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# Effect of Different Cryoprotectants in Cryopreservation of Dog Semen: A Review

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**ABSTRACT**

Several canine practitioners are facing limited success in canine artificial insemination using poorly assessed frozen dog semen due to unestablished set of semen evaluating parameters and semen diluents. The types of cryoprotectants plays import role in formulation of good semen diluents in canine semen cryopreservation which limits spermatozoa structure and viability in assisted reproductive technology. The cryopreservation process causes change in osmotic pressure and ice formation in spermatozoa which lead to cryoinjury and loss of viability and membrane integrity in post-thaw spermatozoa. This causes poor sperm quality and so poor fertility. The efficient cryoprotectants are those which penetrate deeper of spermatozoa and non-toxic. Glycerol and ethylene glycol are the most commonly used cryoprotectant for dog spermatozoa and other cryoprotective agents have been tested only sporadically. Hence, selection of better cryoprotectants based semen diluents and evaluation parameters of post-thaw semen is very much important for successful canine breeding to achieve dissemination of genetic material even after death, overcome quarantine restrictions, prevent venereal infections and semen exchange without moving stud dogs for breeding, etc. Therefore, establishing an efficient cryopreservation technique for dog sperm would be an essential resource for better dog breeding systems. The present article present the details reviews on cryopreservation of dog semen, different cryoprotectants and semen evaluation parameters for determining better production of quality dog semen.

**HIGHLIGHTS**

- Cryopreservation helps to preserve semen to the long period of time.
- Cryoprotectants includes all the required constutuents which protects the sperms from several sperm abnormalities.

**Keywords:** Cryopreservation, DNA integrity, Dog, Semen

The fertility data with the use of cryopreserved semen are still scarce, preventing the homogenous evaluation and there is standardization of the technique for dogs (Farstad, 2009). The semen cryopreservation in canine has gain importance in modern days for efficient assisted reproductive technology in canine breeding (Thomassen and Farstad, 2009; Milani *et al.,* 2010; Leroy *et al.,* 2011), thus reduces transportation cost. The success of insemination is closely related to the semen quality and evaluation of suitable parameters representing spermatozoal integrity and viability. The semen cryopreservation leads to the production of Reactive Oxygen Species (ROS) by the spermatozoa and causing oxidative stress of sperm cells, which is necessary for sperm capacitation and, therefore, fertilization (Lenzi *et al.,* 2002). However, oxidative stress can be premature during the cryopreservation process (Halliwell, 1991; Sharma *et al.,* 2012), carbohydrate

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moieties alternating proteins, sperm motility, plasma membrane functionality, and sperm DNA (Birben *et al.,* 2012). The damage induced by cryopreservation on spermatozoa is multimodal because in several studies there was a combination of cold shock, peroxidation (Slaweta *et al.,* 1988) and osmotic stress (Watson, 2000). Further, the temperature reduction is beyond the freezing point for semen, water forms ice crystals first in the extracellular compartment. This increases the solute concentration in the free uncrystallised water outside the cell, inducing hyperosmotic stress. During thawing, however, the ice crystals melt in the free water that enters the plasma membrane, thus sperm undergo hypo-osmotic stress (Sieme *et al.,* 2016). Hence, cryopreservation may promote loss of membrane integrity, decreased sperm motility and sperm DNA damage (Lucio *et al.,* 2017). Consequently, various studies were undertaken to examine various cryoprotectants to improve sperm quality by preventing the ROS attack during cryopreservation (Bilodeau *et al.,* 2001; Keskes-Ammar *et al.,* 2003; Domoslawska *et al.,* 2013; Manson and Rous 2014; Romagnoli and Lopate, 2014). The semen 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 7 and there are provided the electrolyte balance and osmolarity to the medium (Eilts, 2005). Therefore, selection of better cryoprotectants supplemented semen extender play important role in protecting spermatozoal integrity and viability during cryopreservation. The permeability of cryoprotectant is likely to be different among species since it depends on the structure and composition of the spermatozoal membrane mechanism (Rota *et al.,* 2006; Shalini *et al.,* 2018). The four types of cryoprotectants *viz* glycerol, ethylene glycol, DMSO (*dimethyl sulfoxide*), and propanediol has been chiefly used for dog semen. However, Said *et al.,* (2010) was used dimethyl formamide and different level of glycerol and ethylene glycol. Ethylene glycol had better result, because, it penetrate more quickly in the tissue, probably due to its lower molecular weight. Candy *et al.,* (1997) reported that the ethylene glycol is highly permeable and less toxic than glycerol. Ethylene glycol permeability was found to be higher than glycerol permeability in spermatozoa. Among them, glycerol and ethylene glycol has been used as a more suitable cryoprotectant (Bessa *et al.,* 2006). Ethylene glycol has highest permeability in human (Gilmore *et al.,* 1998) and mouse (Phelps *et al.,* 1999) spermatozoa. This paper also reviewed the suitable spermatozoal quality parameters to be assessed for quality check of semen membrane integrity and viability viz. post-thaw longevity, motility parameters and plasma membrane functional integrity through the Hypo-osmotic sperm swelling test and DNA integrity (Kusum *et al.,* 2012 ; Choudhary *et al.,* 2016; Mango *et al.,* 2019).

**Effect of different cryoprotectants on dog spermatozoa**

There are constituted of sperm extenders by extracellular cryoprotectant (Milk and egg yolk) and intracellular Cryoprotectant (Ethylene glycol, glycerol or dimethyl sulfoxide), or other agents are included buffer agent (tris), sugar (sucrose, lactose, glucose fructose), salts (Sodium citrate and citric acid) and antibiotics (Streptomycin, Amikacin Penicillin). The several extenders recommended for semen cryopreservation in the canine species, such as soya lecithin, decreased density lipoproteins (LDL), reduced glutathione, milk, liposomes and the powder coconut water (Kmenta *et al.*, 2011; *Ogata et al.,* 2015; Lucio *et al.,* 2017; Belala *et al.,* 2016; Das *et al.,* 2018).

Watson (1979) reported that addition of higher concentration of glycerol may also affect the fertilizing capacity of spermatozoa. Olar (1989) found that the post-thaw motility was higher when 3-4 percent glycerol was used in an egg-yolk Tris-based diluent. Although, Smith (1984) found that 9 percent glycerol in an egg-yolk-pipes extender was optimal., In cryopreservation of dog semen, concentration of glycerol have been added to varying levels of 2 and 5 (Olar *et al.,* 1989); 6 percent (Nair, 1996). This decreased sperm motility was reduced when glycerol was initially incorporated in the extender compared to its addition after cooling. The addition of 8 percent v/v glycerolated extender (final glycerol concentration 4 percent) compared to no glycerol addition, had shown to cause a decrease in the number of sperm penetrating homologous oocytes after pre-freeze cooling (Hay *et al.,* 1997). Pereira *et al.* (2002) also found temperature of glycerol addition had no effect on post-thaw sperm quality, however, they found that both post-thaw motility and acrosomal integrity were superior following use of 8 percent glycerolated extender compared to 2, 4 and 6 percent Gharajelar *et al.* (2016).

Silva *et al.* (2003) compared the effect of single and fractionated glycerol on cryopreservation of dog semen

and reported that the single method of glycerol addition found to be easier and most practical to use than the addition of fractionated glycerol. Alvarez and Story (1993) reported that the effects of ethylene glycol as cryoprotectants may be species specific. Ethylene glycol and glycerol are different cryoprotectants due to difference in their permeability coefficient. Storrey *et al.* (1998) reported that chemical structure of glycerol and ethylene glycol are quite similar, having the same ratio of carbon atoms and hydroxyl groups which is the indication of molecule lipophilia and hydrophilia. Ethylene glycol and glycerol is consistent with the measure permeability of these cryoprotectants as reported in boar (Gilmore *et al.,* 1998) and mouse (Phelps *et al.,* 1999) spermatozoa in those species in which estimate the permeability. The permeability of ethylene glycol is approximately 1.5-3 times that of glycerol. Pantano (2000) reported that Ethylene glycol minimizes the detrimental effect of the dehydration and rehydration during the freezing and thawing processes. Spermatozoa had a higher osmotic tolerance to quick addition and removal of ethylene glycol than to the glycerol. Massip (2001) reported that ethylene glycol has smaller molecular weight, a characteristic that may result in lower toxicity and higher permeability to cell. Ball (2001) reported the possibility of ethylene glycol could cause less osmotic lesions has already been suggested for stallion spermatozoa. Guthrie *et al.* (2002) observed the effects of using ethylene glycol in frozen thawed semen varied among species with bull semen. Ethylene glycol resulted in higher post thaw motility when compared with glycerol because of reduction of the osmotic lesions. Pereira *et al.* (2002) reported that ethylene glycol can readily cross cell membrane and hence penetrate and leave the cell faster than glycerol. Mantovani *et al.* (2002) when used to cryopreserve stallion semen observed that ethylene glycol had similar results to those of glycerol, and successfully replaced it when used in same or lower concentrations. Petrunkina *et al.* (2004) reported that dog sperm volume response to osmotic shock is regulated by the activity of potassium channel and in minimized by the presence of an intact cytoskeleton. Ethylene glycol might affect the functionality of potassium channel, their activation mechanism of fluxes of ions and organic osmolytes. Spermatozoa show different volumetric variations under similar hypo osmatic condition, reflecting individual difference in cytoskeleton characteristic and potassium channel activity. Rota *et al.* (2006) reported that progressive motility was significantly higher in ethylene glycol samples than post thaw dog spermatozoa. Ethylene glycol showed higher path velocity and increased straight line velocity. However, ethylene glycol semen samples also showed higher curvilinear velocity, which may indicate a capacitation like condition affecting sperm membranes and possibility of reducing post thaw longevity. Bessa *et al.* (2006) reported the combination of 4 percent ethylene glycol and 4 percent glycerol maintains sperm motility, viability and acrosomal integrity, respectively. Alvarez and Story (1993) observed the effect of ethylene glycol as a suitable cryoprotectant which was species specific. In our study, ethylene glycol showed higher motility because ethylene glycol penetrated more quickly than glycerol (Newton *et al.,* 1996). Ethylene glycol is highly permeable and less toxic than glycerol. Ethylene glycol and glycerol are different cryoprotectant due to variation in their permeability coefficient (Candy *et al.,* 1997).

Chemical structure of glycerol and ethylene glycol are quite similar, having the same ratio of carbon atoms and hydroxyl groups which is the indication of molecule lipophilia and hydrophilia (Storrey *et al.,* 1998). Ethylene glycol and glycerol are consistent with the measure permeability as reported in boar (Gilmore *et al.,* 1998) and mouse (Phelps *et al.,* 1999) spermatozoa. The permeability of ethylene glycol is approximately 1.5-3 times more to that of glycerol. Ethylene glycol minimizes the detrimental effect of the dehydration and rehydration during the freezing and thawing processes (Pantano *et al.,* 2000). Spermatozoa had a higher osmotic tolerance to quick addition and removal of ethylene glycol than to the glycerol. Ethylene glycol has smaller molecular weight, a characteristic that may result in lower toxicity and higher permeability to cell (Massip, 2001). The effects of using ethylene glycol in frozen thawed semen varied among species. Ethylene glycol can readily cross cell membrane and hence penetrate and leave the cell faster than glycerol (Pereira *et al.,* 2002). Dog sperm volume response to osmotic shock is regulated by the activity of potassium channel and is minimized by the presence of an intact cytoskeleton. Ethylene glycol might affect the functionality of potassium channel, their activation mechanism of fluxes of ions and organic osmolytes. Spermatozoa showed different volumetric variations under similar hypoosmatic condition, reflecting individual difference in cytoskeleton characteristic and potassium

channel activity (Petrinkina *et al.,* 2004). Addition of 5 percent ethylene glycol as a cryoprotectant resulted in 63 percent post-thaw motility in canine semen (Rota, 2006). Ethylene glycol showed increase path velocity and there is higher straight line velocity. However, ethylene glycol semen samples also showed higher curvilinear velocity, which may indicate a capacitation like condition affecting sperm membranes and possibility of reducing post-thaw longevity. The combination of 4 percent ethylene glycol and 4 percent glycerol maintain sperm motility, viability and acrosomal integrity (Bessa *et al.,* 2006; Mota, 2014).

The addition of 8 percent glycerolated extender at 5°C, compared to 4 percent glycerol, had shown to be more harmful to domestic dog sperm longevity, when held at 5°C. The addition of higher concentration of glycerol may also affect the fertilizing capacity of spermatozoa (Watson, 1979). Olar *et al.* (1989) found that the post-thaw motility was higher when 3-4 percent glycerol was used in an egg-yolk Tris-based diluent. Although, Smith (1984) found that 9 percent glycerol in an egg-yolk-pipes extender it was optimal. In cryopreservation of dog semen, concentration of glycerol has been added to varying levels of 2, 5 and 6 percent (Nair, 1996). The decreased sperm motility was reduced when glycerol was initially incorporated in the extender compared to its addition after cooling. The addition of 8 percent v/v glycerolated extender (final glycerol concentration 4 percent) compared to no glycerol addition, had shown to cause a decrease in the number of sperm penetrating homologous oocytes after pre-freeze cooling (Hay *et al.,* 1997). Pereira (2002) also found that the temperature of glycerol addition had no effect on post-thaw sperm quality and both the post-thaw motility and the acrosomal integrity were superior following the use of 8 percent glycerolated extender compared to 2, 4 and 6 percent. A single method of glycerol addition found to be easier and most practical to use than the addition of fractionated glycerol (Silva *et al.,* 2003; Shalini and Antoine, 2018).

The value of dog spermatozoa livability as observed in four different group of cryoprotectant in post-thaw spermatozoa was in the range of 55.23 ± 2.21 to 57.00 ± 1.60 percent (Kusum *et al.,* 2012). Pefia *et al.,* (1999); Cordoso *et al.* (2003) and Pena (2003), reported almost similar report. The values were lower than that recorded by Pefia *et al.* (1999) and Kurien (2012) who used 6 to 8 percent glycerol levels and relatively higher than reported by Verstegen *et al.* (2005). These variations might be due to the use of different cryoprotectants and the method of freezing.

## Evaluation of Important Parameters of Frozen Semen

### Post-thaw motility

The value of post-thaw motility in canine semen with 5 percent ethylene glycol as cryoprotectants was recorded as 36.83 ± 1.26 and with 4 percent and 8 percent glycerol and was 32.75 ± 1.88 and 28.50 ± 0.81, respectively (Kusum *et al.,* 2012). While 4 percent glycerol and 4 percent ethylene glycol showed 29.25 percent similar findings were reported by Kurien (2000). Morton and Bruce (1989) obtained 41 percent post-thaw motility in frozen thawed dog semen, while Dobrinski *et al.* (1993) recorded 32.2 ± 1.5, 33.7 ± 1.6, 30.9 ± 1.6 and 30.3 ± 1.4 percent post-thaw motility in Triladyl, Pipes, IMV Universal and Tris extenders, respectively. Nothling and Volkmann (1993) recorded post-thaw 35 percent motility in Triladyl extender while, Silva and Verstegen (1995) observed post-thaw motility of 65, 65 and 50 percent in Laiciphos, Tris and Baciphos extenders respectively. Strom *et al.,* (1997) recorded 69.7 ± 8.2 and 73.7 ± 3.2 percent post-thaw motility at 37°C in Anderson and Clone methods of semen cryopreservation whereas (Pefia *et al.,* 1999) observed 33.6 ± 16.2 and 60.0 ± 8.2 per cent post-thaw motility in 6 and 8 percent glycerol levels, respectively.

Whereas, Kurien (2000) obtained the average post-thaw live sperm percentage of 39.56 ± 1.0, 37.43 ± 1.08 and 31.01 ± 0.68 in Tris, Trialdyl and Laiaphas - 488 extenders respectively. Pena *et al.* (2003) obtained 54.6 ± 10.7 percent of live spermatozoa in frozen thawed dog semen. Cardoso *et al.* (2003) found dog spermatozoa motility after thawing 49.2 ± 26.0, 44.2 ± 18.3 and 35.8 ± 26.8 percent for three groups with 4, 6 and 8 percent glycerol respectively. Nizanskin (2006) reported 49.5 ± 8.1 percent progressive motility in frozen thawed dog spermatozoa added with 4 percent glycerol. Rota *et al.* (2006) observed that progressive motility was significantly higher with ethylene glycol than glycerol (51.5 ± 4.7 percent and 31 ± 5.6 percent). According to earlier report of Strom *et al.,* (1997) who recorded 69.7 and 73.7 percent post-thaw motility at 37°C in Anderson and clone methods of semen cryopreservation. Whereas, Pefia *et al.* (1999) observed

60.0 percent post thaw motility in 6 and 8 percent glycerol level, respectively.

**Abnormal spermatozoa**

The value of abnormal spermatozoa in dog semen estimated in different groups of cryoprotectants ranged from 25.42 ± 0.80 to 28.83 ± 1.92 percent. Nair (1996) observed post-thaw abnormality of 35.84 ± 1.64 and 42.74 ± 1.15 percent in 9 and 6 per cent glycerol levels, respectively. Kurien (2012) recorded an average post-thaw abnormal sperm percentage of 18.73 ± 0.17, 18.98 ± 0.15 and 19.60 ± 0.16 in Tris, Triladyl and Laiciphos-488 extenders, respectively, whereas, Silva *et al.,* (2003) obtained 24.9 ± 20.0 percentage of abnormal sperm in frozen thawed semen.

The normal and abnormal spermatozoa morphology completely depends up on the phase of spermatogenesis, where spermatid is converted to spermatozoa. It is regulated by sertoli cells which are the only non-germinal cell in the seminiferous tubules. Sertoli cells regulate the acrosomal phase of spermatogenesis where there is condensation and elongation of nucleous and acrosome and some minor changes in tail portion occurs. So any alteration in sertoli cell structure or function may affect morphology of sperm Medeiros *et al.* (2002).

**Hypo-osmotic swelling test (HOST)**

HOST response measures the membrane integrity and membrane stabilizing action of spermatozoa. The mean value of HOST response observed in all cryoprotectants was in the range of 45.33 to 51.50. It is a known fact that sperm motility and viability is dependent upon membrane transport. These facts find support from the result of present study in which higher motility and viability was observed. The plasma membrane is regarded as the primary site of freezing injury and the types of damage depends on different freezing conditions. Kurien (2000) observed an average hypo-osmotic swelling response as 49.88 ± 0.71, 48.05 ± 0.90 and 43.03 ± 0.57 percent in Tris, Triladyl and Laiciphos-488 extenders respectively in Mongrel dog semen. Fertilization will not occur, if the sperm membrane is physically intact, but is inactive biochemically. Therefore, HOS test could be regarded as more conclusive method to detect membrane integrity and fertilizability of spermatozoa (Thundathil, 2002). Ponglowhapan *et al.* (2004) obtained the mean percentage of 54.2 ± 6.7 damaged plasma membrane in frozen thawed dog semen. During the HOS test, spermatozoa with a biochemically active plasma membrane, when exposed to the hypo-osmotic solution, will increase in volume due to intracellular influx of water, which is the sign of membrane integrity and normal activity of spermatozoa (Dobrinski *et al.,* 1993; Rota *et al.,* 2006; Cheema, 2012).

**Acrosomal integrity**

Kurien (2000) recorded an average post-thaw intact acrosome percentage of 64.78 ± 0.33, 64.43 ± 0.26 and 62.86 ± 0.41 in Tris, Triladyl and Laiciphos-488 extenders, respectively in Mongrel dog semen. Yildiz*et al.,* (2000) obtained the mean percentage of 44.6 ± 3.2 and 47.9 ± 3.1 damaged acrosome in frozen thawed semen using fructose and glucose, respectively. Ponglowhapan *et al.* (2004) reported 34.7 ± 15.0 percentage of acrosomal loss in frozen semen. Umamageswari (2005) showed post-thaw acrosomal integrity 46 percent.

Ultra structurally, cold shock is manifested by loss of selective permeability and integrity of plasma membrane, release of intracellular enzymes and lipids from the spermatozoa and there after loss of motility and diminished metabolism. Therefore, a more complete assessment of function was obtained by evaluating spermatozoal motility, membrane integrity and acrosomal morphology in this study. Mammalian sperm are very sensitive to cooling from body temperature to freezing. Damage to sperm due to freezing known as cold shock was observed as an irreversible loss of motility (Medeiros *et al.,* 2002).

**DNA integrity**

DNA integrity of spermatozoa (Double strand breaks) was estimated by single cell alkaline micro gel electrophoresis method (comet assay) as described by Singh *et al.* (2003). Cryopreservation is widely used in many assisted conception unit to preserve male fertility (Aitken, 1995; Serafini *et al.,* 2017; Ferreira *et al.,* 2018). Maintenance of sperm DNA integrity is crucial to the health of future generations (Evenson, 1999; Hamilton *et al.,* 2019). In contrast to the relatively dormant female gametes, spermatozoa are produced by the testes as male germ cells

and there are undergo lifelong cell replication, meiosis and spermiogenesis. Male gametes as corresponds to female gametes have a greater possibility of damage to nuclear DNA of Y chromosomes. This is due to lack of recombination repair process, as there is only one Y chromosome available during meiosis (Ward Zalelsky, 1996; Aitken, 2011; Stuart *et al.,* 2019). Therefore, analysis of spermatozoa for DNA integrity is more important before freezing and thawed DNA damage does occur in both developing and mature sperm. So, high proportion of sperm with DNA damage might be a cause of infertility in animals (Shen *et al.,* 1999). Although, ideally the healthiest sperm (with intact DNA) will fertilize the ovum, sperm with damaged DNA may accomplish fertilization, this might be the reason of poor pregnancy outcome (Wdowiak *et al.,* 2015).

Sperm membrane disruption caused during cryopreservation may be a consequence of liquid phase transition change and lipid peroxidation (LPO) and further lead to DNA damage due to oxidative stress (Alverage and Storey, 1993). Sperm head mainly comprise the sperm DNA, so change in sperm morphology may be related to abnormal DNA content (Martinez *et al.,* 2005; Gloria *et al.,* 2018). Macias (2006) also reported low DNA integrity due to freezing-thawing process in dog spermatozoa. In recent days, there is a burgeoning interest in the implication of spermatozoal DNA damage during semen preservation. As DNA fragmentation of animal spermatozoa was negatively correlated with fertilization, as it did not preclude pronucleous formation after ovum penetration (Macias *et al.,* 2006).

Therefore, assessment of spermatozoal DNA integrity is a necessary requirement for selection of better cryoprotectants and a thorough knowledge of spermatozoa achieving capacity in fertilization and embryo development (Evenson *et al.,* 1999; Cho and Agarwal, 2018). The ethylene glycol showed higher percentage of intact DNA in frozen semen. High DNA intactness in ethylene glycol as compared to other cryoprotectants might have caused less LPO so reduced the effect of oxidative stress on DNA damage (Baumer *et al.,* 2003; shahoo *et al.,* 2015). This might be due to more stability of spermatozoal membrane and less toxicity of ethylene glycol to spermatozoa (Candy *et al.,* 1997; Samoylova, 2010).

## CONCLUSION

This reviews article on different cryoprotectants for canine semen preservation revealed ethylene glycol is better as compared to other cryoprotectants. Further, post thaw semen evaluation should include batch wise testing for spermatozoal DNA integrity, membrane integrity, progressive motility, and viability to assess efficiency of semen diluents. However, more studies are required to establish universal semen extender for different breeds of dogs.

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