Cryopreservation of dog semen as an alternative method to improved fertility in bitches: A review article

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SUMMARY

Cryopreservation of dog semen facilitates the exchange of genes between populations and makes it possible to preserve genes from valuable males for an extended time. Although AI in dogs can help avert sexually transmitted diseases and reducing the chances of inbreeding, the technique is not routinely practised in most developing countries including Tanzania. Among the reasons are problems associated with cryopreservation. Cryopreservation of semen has a number of problems limiting its success and thus, affecting fertility in dogs as a whole. The common assisted reproductive technologies like AI in dogs are constrained by challenges in semen cryopreservation which partly is caused by freezing temperature, cryoprotectants and lifespan of spermatozoa. The purpose of this review is to study the possible ways which can improve cryopreservation of semen in dogs. Improving semen cryopreservation is not a one process; it involves a combination of factors with the ultimate goal of improving the outcome of semen cryopreservation in terms of conception rates in bitches, and thus, fertility in general. Cryopreservation parameters to improve include: type of ejaculate, insemination technique, storage, post thawing procedures, extenders, cryoprotectants, collection method and antioxidants. Several approaches have been recommended, including cryopreservation protocols, for instance freezing rates should be slow enough to allow the cells to minimize chemical potential and osmolality gradients across the plasma membrane, but fast enough to dehydrate the cell without exposing it to lethal salt concentrations. Moreover, the first ejaculates and the ejaculates from old dogs are to be discouraged if we need to improve fertility output under cryopreservation method. Thawing temperature of 70°C and above within a few seconds, have shown promising results. Possibly, this temperature is helpful to reduce the toxic nature of cryoprotectants. However, this review suggests the use of intrauterine method, and possibly innovation of new modification and training expertise would help to improve fertility in bitches. Nevertheless, more work is needed to accurately predict fertility of cryopreserved canine semen.

Keywords: cryopreservation, semen, dogs

INTRODUCTION

The domestic dog (*Canis* familiaris) is one of the first domesticated species (Morey, 1994), and results from at least 14,000 years of domestication that started with the grey wolf (*Canis lupus*). Dogs are used for several purposes, including as hunters, guardians, shepherds, guide dogs for the blind, service and hearing dogs, rescue dogs, and dogs for police and military purposes, e.g. in drug and explosives detection. The largest numbers of dogs are, however, kept as companion animals. The expanding use of dogs for different purposes and the selection by humans have resulted in a large diversity of this animal, with varying appearance and behaviour. The domestic dog can be useful models for research in reproduction of endangered wild *canids*. There is also a great demand to exchange genetic material in domesticated dogs, since many breeds of dogs are small in number and transportation of semen and successful artificial insemination (AI) would help to widen the gene pool of minority breeds. Successful preservation of canine semen is therefore important for improving the results of the main assisted reproductive technologies (ARTs), such as in vitro fertilization and (most commonly) AI in dogs (Luvoni, 2006).

Cryopreservation is a process where cells or whole tissue are preserved by cooling to low sub-zero temperature such as -196ºC. The process usually involves adding cryoprotectants, freezing, storage and thawing. In the modern breeding world use of cryopreserved semen is in demand but there have been a number of challenges with efficiency. The process of cryopreservation is reported to decrease the ability of spermatozoa to fertilize (Bilodeau*et al*., 2000; Cerolini*et al*., 2001), which reduces the balance of antioxidants enzymes of canine spermatozoa and its general biological properties (Strzezek*et al*., 2012).

In an attempt to achieve better conception rates, studies in various species advocate intrauterine deposition of semen (Fontborne and Badinand, 1993: Linde-Forsberg,1999) though in dog anatomical factors limit its use. But the good thing is sperm cell distribution occurred evenly along both horns, independent of the site of semen deposition (Fukushima *et al*., 2009). Moreover, identifying an optimum time of fertilization, remains as a

stumbling block to achieving high conception rates in dogs. Although in other canines such as brown bear, problems of sperm agglutination has been addressed in the ejaculates, the problem which impairs freezability. Gomes-Alves *et al*. (2014) described the usefulness of using Test-tris-fructose-egg-yolk –glycerol as a suitable extender for use in brown bears to reduce agglutination in fresh semen samples.

Ovulation in bitches occurs 1 or 2 days after the preovulatory peak of the luteinizing hormone (LH), still in the beginning of the estrus stage of the estrous cycle, and the ovarian follicles start luteinization before ovulation (Pereira *et al*., 2012). Oocytes are ovulated still immature, in the beginning of the first meiotic division (GV). Therefore, the oocytes require 2 to 5 days to reach maturation after ovulation (Reynaud *et al*., 2012). The environment in the uterine tubes plays an important role in canine oocyte maturation. The uterine tubes in canids, unlike other species, is responsible for maintaining, during an extended period of time, the survival of still immature oocytes until they complete their development, are fertilized and reach the blastocyst stage (Luvoni*et al*., 2005). This necessitates timing of insemination to tally with ovulation.

Additionally, dog's sperms respond differently to cryopreservation as compared to other animal species. Normally, it is associated with high individual differences and poor conception rate post thawing (England, 1993; Yu*et al*., 2002).Other obstacles include poor post thaw semen quality and short lifespan post thawing (Oettle, 1986; England, 1993). Furthermore, it has recently been found that sperm oxidative damage intensifies post thawing (Lucio *et al*., 2016). Therefore, the use of cryopreserved semen is impeded by these setbacks highlights a need for a research on ways to improve cryopreservation. Successful artificial insemination in bitches requires understanding of semen collection method, types of extenders, cryoprotectants, storage, and insemination techniques. This review aimed at discussing the main factors related to cryopreservation of dog's semen as ways of improving artificial insemination in bitches.

Principles of cryopreservation

Spermatozoa cryopreservation is a method for preserving genetic material and maintaining genetic diversity in several species, including dogs. This extends the storage time and facilitates semen transportation over distance. Since the introduction of glycerol as a cryoprotectant agent by Polge*et al*. (1949) and the discovery of dimethyl sulfoxide (DMSO) by Lovelock and Bishop, (1959) many cryopreservation techniques developed, mostly through empirically derived methods. In the principles of cryopreservation, the cooling rate is very important as when it is slow then most of the sperm cells will be functional after thawing.However, cryopreservation has negative effects on sperm viability, which could be related to injury of the plasma membrane, associated to changes in lipid phase transition, mechanical stress, efflux of water and high salt solutions and, possibly, by interfering with intracellular ice crystals formation, and thus decreasing the fertilizing ability of spermatozoa (Bilodeau*et al*., 2000; Cerolini*et al*., 2001). The above could result from enhanced peroxidation of sperm cell membrane lipids which is largely determined by excessive generation of reactive oxygen species (ROS) in semen (Alvarez and Storey, 1992; Wang *et al*., 1997). The membrane of a sperm cell contains large amounts of polyunsaturated fatty acids (PUFAs), which make them highly susceptible to lipid peroxidation (LPO) in the presence of ROS, resulting in impaired sperm function (Aitken *et al*., 1996). The main factors which influence the survival of cryopreserved spermatozoa are: gamete's osmotic properties, cooling and warming rates and formation of intracellular ice crystals (Mazur *et al*., 1972; Stănescu and Bîrţoiu, 2012). Since canine spermatozoa act as a perfect osmometer(Songsasen*et al*., 2002), the responsiveness of spermatozoa to osmotic challenge and the ability to regulate cell volume is critical to cryopreservability(Petrunkina*et al*., 2005). Different canine semen cryopreservation techniques include as a first step the centrifugation of the sperm. The goals of centrifugation are to remove the excess of prostatic fluid which has a negative effect on motility and vitality of frozen-thawed spermatozoa (England and Allen, 1992; Rota *et al*., 1995; Sirivaidyapong*et al*., 2001) and to standardize the extension of the semen to a controlled final sperm and glycerol concentration. The most used regimen in the cryopreservation protocols is centrifugation at 600 – 700 g for 5 – 10 minutes (Rijsselaere*et al*., 2002; Schäfer-Somi *et al*., 2006). The main disadvantage of this method is the elimination of natural antioxidants from seminal plasma. Thus, freezing rates should be slow enough to allow the cells to minimize chemical potential and osmolality gradients across the plasma membrane, but fast enough to dehydrate the cell without exposing it to lethal salt concentrations.Therefore, for better results in cryopreservation, freezing and thawing procedures have to be optimally maintained to

ensure maintenance of pH, osmolality, as well as energy provision and cryoinjury prevention.

Semen collection and evaluation

Prior to semen collection a thorough and complete historical review of the dog's previous health and breeding experiences should be obtained.Inaddition, information regarding the medication or supplements administered over the previous 6 months (at the minimum) and on the genetic or familiar background are important (Johnson, 2006). There should also be collection of information about the status of vaccinations, dewormings and heartworm protection history, as well as on the duration of the ownership and accuracy of the history of the animal even before the establishment of the ownership (Freshman, 2002; Ettinger*et al*., 2010). Many factors influence semen quality, including the animal's age, the size of the testicles, the degree of sexual arousal, the frequency of ejaculation, the collection procedure and the amount of seminal fluid collected (Kutzler, 2005; Johnson, 2006).Different techniques are used to collect semen in dog and these include the use of electroejaculator, artificial vagina andpenis massage (Ortega-Pacheco *et al*., 2006).

For preservation reasons, either semen freezing or chilling, or for fresh semen AI, it may be of benefit to perform two collections, with an interval of 45 to 75 minutes among them. Although the number of spermatozoa is rather low in the second collection comparing with the first, the amount of both collections is in average, 70% more than if the collection is performed only once (England, 1999). The total volume of a dog's ejaculate may vary from 1.0 mL up to 30.0 mL (England *et al*., 2010; Ettinger*et al*., 2010). The canine ejaculate is composed of 3 distinct fractions (Johnston *et al*., 2001; Kustritz, 2007). The first or pre-spermatic fraction is composed of clear seminal plasma, devoided of sperm cells, originates from the prostatic gland and its main function is to flush the urethra (England *et a*l., 2006; Nelson *et al*., 2009; Ettinger*et al*., 2010). The volume of the pre-spermatic fraction usually varies between 0.5 and 2.0 mL (Freshman, 2001). The second fraction, also denominated sperm-rich fraction, has a cloudy and opalescent appearance with an opaque consistency, varying in volume between 0.5 and 5.0 mL, depending on the testicular size and on the individual variation; moreover, in its composition there should be no cellular components besides sperm cells (England, 1999; Nelson et al., 2009). The dog can take up to 2 minutes to achieve the emission of the sperm-rich fraction (Kutzler, 2005).

Type of ejaculates

The type of ejaculate collected can be influential in terms of improving post thawing viability in dog's semen. For instance, the first ejaculates have been found to have lower post-thaw motility and velocity as compared to subsequent ejaculates that were collected two days apart (Dobrinski *et al.*, 1993). Interestingly, motility pre-process of all the ejaculates were almost similar but after thawing motility is different. Showing that, for better results the first ejaculates has to be discarded especially the ejaculate collected after long period of reproductive or sexual rest, and the subsequent ejaculates are the ones to be used. Moreover, it is also found out that the best ejaculates come from dogs below eight years of age (Thomassen *et al*., 2006).These studies suggest that time and what semen to collect, as well as age of the animal from which we collect our ejaculates can improve cryopreserved semen. Hence, the first ejaculates and the ejaculates from old dogs are to be discouraged if we need to improve fertility output under cryopreservation method.

Individual dog difference

Individual dog difference in terms of post-thaw sperm quality has been advocated in many studies (Alhaider & Watson, 2009; Dobrinski *et al*., 1993). InDobrinski *et al*. (1993) the difference were obtained in terms of interaction with the extender, as well as the number of ejaculates. Yu *et al*. (2002) captured differences in terms of cooling and warming rates. Individual difference had also been captured in terms of age. Whereas using frozen semen of dogs older than eight years, the whelping rate was found to be lower (than those below eight) (Thomassen *et al*., 2006). Individual differences were also captured by Zakosek *et al*. (2012). The scenario was when dogs showed different results when the same extender was used. But when they tried to use human extenders, the results post-thaw was better. The concept shows that we can manipulate extenders based on the breed of dogs we have at the time. Moreover, increasing sperm number has been advocated as one of the means to compensate differences in the fertility among males (Saacke*et al*., 2000). Hence, these studies reveal that there are individual differences in terms of post-thaw semen quality. Therefore, for better results in cryopreservation choosing as a donor needs a lot of study and examination of the particular stud dog which provides good results in terms of post-thaw viability of spermatozoa. Possibly, there can be development of individual freezing extenders for

those dogs that show problems in terms of semen freezing, and yet are considered as superior breeds.

Insemination technique

Insemination techniques are an influencing factor in improving results after cryopreservation of semen in dogs. The technique can be in terms of manipulating the route of insemination, number of spermatozoa, and number of insemination. Intrauterine route of insemination is found to result in a 85% pregnancy rate when fewer number of insemination dose (30-35 $\times 10^6$) were used using the same dose and the route being non-surgical intrauterine (Linde-Forsberg et al., 1999; Kong *etal*., 2003). Contrary to the above findings, Nothling, Gertsenberg, and Volkmann (1995) found higher whelping rate in intravaginal as compared to intrauterine. But in Nothling's study, bitches were inseminated on average more than five times, therefore, this could not reflect the real case under field situation. Hence, from these studies it shows that in order to improve fertility under the cryopreservation, the technique used for insemination is vital (Table 1 and 2). This research suggests the use of intrauterine method, and possibly innovation of new modification and training expertise would help to improve fertility.

Table 10: Comparisons of vaginal AIs and intrauterine AIs

Total number of spermatozoa (x 10^6)	Vaginal AIs			NIU AIs		
	Number of AIs	Whelping rate (%)	Litter size	Number of AIs	Whelping rate (%)	Litter size
≤100	7	28.6	3.5 ± 2.1	8	100.0	3.0 ± 2.0
101 - 200	24	45.8	2.8 ± 2.1	31	90.3	4.8 ± 3.0
201 - 300	24	50.0	4.5 ± 2.0	30	77.4	5.3 ± 2.5
301 - 400	19	63.2	4.5 ± 3.2	21	76.2	6.4 ± 3.9

Table 11. Litter size and whelping rate under intrauterine insemination versus intravaginal insemination

Items	Intravaginal	Intrauterine
No. of bitches inseminated[a]	15	26
No. of bitches whelping (%)*	6 (40.0)	21 (86.6)
No. of pups born	17	89
No. of pups per litter (Mean±S.E.)	2.8±1.2	4.2±1.6

Semen storage

Semen to be used for AI can be prepared in different ways. It can either be used fresh, or extended fresh or chilled, or frozen-thawed (FT). Fresh semen for AI is usually used when both the male and the female dog are present at the site of semen collection and the AI can be performed straight away. The freshly ejaculated semen can be extended in order to maintain viability or even improve motility, e.g. in male dogs with prostate problems. The fresh semen has the advantage of not being processed and therefore of not being damaged by chilling or freezing procedures. A great disadvantage is of course that the semen has to be used immediately after semen collection. For any further transport or short storage, the semen has to be chilled, while for long-term storage, freezing is the only option. Today AI in dogs is routinely used, with acceptable pregnancy results both with fresh, chilled and with FT semen, although chilled dog semen have given better results for AI, compared withfrozen and thawed semen.

Cryopreservation of spermatozoa is well studied in various species including the dog, and the first pregnancies resulting from FT spermatozoa were

achieved with semen diluted in lactose and Tris (hydroxymethyl) aminomethane (Tris)-based extenders. However, cooling of dog spermatozoa appears to damage the spermatozoa less than freezing and thawing do, and compared with FT spermatozoa, semen quality (expressed as motility, sperm morphology, acrosome status, hypo-osmotic swelling, and longevity at 39ºC) has been reported to be superior for up to 4.9 days of cool storage despite some deterioration during cold storage. As with motility, acrosome reaction in dogs is more affected by freezing and thawing than by cold storage (Oettlé, 1986). Changing of the means of storage of dog semen has been recommendedelsewhere. For instance, use of ultra-freezers (UF) at -152°C rather than liquid nitrogen(Álamo *et al*., 2005). Moreover, the use of the dry shipper for short time storage of dog's semen has also been advocated (Batista *et al*., 2012). However, studies investigating conception rates of sperm stored under these methods have not been done. Possibly, they could result in the improvement it is not yet known. Hence, there can be a possibility that improving the method of storage can help to improve the semen quality of the cryopreserved semen. Overall, more research is needed to establish the best storage improvement for cryopreserved semen.

Post thawing procedures

Various manipulations regarding post thawing procedures are advocated to improve post-thaw motility. They included use of certain extenders such as Equex STM paste though timing in terms of thawing is seen to be more influential where survival and motility was favoured first hour post-thawing (Alhaider and Watson, 2009). Moreover, individual dog interaction with Equexhas been reported, particularly in terms of concentration of intracellular free calcium and membrane fluidity (Alhaider and Wats, 2009). In some studies, thawing temperature has been captured to influence post-thaw spermatozoa quality. Nothling and Shuttleworth (2005a)established that thawing at 70ºC in water was the best approach other than 37ºC, and this was better in terms of having few abnormal acrosomes. Moreover, it is further supported that when 0.5mls straw were put at 70 for 8 seconds, provided better results in terms of post-thaw survival as compared to 30ºC for 15 to 60 sec(Peña and Linde-Forsberg, 2000; Rota *et al*., 1998). Further supported by Lyashenko (2015) that temperature range of 65 ºC to 70 ºC with time range of 6-7 seconds provided better results. Though there seem to be an association between freezing rate and thawing rate where the freezing rate was fast then the thawing rate has to be faster as well. Similarly, a slower freezing rate has to be followed by a slower thawing rate (Farstad, 1996). The scenario can imply that one has to be aware of the freezing procedure, or follow the manufactures manual on the freezing protocol used. Therefore, few studies involving thawing temperature of 70C and above within a few seconds, have shown promising results (Figure 1). Possibly, this temperature is helpful to reduce the toxic nature of cryoprotectants. Hence, this research recommends further studies and modifications to be done to qualify the use of higher temperatures.

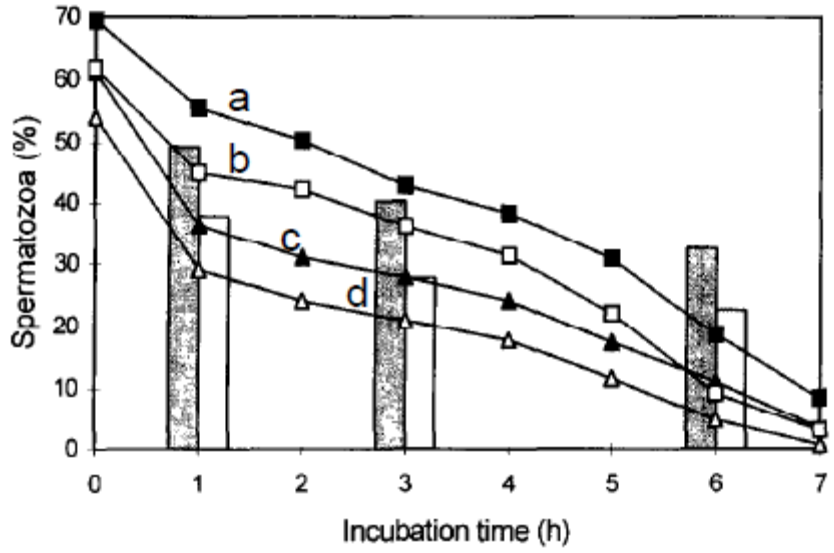

Figure 3. Percentages of sperm motility and live spermatozoa at 37ºC and 78ºC

Extenders

For a long time, the standard semen extenders used in dogs were made with chicken egg yolk. However, egg yolk presents numerous disadvantages. It is associated with a health risk as egg yolk is an excellent microbial culture medium and can promote the onset of uterine infection following insemination of the bitch. Egg yolk also poses problems for analyses using a CERROS type image analyser or during biochemical assays (Wall and Foote, 1999). Lastly, the components of egg yolk can vary as a function of the diet of the chicken, and are not necessarily all beneficial for spermatozoa; the granules are actually deleterious for the respiration and motility of spermatozoa (ksmpshmidt *et al*., 1953; Pace and Graham, 1974).

Currently Tris being extenders is commonly used and found effectively in dog semen (Hewitt *et al*., 2001: Ponglowhapan and Chatdarong, 2008: Martins *et al*., 2012).However, some researchers (Bencharif *et al*., 2010) suggest the 6% low-density lipoproteins (LDL) extender to be the best. While other studies show that equex and 6% LDL extenders are most successful (Bencharif*et al*., 2010). Studies on extenders are still controversial as most extenders present some interesting individual features which are not in the others. Such features are: post-thaw fertility, motility, acrosomal integrity, DNA integrity, etc. Likewise, efficiency of most extenders is still dependent on the concentration and dilutions levels. Hence, achieving the balance of all these parameters allow for determining the best extender. Similarly, (Bencharif et al., 2012) found that each extender to have better results in a specific aspect and its own advantage. For instance, the mixture of 6% LDL with glutamate medium was found to improve the aspect of spermatozoa motility.the aspect of where the semen is collected is also captured. In canine epididymal sperm ACP-106c extender is found useful especially immediately after sperm recovery (Filho*et al*., 2014).

Furthermore, it has found that when extenders and enhancers are added to cold stored semen help to improve spermatozoa viability and progressive motility (Kmenta *et al*., 2011). Hence, the argue is to find more in terms of fertility of these spermatozoa likewise as to whether enhancers can be helpful in other extenders other than lecithin-based extenders. Nevertheless, this is supported by studies comparing commercial versus laboratory prepared extenders. In addition, laboratory preparedextenders are found to have better preservation characteristics as compared to the commercial ones (Iguer-ouada and Verstegen, 2001)(Figure 2). Therefore, this shows that the modification in terms of extenders can provide a way to improve cryopreservation. Possibly use of own prepared extenders other than commercial ones will be useful as they will consider the need of the breeder. They might take note of individual dog difference and the environment in question as the commercial ones were made under different settings.

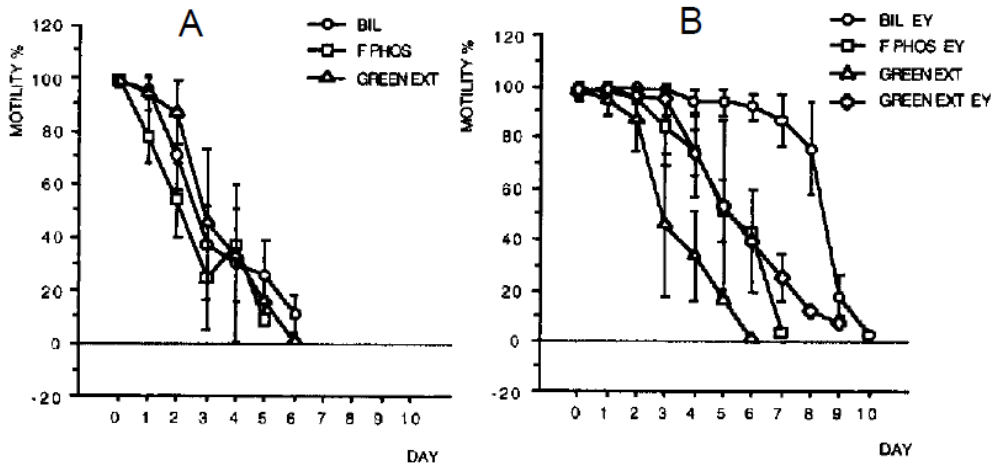

Figure 4. Percentages of spermatozoa motility over time

Figure 2: (A) shows changes in percentages of spermatozoa motility over time when semen was diluted in commercial extenders (Biladyl, fresh-phos, and green-extender). B shows improved motility when modifications were done on the three commercial extenders especially under biladyl. The

modification in this case was addition of 20% egg yolk (taken with modification from Iguer-ouada and Verstegen (2001).

Cryoprotectants

Semen cryoprotectants improve cell survival after the freezing process. Cryoprotectants are divided in to two groups. First are intracellular cryoprotective agents like glycerol and dimethylsulfoxide. Second are extracellular ones such as proteins and sugars. Glycerol is the cryoprotectant often used to freeze semen of different species (Silva *et al*., 2003). As a permeable cryoprotectant, glycerol prevents the formation of ice crystals inside the cells. However, glycerol has been reported to has toxic effects on spermatozoa, such as physicochemical alterations that can lead to rupture of the plasma membrane or removal of important membrane proteins, and cause acrosomal damage, which will be reflected in reduced fertility (Curry, 2000; Holt, 2000).

The ideal cryoprotectant should have a low molecular weight with high water solubility and low toxicity (Medeiros *et al*., 2002). However, there is a great diversity in cryobiological responses of different cell types or given cells among different mammalian species. Cryosurvival requires that cell freezing and thawing be done within certain biophysical and biological limits defined by the following cryobiology principles: cells should be frozen in such a way that little or none of their intracellular water freezes. They should be warmed in such a way that unfrozen intracellular water remains unfrozen during warming, or that small ice crystals formed during cooling remain small during warming. Even the aforementioned conditions are important; most cells will not survive unless substantial concentrations of cryoprotectants are used. It is well known that milk and egg yolk are nonpermeable cryoprotectants. They prevent formation of ice crystals outside the cells, provide enough energy for the spermatozoa and also play the role of phosphate buffer for them (England *et al*., 2000).

Egg yolk has been an important ingredient in extenders used for preservation of dog semen. The low density proteins in egg yolk provide protection of thesperm membranes against cold-shock and cryo-induced cell damages (Farstad 2009). Egg yolk is, however, a biological product with associated disadvantages of a high risk of contamination with bacteria (Bousseau*et al*., 1998). Furthermore, the fears of interspecies transmission of avian influenza, hygienic risks associated with the use of egg yolk and the difficult to standardize the quality of the extenders (Bousseau*et al*., 1998), there is an interest in using animal-free protein extenders for preserving semen from different species. Recently, it has been demonstrated that soya bean lecithin might be an interesting alternative to egg yolk for liquid cold preservation as well as cryopreservation of dog semen (Beccaglia*et al*., 2009a, Beccaglia*et al*., 2009 b; Kmenta*et al*., 2011; Kasimanickam*et al*., 2012). However, Soya lecithin contains different phospholipids concentrations which have negative effects on post-thaw quality of canine sperms (Axner and Lagerson, 2016).

Usually with the use of cryoprotectants agents (CPAs) there are certain levels or concentration that are advised to be used around 2-8%, but beyond which, the cryoprotectants become toxic. In dogs using 0.5ml straw, it has been found that the use of CPA-free freezing is difficult to be achieved. But there is the possibility of CPA-free methods of cryopreservation to be used in large volume of sperm in dogs. Therefore, it is suggested to use methods that involve low concentration of glycerol without cooling stages to 6^0C. Likewise, a brief exposure to the liquid nitrogen vapour can be used as it provides better post freezing and thawing sperm characteristics (Kim *et al*., 2012). Concentration of glycerol of about 4 to 11% has been widely used (Peña and Linde-Forsberg, 2000). However, high glycerol concentrations of from 4 to 8% have been studied (Cardoso*et al*., 2003) providing better results like inclusion of 20% coconut and egg yolk 20% extenders. Since there is limited research to whether CPA-free can be a solution in dogs then, the use of low glycerol concentration can be adopted. Hence, this review recommends the use of concentration of about 4 to 8% for better results. This might be helpful to control toxicity levels of cryoprotectants.

Method of collection

The most commonly used method of semen collection in dogs is manual. Sperm can also be collected from the epididymis after surgical sterilization, post-mortem or vaginal lavage after natural mating. Dogs ejaculate in three fractions. The first fraction is termed as pre-sperm fraction which originates from the prostate gland. Normally it is clear or slightly cloudy and volume ranging from 0.5 to 20 ml or more (Freshman, 2001). The second fraction is called as sperm-rich fraction which is normally opaque, milky-white in colour and ranging from 0.5 to 2.0 ml (Johnston *et al*., 2001). The third or prostatic fraction is normally clear and may consist of more volume, depending on how long pressure is maintained proximal to the

bulbusglandis (Johnston *et al*., 2001). This prostatic fraction is also useful for evaluating the prostate Interestingly, the method of collection can limit sperm quality. For instance, dog's semen quality varies between manualand electro-ejaculation methods.Although spermatozoa motility is almost the same in both manual and electro-ejaculation but individual sperm motility does not last longer in manual collection as compared to electro-ejaculation method (Christensen *et al*., 2011). Moreover, direct collection of ejaculates to the extender in sought as an attempt to reduce agglutination (Gomes-Alves *et al*., 2014). Additionally, modification such as collection into a warm extender help to reduce the impact of pH shift and cold shock (Johnson, 2000). Hence, improve parameters such as viability and motility (see figure 3). The site of where semen is collected for instance in the epididymal spermatozoa when fluids from accessory glands has much improvement in terms of semen quality. Addition of prostatic fluid has also been found to improve sperm fertilising ability (Nothling*et al*., 2005), conception rate (Nothling*et al*., 2007), motility (Hori *et al*., 2005; Korochkina*et al*., 2014), viability (Hori *et al*., 2005), and distance average path and velocity (Korochkina*et al*., 2014). These positive effects of prostatic fluid could be due to secretions produced by the prostate which contains citric acid, calcium, and a number of enzymes that can protect the sperm cell and improve the survival, motility and fertilizing ability.

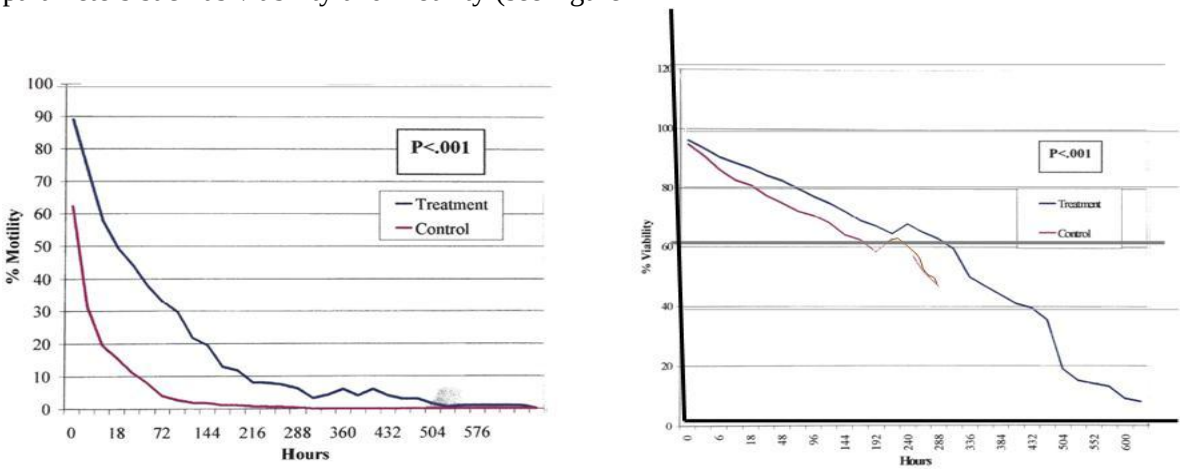

Figure 5. Motility and viability when collection of semen is modified

Figure 3: Treatment group are dogs where modification in collection was undertaken i.e. the sample was collected into warmed extender. The results were better in the treatment group than those where semen were collected into dry container without any modification (Control). Hence, improved motility and viability of spermatozoa (source: Johnson, 2000).

Anti-oxidants

Antioxidants are found to control the balance between the reactive oxygen species and hence, control the sperm cell membranes and DNA. It is worthwhile noting that normally the physiological concentration of antioxidant in the spermatozoa cytoplasm is not that sufficient to protect against the cryopreservation procedures (Bansal, 2010). Antioxidants like catalase were reported to have a pronounced effect on the improvement of sperm quality after thawing (Michael e*t al*., 2007). Kmenta *et al.* (2011) used antioxidant catalase as an extender with 0.8% lecithin and reported superior preservation of cold stored spermatozoa in canine (Kmenta *et al.*, 2011). Though still the concentration of these antioxidants is found to be necessary to achieve certain results in terms of improvement of sperm quality that would also vary from one antioxidant to another. For instance, in studies involving glutathione and ascorbic acid, glutathione at 5mM was found to give better results when supplemented (Monteiro *et al*., 2009) (see table 3). Farstad, (2009) suggested that when these extenders are supplemented with defined compounds such as cryoprotectants and antioxidants they can be helpful to protect sperm membrane damage and also DNA damage. Therefore, addition of antioxidant is very useful towards protecting sperm from damage and hence, improving cryopreservation. The use of antioxidants can be of value if the right type of antioxidant and the right concentration of antioxidant can be applied in order to give better results. Hence, this area requires a need for further research to ascertain the best antioxidant and their respective concentration.

Table 3: Showing forward progressive velocity of sperm (SFPV 0-5). Glutathione (Glu) at

concentration of 5 had a higher velocity in almost all time intervals. Varying concentration of both ascorbic acid (AA) and glutathione gave different results in terms of SFPV. Sperm involving glutathione at a concentration of 5mM showed higher forward progressive velocity (taken from (Monteiro *et al*., 2009).

Table 12. Showing forward progressive velocity of sperm

Sperm forward progressive velocity (0–5)					
Groups	0 min	30 min	60 min	90 min	120 min
Control	2.9 ± 0.1	2.4 ± 0.3*	1.5 ± 0.6^{ad}	0.8 ± 0.9^{b}	0.2 ± 0.3
AA-50	3.1 ± 0.1	2.7 ± 0.3^{c}	2 ± 0.4^{d}	1.4 ± 0.6^{d}	0.4 ± 0.7
AA-250	3.1 ± 0.1	2.8 ± 0.3^{c}	2.1 ± 0.4^{c}	1.1 ± 0.7	0.3 ± 0.7
Glu-1	3.2 ± 0.4	2.6 ± 0.4^{c}	2.1 ± 0.7^{d}	1.3 ± 1.2^{d}	0.4 ± 0.9
Glu-5	3.4 ± 0.5	3.2 ± 0.5^{c}	2.6 ± 0.7^{ad}	1.8 ± 1.2^{bd}	0.8 ± 0.8

Conclusion

Cryopreservation of dog semen facilitates the exchange of genes between populations and makes it possible to preserve genes from valuable males for an extended time. However, cryopreservation of dog semen has a number of limitations which are partly caused by freezing temperature, cryoprotectants and post thawing lifespan of spermatozoa. As an attempt to improve the output of cryopreservation, the time, type of semen to collect, as well as age of the animal from which we collect our ejaculates are important. Nevertheless, storage is still a stumbling block but there seems a possibility that improving the method of storage, can help to improve the semen quality. Therefore, more research is needed to find out the appropriate storage improvement for cryopreserved semen.

There are individual differences in terms of post-thaw semen quality. Involvement of thawing temperature of 70ºC and above within few seconds presents promising results. Modification in terms of extenders can provide a way to improve cryopreservation. There is a need for further study and examination of the particular stud dog which provides good results in terms of post-thaw viability of spermatozoa. Possibly, there can be development of individual freezing extenders for those dogs that show problems in terms of semen freezing and yet are considered as superior breeds. Alternatively, increasing sperm number can be one of the options to compensate differences in fertility among males. Moreover, we recommend further studies and modifications to be done to qualify the use of higher temperatures as an attempt to improve results under the cryopreservation.

Modification in terms of extenders can provide a way to improve cryopreservation. Possibly, use of own prepared extenders other than commercial ones will be useful as they will consider the need of the breeder. They might take note of individual dog difference and the environment in question as commercial ones were made under different settings. Since there is limited researchon the use of CPA-free in cryopreservation in dogs then, therefore, we recommend the use of glycerol at 4 to 8% concentration for better results. Possibly, as the concentration of cryoprotectants increases also the thawing temperature has to increase as well. This might be useful for controlling toxicity levels of cryoprotectants, and thus improving conception rates.

The collection method can be a tool to improve the results of the cryopreservation process. Therefore, use of methods that do not confer to long exposure of sperms to prostate fluid will be of value here. Furthermore, collection of ejaculates into extension media together with modification of the environment of extension media can be of value. The addition of antioxidants is very useful for protecting sperm from damage and thus, improving cryopreservation. The use of antioxidants can be of value if the right type of antioxidant and the right concentration of antioxidant can be applied in order to give better results. Therefore, the review recommends further research to ascertain the best antioxidant and their respective concentrations.

Finally, improving cryopreservation is not one process. It involves a combination of a lot of factors with the ultimate goal of improving the outcome of cryopreservation. This is in terms of conception rates in bitches and thus, fertility in general. Factors

to be improved include: type of ejaculate, insemination technique and storage also, post thawing procedures, extenders, cryoprotectants, collection method and antioxidants.

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