., Han, Y.B., Hershlag, A and Zheng, L.J. (2007). Impact of Ca^{2+} flux inhibitors on acrosome reaction of hamster spermatozoa. Journal of Andrology. 28: 561-64.

Forero-Gonzalez, R.A., Celeghini, E.C.C., Raphael, C.F. andrade, A.F.C., Bressan, F.F. and Arruda, R.P. (2012). Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia. 44: 154-59.

Futino, D.O., Mendes, M.C., Matos, W.N., Mondadori, R.G. and Lucci, C.M. (2008) Glycerol, methyl-formamide and dimethyl-formamide in canine semen cryopreservation. Reproduction in Domestic Animals. 45: 214-20.

Gonzalez-Fernandez, L., Morrell, J.M., Pena, F.J. and Macías-García, B. (2012). Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology. 78: 415-22.

Hernandez-Aviles, C., Gomez-Romero, M., Buitrago-Horta, R., Lozano-M_arquez, H., Jim_enez-Escobar, C. and Zambrano-Var_on, J. (2018). Evaluation of post-thaw sperm functionand integrity parameters under different freezing regimens in Colombian Paso Fino stallions. J. Equine Vet. Sci. 67: 7-14.

Hernandez-Aviles, C., Ruíz-Cristancho, A., Vergara-Galv M., Zambrano-Varon, J. and Jimenez-Escobar, C. (2020). The Effect of N-N-Dimethylformamide on the membrane characteristics of canine spermatozoa after cryopreservation and its Relationship with post thaw motility. Tropics in Companion Animal Medicine. Topics in Companion Animal Medicine. 38: 100372, DOI: 10.1016/j.tcam. 2019.100372.

Lopes, K.R.F., Costa, L.L.M., Lima, G.L., Souza, A.L.P. and Silva, A.R. (2009). Dimethylformamide is no better than glycerol for cryopreservation of canine semen. Theriogenology. 72: 650-54.

Lucio, C.F., Regazzi, F.M., Silva, L.C., Angrimani, D.S., Nichi, M. and Vannucchi, C.I. (2016). Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. Theriogenology. 85: 1568-75.

Marcocci, L., Maguire, J.J., Droy-Lafaix, M.T. and Packer, L. (1994). The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. Biochemical Biophysics Research Communication. 201: 748e755.

Martins-Bessa, A., Rocha, A. and Mayenco-Aguirre, A. (2006). Comparing ethylene glycol with glycerol for cryopreservation of canine semen in egg-yolk TRIS extenders. Theriogenology. 66: 2047-55.

Mota Filho, A.C., Teles, C.H.A., Jucá, R.P., Cardoso, J.F.S., Uchoa, D.C., Campello, C.C., Silva, A.R. and Silva, L.D.M. (2011). Dimethylformamide as a cryoprotectant for canine semen diluted and frozen in ACP-106C. Theriogenology. 76: 1367-72.

Olar, T.T., Bowen, R.A. and Pickett, B.W. (1989). Influence of extender, cryopreservative and seminal processing procedures on Post thaw motility of canine spermatozoa frozen in straws. Theriogenology. 31: 451-61.

Oldenhof, H., Blasse, A.K., Wolkers, W.F., Bollwein, H. and Sieme, H. (2012). Osmotic properties of stallion sperm subpopulations determined by simultaneous assessment of cell volume and viability. Theriogenology. 76: 386-91.

Oliveira, E.C.S., Juliani, G.C., Marques, Jr A.P. and Henry, M. (2006). *In vitro* evaluation of canine spermatozoa cryopreserved in different extenders. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 58: 1116-22.

Ortega-Ferrusola, C., Anel-Lopez, L., Ortiz-Rodriguez, J.M., Martin-Munoz, P. and Alvarez, M., *et al.* (2017). Stallion spermatozoa surviving freezing and thawing experience membrane depolarization and increased intracellular Na. Andrology. 5: 1174-82.

Peña, A. and Linde-Forsberg, C. (2000). Effects of Equex, one-or two-step dilution and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology. 54: 859-75.

Pena, A.I., Barrio, F., Quintela, L.A. and Herradon, P.G. (1998). Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. Theriogenology. 50: 163-74.

Pena, A.I., López-Lugilde, L. and Barrio, M. *et al.* (2003). Studies on the intracellular Ca^{2+} concentration of frozen-thawed do spermatozoa: Influence of Equex from different sources, two thawing diluents and post-thaw incubation in capacitating conditions. Reproduction in Domestic Animals. 38: 27-35.

Prieto-Martínez, N., Morato, R., Muino, R., Hidalgo, C.O. and Rodríguez Gil, J.E., *et al.* (2017b). Aquaglyceroporins 3 and 7 in bull spermatozoa: identification, localization and their relationship with sperm cryotolerance. Reproduction Fertility and Development. 29: 1249-59.

Prieto-Martínez, N., Vilagran, I., Morato, R., Rivera del Alamo, M.M. and Rodríguez-Gil, J.E. *et al.* (2017a). Relationship of aquaporins 3 (AQP3), 7 (AQP7) and 11 (AQP11) with boar resilience to whit stand freeze-thawing procedures. Journal of Andrology. 5: 1153–64.

Ramu, S. and Jeyendran, R.S. (2013). The Hypo-osmotic Swelling Test for Evaluation of Sperm Membrane Integrity. In: Spermatogenesis, Methods in Molecular Biology (Methods and Protocols Protocols), [Carrell, D., Aston, K., (eds)]. Vol 927, Totowa, NJ: Humana Press.

Robak, J. and Gryglewski, R.J. (1988). Flavonoids are scavengers of superoxides anions. Biochemical Pharmacology Journal. 37: 837e841.

Rota, A., Milani, C., Cabianca, G. and Martini, M. (2006). Comparison between glycerol and ethylene glycol for dog semen cryopreservation. Theriogenology. 65: 1848-58.

Ruch, R.J., Cheng, S.J. and Klaunig, J.E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 10: 1003-08.

Silva, A.R., Cardoso, R.C.S., Uchoa, D.C and Silva, L.D.M. (2003). Quality of canine semen submitted to single or fractionated glycerol addition during the freezing process. Theriogenology. 59: 821-29.

Silva, A.R., Cardoso, R.C.S. and Silva, L.D.M. (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.