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Factors Affecting the Quality of Cryopreserved Dog Spermatozoa

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ABSTRACT

Limited research is done on canine semen as compared to dairy animals. Semen evaluation is very important to confirm the pregnancy in bitch and most of the factors are contributed by male, if used chilled or frozen. Dog's health, semen collection procedure, use of extender, cooling and freezing time, thawing technique and insemination, all plays very important role while dealing with the breeding operation. Standardized procedures are to be adopted for successful results. This review covers the various observations pointed out by the scientists for semen handling during Artificial Insemination (AI).

Keywords: Semen evaluation, Dog Semen, Factors effecting semen quality, Successful AI in dogs

INTRODUCTION

Health concerns and legal formalities in physical transportation of dogs led to introduce the use of AI in dogs for breeding. Use of AI facilitate the selection of breeding males worldwide by using frozen semen without moving the stud dogs from a long distance to utilize the breeding potential. This navigated to look for the techniques of cryopreservation with focus on quality of post thawed semen offering practical advantages because of preserving the genetic potential of desired traits, however, sperm damage during cooling and thawing increases the risk of sperm loss and require strict monitoring (1). Cryopreservation techniques of epididymal canine sperm is almost parallel to the protocols used for ejaculate collection and spermatozoa retain their functional ability despite some damage (2). Dog semen has a complex interaction of diluent, seminal plasma, cell membrane, and freezing / thawing procedures (3). Dog semen contains variable quantities of antioxidant in different dogs influenced by age, size and season (4,5). Freezing is broadly considered to cease the enzymatic reactions and cell metabolism. Damage is also reported due to lipid transitions, water movement, chemical stress, salt concentration (6). Sperm is exposed to cold shock above freezing temperature, that directly affect the sperm membrane integrity. Naturally available cholesterol in semen maintains the intactness of cell membrane (7). Dog kept under cold storage is safe to freeze after 1-2 days without compromising the post thaw motility and sperm survival (8). The first AI following storage of dog semen was reported in the 1950s (9). A.I results of cryopreserved semen have been limited due to identifying proper time of insemination for the bitch and achieving accurate intrauterine deposition of spermatozoa (10). Factors influencing the quality of frozen semen are: osmotic environment, composition of extenders, freezing and thawing protocols (11). Adverse effects of cryopreservation on canine semen include the damage of acrosomal membrane. It was reported that the 85% of normal acrosomes initially, upto 65% after cooling and around 20% after cryopreservation / thawing, however, no adverse effects observed on membrane integrity if standardized extenders are used (12). Generally, a comprehensive knowledge of freezing and thawing protocols is necessary to achieve the better fertility results of A.I in canines.

Sperm Osmotic Properties

Dog sperm acts as a perfect osmometer since the cell releases water and shrink to avoid crystal formation during freezing (13). The ability to regulate cell volume and osmotic

challenge is serious to cryopreservation (14). The extra cellular osmotic pressure overcomes the intracellular one by outward movement of water (15). It was revealed that sperm become susceptible to cold shock during dilution from hypotonic to isotonic solution and rapid freezing. Osmotic factor leads to physical swelling or shrinkage of cell (16). Cell injury may be the result, if shrinkage or swelling goes beyond a certain limit. Volume of cell must be to a tolerable limit during freezing and thawing (14).

Prostatic Fluid

First step during cryopreservation of canine spermatozoa, is centrifugation. Excessive prostatic fluid adversely affects the motility and vitality of frozen semen (16). Removal of prostatic fluid by 5 min 720xg centrifugation is recommended to preserve the functional parameters of sperm. Increasing the centrifugation time to 10 min 600xg decreases mitochondrial membrane potential (17,18). Centrifugation is more useful in low quality ejaculates and old dogs (19). It has been observed increased fertility by supplementation of prostatic fluid in thawed canine semen (20). It was reported that breed variation in seminal plasma composition, these changes can influence freezability and post thaw results (21). In dogs, the interaction of seminal plasma membrane vesicles with the spermatozoa has not yet been proven as evident in human and some other species by fusion with membrane of spermatozoa.

Composition of Extender

Extenders are isotonic and added to the semen as energy source. A good extenderact as buffer against pH changes and maintain electrolyte concentration with antimicrobial effects. Normal composition of extender contains egg yolk, buffers, sugar (glucose or fructose etc) as energy source and a cryoprotectant glycerol. pH of an extender ranges between 6.9 to 7.1. Each ingredient is added for its essential and protective function. Buffer is required for ion removal as hydrogen ions built-up during metabolic activities of sperm. Increased hydrogen concentration reduces the pH which compromises the survival of sperm. Currently, extenders with different proportion of ingredients are in use. Commercially prepared bovine extender found useful for cryopreservation of dog semen (22). Tris-fructose-citric acid extender is better in comparison to tris-fructose-citrate, glycine and lactose. Lowest progressive motility observed with skim milk, lactose and tris-fructose citrate extenders (23). The egg yolk-Tris

(EYT) extender without glycerol is common for cold storage while glycerol added as cryoprotectant for freezing (24).

Commonly used diluents for canine semen have 10-20% egg yolk for nutritional support and sperm membrane protection due to a natural source of phospholipids and low density lipoproteins (LDL) (25). Use of 10% EY was suggested for best initial pst-thaw motility and live sperm percentage (26). However, up to 20% mixing of EY is also found in some experiments without any significant difference. EY is being replaced by purified low density lipoproteins having advantage of maintaining sperm motility and membrane integrity (27).

Sugars (Fructose or Glucose) are added as energy source. It has been established that fructose is more efficient in providing energy source than glucose. Furthermore, fructose has also been reported to play a role in sperm activation mechanism (28). Glycerol having intracellular and extracellular actions is frequently used as cryoprotectant for dog semen. It lowers the freezing point by reducing the salts and protects the membrane damage. Use of 4-11% glycerol concentrations is recommended but most common protocol range is between 5% to 8% use of glycerol (29,30). Antibiotics are added to the semen to avoid bacterial contamination and kill existing contamination from animal ejaculates. Antibiotics may have detrimental effect on spermetazoa (31). There has been no significant effect, whether dilution with extender is done in one or two steps. If Sodium Dodenil Sulphate (SDS) is being used as cryoprotectant, it should be done in two steps; SDS to be added in second step (32).

Equilibration

Before the start of freezing process, diluted semen is kept at 4 C° in order to produce resistance in the sperm for subsequent cold shock. Range of45 min to 5 hrs equilibration timesreported for dog semen. It was achieved best post-thaw motilitywhen spermswere cooled from 37°C to 5°C for 1 hour and equilibrated at 5°C for 1 hour using Tris-EY with4% glycerol (29). It was reported that greatest post-thaw motility using Tris-EY extender (+6.5% glycerol) with equilibration time of 4 hours at 5°C (26), however, decreased motility found after fast cooling to 0°C in 40 min with > 2 h equilibration (33). Glycerol was considered a slow chemical to permeate that requiring an extended equilibration time before freezing but found its rapid membrane infusion and negated the long equilibration time (34).

Freezing ang Packing

Freezing is suspended animation for the cell to slow down the biological process of the cell in low temperature (15). No doubt sudden exposure to increase or decrease in temperature causes shock to the spermatozoa leading to cell injury. Therefore, freezing rate has to be fixed in order to maintain post thaw viability of sperms. Freezing rate varies among species and the media being used. A slow cooling procedure is preferred in canine's semen freezing progression (35). Clinical trials and studies on acrosome integrity have proved more influence of freezing rates than the of extenders (22).The time between the packaging and thawing is most critical for reduction of progressive motility percentage (36).

It was found that the best freezing rates of 10 to 50 C° per minute in the critical range of -15 / - 60 C° among different cooling rates (29). Excessively fast freezing (-99 C° / min) and a extremely slow freezing rates (-0.5 C° /min) have a detrimental effect, whereas, cooling at of –12 C° /min or – 28 C° /min delivered better results (33). Maintaining the frozen semen in straw at 8 cm above liquid nitrogen and thawing at 70 °C in water is supported for best results (37).

Spermatozoa are usually frozen in 0.5 ml straws by manually dropping the straws into a liquid nitrogen tank in three steps (8) but best procedure is use of an automatic programmable freezer that allows two step cooling curve comparatively faster and controlled decreases in temperature (38). Use of ultra-freezers is a viable alternative maintains inner cabinet temperature at -152 C° without o programmable cooling. The main drawback of this system is fluctuation between -100 and -152 C°. The same protocol is used as in case of liquid nitrogen (39). The last stage of cryopreservation is the plunge into liquid nitrogen (-196 C°).Up till the end of last century, straws 2.5 ml semen straws were commonly in use with a high pregnancy rates but would require higher volume of semen and more quantity of extender, thus reducing the advantages of AI. Some authors also used 0.25 ml of straw size but it did not give good results. It was conducted an experiment to study the effects of straw size and concluded that 0.5 ml resulted in 5.7% more progressive motility 60 min after thawing than 0.25 ml straws. Semen is also frozen in pellets form (37). It was found that the better post thaw motility in pallets than straws (40).

Thawing

Water bath at 70ºC is used 8 seconds and semen is thawing media used at 37ºC to re-extend semen dose before insemination (8). Thawing at 70 °C found to give more motile sperms 60 min after thawing, compared to at 37 °C and 6.6% fewer abnormal acrosomes (37). Perhaps, thawing is the most critical factor in maintaining quality of frozen semen. This is a step, where most of the cell death may occur, being a reversal of cryopreservation (15). The thawing speed should be such that no recrystallization of cellular material occurs and cell goes normal without any hypnotic stress. The maximum thawing rate is directly related to cooling rate. However, the sperm frozen by 10°C/min and thawed at 37°C for 30 seconds (fast thawing) shows better post thaw motility as compared to slow thawing rate signifying faster thawing has improved sperm viability than slower one. The results have been improved by thawing the frozen in a medium like Tris buffer solution (35). The mechanism of improvement is not known but it also increases the volume for insemination. This may reduce the toxic effects of glycerol (41). Some freezing protocol also recommend the thawing process to be carried out in Homologous Prostatic fluid and is thought to improve post thaw survival of the sperms (20). Addition of antioxidants improves post thaw semen quality (42).

CONCLUSIONS

Use of frozen- thawed semen has facilitated promoted the A.I due to its convienience and gene propagation with maximum use of a stud dog at long distance without involving its physical transport. However, the whole process from semen collection to insemination is a highly sensitive procedure to be monitored carefully and require step wise regular monitoring under scientific environments. Human errors, procedure awareness and dog itself are the main contributing factors while dealing whit frozen semen. A.I is the best tool and a valuable service for breeding, if taken seriously.

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