CRYOPRESERVATION OF BUFFALO *(BUBALUS BUBALIS)* SEMEN-LIMITATIONS AND EXPECTATIONS

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ABSTRACT

Artificial insemination with cryopreserved semen is the most viable biotechnology for faster and increased genetic improvement in many species allowing for improved herd performance and productivity. Pakistan has 34.6 million buffalo which have major share in total milk produced in the country. Rapid increase in human population and demand for animal products motivated the researchers to increase per animal milk production using this biotechnology. In buffalo, natural breeding practice is common in the country compared to artificial insemination, low fertility rate with cryopreserved semen is main hindrance in its propagation. It is need of the hour to disseminate the knowledge of various factors and components contributing in buffalo semen cryopreservation to improve milk and fertility rate of this breed.

Keywords: buffalo, cryopreservation, extender, processing, semen

INTRODUCTION

The genetic improvement and disease control in livestock have primary importance in the success of agri-food industry. In this contribution, artificial insemination (AI) is possibly the most decisive tool for the progression of modern animal production. By means of AI, each ejaculate collected from genetically superior male is used to inseminate females at a large scale and also controls sexually transmitted diseases.

The development of cryopreservation protocols in dairy industry began in 1950s. Bratton *et al*. (1955) demonstrated that bovine sperm frozen to -79^{o}C and packed on dry ice could still yield high fertility. Since then, cattle and buffalo industry have seen progressive improvement in tropical developing countries including Pakistan and AI gained wide acceptance in the development of the livestock resources (Cheema and Samad, 1986). The development and application of AI in the last four to five decades especially in cattle has been striking. Extensive research has been stimulated by the rapid expansion of this practice (Andrabi *et al*., 2001; Anzar *et al*., 2003).

Semen cryopreservation is a complex process which involves many steps: extension, cooling, freezing, storage and thawing. During each step sperm structure and function are affected (Bailey *et al*., 2003) resulting in reduced sperm

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motility (Tuli *et al*., 1981), acrosomal damage and alteration in sperm membrane integrity (Rasul *et al*., 2001).A thorough knowledge of each step is vital in order to attain a maximum conception rate. Successful cryopreservation varies highly among species, individuals within species and even within ejaculates of individuals, which is largely attributed to the differences in biophysical characteristics among cell types (Thurston *et al*., 2001; Waterhouse *et al*., 2006). In general, the plasma membrane is considered to be the primary site of cryo-injury and the principal damage occurs during freezing and thawing (Parks and Graham, 1992**)** resulting in substantial loss of viable spermatozoa. This vulnerability has been documented to be higher for buffalo bull semen owing to a higher oxidative stress though higher lipid oxidation rate, reduced activity of naturally present antioxidant enzymes, higher membrane contents of unsaturated fatty acids and lower osmotic pressure of buffalo semen (Raizada *et al*., 1990; Khan and Ijaz, 2008). Comparatively, AI in buffalo with cryopreserved semen has been limited due to poor semen freezability (Kumaresan *et al*., 2006; Andrabi *et al*., 2008) and conception rate (Chohan *et al*., 1992; Bhosrekar *et al*., 2001). The purpose of this article is to point out various factors affecting the efficacy and fertility using cryopreserved semen.

EXTENDER COMPONENTS

Buffer

A buffer solution resists changes in pH when small quantity of an acid or an alkali is added to it. In various species considerable work has already been done on this aspect for extender preparation. The composition of the extender is decided on the basis of duration and storage temperature of the semen and need a suitable buffer for this purpose (Rasul *et al*., 2000). Ideally, a buffer should possess following characteristics (i) Maximum water solubility (ii) pH between 6 to 8, favorably 7 (iii) Minimum salt/temperature effects/ buffer concentration (iv) Well behaved cations interaction(v) Better ionic strengths and chemical stability (Andrabi, 2009).

Efforts are being made to develop a most suitable buffering system for buffalo bull semen cryopreservation which should have composition close to natural medium and help in maintaining fertility of the frozen semen. Starting from the use of various organic buffers for the cryopreservation of the bull semen (Foote, 1970), chemically defined buffers gotattention. Matharoo and Singh (1980) found a least loss of post-thawed motility with Tris-based extender. However, Chinnaiya and Ganguli (1980) reported that spermatozoa cryopreserved in citrate, citric acidor Tris-based extender had similar acrosomal damage, while Dhami and Kodagali (1990) reported improved freezability using Tris-based extender.

Similarly, in buffalo bull spermatozoa, Singh *et al*. (1991) reported least release of sorbitol dehydrogenase and lactic dehydrogenase during cryopreservation using Tris-based extender followed by citrate and citric acid extenders. Singh *et al*. (2000) reported better results with Tris-based buffer as compared to Laiciphos and Biociphos. While, Rasul *et al*. (2000) also reported improved motility rate using Tris citrate as compared to tri-sodium citrate, Tris-Tes or Tris-Hepes. On the other hand, Oba *et al*. (1994) and Chachur *et al*. (1997) reported similar effects on motility rate, acrosomal and plasma membrane integrity using Tesor Tris-based extender.

All these studies clearly document the use of zwitterions buffers as a better option for bubaline

semen extension and Tris-citric acid seems to be the most appropriate buffering system for the freezability of buffalo bull spermatozoa. The pH of zwitterions buffers is closer to the pKa (acid dissociation constant) and it is least affected by temperature (Graham *et al*., 1972). As compared to cattle spermatozoa, the prone inclination of buffalo spermatozoa towards freezing stress makes it a dire need of time to study the effects of buffers on spermatozoa pre and post-cryogenic membrane stability at molecular and biochemical levels.

Antibiotics

The viability and fertility potential of cryopreserved spermatozoa is highly affected by presence of bacteria (Thibier and Guerin, 2000) either directly or indirectly. Hence, control of these bacteria through the use of various antibiotics in the extenders becomes one of the vital measures to attain in maxima results of AI. Benzyl penicillin alone or in combination with streptomycin sulphate is generally added in semen extenders of buffalo bulls (Akhter *et al*., 2008). However, these antibiotics have not been effective in controlling bacteriospermia of buffalo semen (Aleem *et al*., 1990). Ahmed and Greesh (2001) reported norfloxacin (200 µg / ml) or gentamicin or amikacin (500 µg / ml) as drug of choice for efficient preservation of buffalo bull semen. In the recent years, a new combination of gentamicin tylosin and lincospectin, GTLS has shown maximum capacity to control bacteria present in buffalo bull semen (Hasan *et al*., 2001; Akhtar *et al*., 2008). The testing of a wider range of antibiotics alone and in combinations is recommended to enhance the quality of cryopreserved buffalo bull semen.

Cryoprotectants

In order to protect the spermatozoa from the cryoinjuries, various cryoprotectants are added in the extenders (Purdy, 2006). On a broad based level, these cryoprotectants are classified into two categories (permeable and non permeable). Permeable cryoprotectants include glycerol, methanol, butanediol, 1,2-proparediol, ethylene glycol, dimethylsulfoxide (DMSO) and propylene glycol. These cryoprotectants have ability to pass through the sperm plasma membrane and act intra-cellularly and extra-cellularly, rearrange the membrane proteins, reduce formation of intracellular ice and thus protect the sperm from damage (Holt, 2000). Most penetrating cryoprotectants serve as both solvent and a solute. Non permeable cryoprotectantsinclude egg yolk, amino acids, trehalose, dextran, sucrose, xylose, fructose, lactose. mannose, raffinose, synthetic polymers such polyvinyl pyrollidone (PVP), amides and skimmed milk (without fat) which do not penetrate the sperm membrane and act outside the sperm (Aisen *et al*., 2000).

Cryoprotectants have a property to lower the freezing temperature and ultimately reduce extracellular ice formation (Kundu *et al*., 2002). Glycerol (6 to 7%) is commonly used as cryoprotectant for buffalo bull semen. However, addition of glycerol at 2 to 3% or more than 7% reduced post-thaw sperm motility (Ramakrishan and Ariff, 1994; Nastri *et al*., 1994). Similarly, Fahy, (1986) reported 2.25 to 9% glycerol to be safe. Rasul *et al*. (2007) observed synergistic outcome of dimethyl sulfoxide (DMSO) and glycerol on the post-thaw buffalo sperm quality in terms of motion characteristics, plasma membrane integrity and acrosome morphology using tris citric acid extender differing in glycerol and DMSO concentrations. Glycerol is beneficial for the sperm as its freezing point is much lower than water. Hence, 5 to 7% concentration of glycerol in the

semen extender may be suitable for buffalo bull semen cryopreservation. Development of less toxic and more efficient cryoprotectant will further make an ample contribution in improving buffalo bull semen characteristics.

Glycerol and egg yolk are used in combination as cryoprotectants in cryopreservation. Proteins and lecithin are found in egg yolk which maintain and preserve the lipoprotein sheath of spermatozoa (Kumar *et al*., 1992). Egg yolk possess ability to stimulate enzymes found in the spermatozoa which cause deamination of amino acids causing damage to spermatozoa during storage period (Sahni and Mohan, 1990). To avoid production of hydrogen peroxide, egg yolk must be dialyzed before adding it to the extender. In past, limited attention was levied upon the concentration of egg yolk for freezing buffalo bull semen. Most of the researchers have used 20% concentration (Sansone *et al*., 2000; Andrabi *et al*., 2008) while Sahni and Mohan (1990) reported that the egg yolk concentration could be reduced to 5% in tris glycerol medium without affecting the post-thaw sperm motility. Andrabi *et al.* (2008) reported improved freezability of buffalo bull spermatozoa by adding duck egg yolk in extender as compared to other avian egg yolks.

Cheshmedjieva *et al*. (1996) studied the effect of addition of polyethylene glycol (PEG 20) to egg yolk based freezing medium and concluded that addition of PEG 20 to semen extenders preserves the lipids of frozen buffalo bull spermatozoa. Further studies on PEG20 in future may find it a better choice for semen cryopreservation.

Sugars like xylose, galactose, raffinose, fructose, maltose, glucose and sucrose are non permeable cryoprotectants, these are also added in semen extender (Nagase *et al*., 1964). Sugars and polyols have the ability to replace water molecules with normal hydrated polar groups and hence stabilize the spermatozoa membrane by protecting the sperm from damage during cryopreservation (Woelders *et al*., 1997). As sugars have high molecular weights, which changes the cell membrane permeability and maintains the electrolyte balance during cryopreservation. The newer international trends in disease control consider the ingredients of animal origin (egg yolk) to be a source of contamination to the semen (Bousseau *et al*., 1998), hence emerges the need of using non-animal sources.

Other additives

Attempts have been made to ameliorate the freezability of buffalo bull semen by adding different substance like antioxidants, metabolic stimulants, detergents and chelating agents. Bhosrekar *et al*. (1990) added tri ethanolamine laurel sulphate in extender having Tris-citric acid base and reported to improve post thaw spermatozoa motility. Detergents are believed to exert directly their protective effect on the membrane of the spermatozoa, or by emulsifying the egg yolk lipids which become readily available to spermatozoa membrane during cryopreservation (Arriola and Foote, 1987; Buhr and Pettitt, 1996).

The addition of cysteine or ethylenediamine tetraacetic acid (EDTA) at 0.1% in semen diluents during buffalo bull semen freezing did not improve semen quality in terms of release of lactate dehydrogenize (Dhami and Sahni 1993). Singh *et al*. (1996) reported that addition of 2.5 mM ascorbic acid in buffalo bull semen diluents significantly improve post-thaw spermatozoa motility and livability. Whereas, Kolev (1997) reported 0.3 mg/ml addition of vitamin E in extender had best effects on buffalo bull spermatozoa motility, acrosomal integrity and survivability during cryopreservation.

Report from Fabbrocini *et al*. (2000) revealed that sodium pyruvate at 1.25 mM concentration in the extender significantly improved post-thaw progressive motility and viability of buffalo bull semen. It is also documented that by adding oviductal proteins of different stages of estrous cycle also effect post-thaw semen characteristics by improving functions and reducing lipid per-oxidation during cryopreservation (Kumaresan *et al*., 2006). Bradykinin (2 mg/ml) in tris-based extender is also reported to improve the buffalo bull semen quality by Shukla and Misra (2007). Butylatedhydroxytoluene (BHT) at concentration 1.0 and 2.0 mM in tris citrate egg yolk extender gave best results for cryopreservation of buffalo bull semen (Ijaz *et al*., 2009).

Osmotic pressure

All solutes or colloids present within or outside spermatozoa contribute towards the osmotic properties of the solutions and when the concentration of the solutes become high, the osmotic pressure is raised. In this way, number of solute particles in a solution not only affect the osmotic pressure but also change the freezing point of the solvents. When spermatozoa are exposed to hyperosmolal solution, more extra cellular ice crystals are formed (Watson, 1995) and vice versa. So, any change in spermatozoa volume during extension, freezing and thawing processes result in sperm injury (Mazur, 1985).

As water and polyhydric alcohols have high osmotic permeability coefficient which enhances the shifting of these substance across the cell membrane of the spermatozoa during freezing (Noiles *et al*., 1993). Under hypotonic/ hypertonic solution, spermatozoa either swell or shrink (DU *et al*., 1994; Gilmore *et al*., 1996) and change its size. Changes in osmotic pressure during cryopreservation exert stress and damage spermatozoa plasma membrane. Therefore, osmotic pressure plays a vital role during cryopreservation of the buffalo bull semen and ultimately it affects the frozen semen quality (Khan and Ijaz, 2008). Different osmotic pressures of the buffalo bull semen have been reported, which include 293.33 mOsm/kg (Sansone *et al*., 2000), 268.81 mOsm/L (Khan and Ijaz, 2008) and 289.4 mOsm/kg (Mughal *et al*., 2013) From these findings, it is clear that buffalo bull semen has osmotic pressure lower than cattle semen. Therefore, buffalo bull semen diluents should have osmotic pressure close to their original values.

SEMEN PROCESSING

Initial processing

Methods of semen processing after collection are different from organization to organization. Changes in the sperm motility, morphology and freezability were not observed when ejaculate was processed within an hour of collection (Fabbrocini *et al*., 1995). Usually semen dilution is done at 30^{o}C to 37^{o}C with a media having all necessary constituents. Vale *et al.* (1991) recommended that ejaculates should be kept for 10 to 15 minutes, sometimes under these circumstances semen of buffalo bulls may show agglutination. To avoid this condition in such cases addition of diluents soon after the collection prevents irreversibly agglutination and also maintains sperm motility. Whenever there is delay in semen processing, then immediately after semen collection it should be diluted with freezing medium and stored at 5^{o}C (Televi *et al*., 1994). Under these circumstances spermatozoa motility up to 6 h at 5^{o}C remains constant in such diluents.

Dilution of collected semen depends on the demand and concentration of the semen of the sire.

Semen dilution

One step or two steps dilution methods are being used. Del Sorbo *et al*. (1994) compared both of these methods using tris-egg yolk extender and reported better results with two step method with equilibration duration of 6 h. One step method needs shorter equilibration time (2 to 4 h) before freezing. Addition of glycerol in two step dilutions method showed higher sperm motility when glycerol was added 1 h before freezing. (Fabbrocini *et al*., 1995). Del Sorbo *et al.* (1995) also suggested two step dilution methods using sodium pyruvate with second dilution 1h prior to freezing the semen.

Cooling rates, equilibration time and freezing

Slow and fast cooling rates are being used for semen cryopreservation. D during slow cooling process spermatozoa are exposed to high salt concentration and osmolality with changes in PH is observed. While during fast cooling, intracellular water may not pass out of membrane, resulting in intracellular ice crystals formation (Mazur *et al*., 1972; Mazur, 1977). Osmolality of the medium and rate of cooling also had a significant interaction (Woelders *et al*., 1997). Singh *et al*. (1989) and Sahi and Mohan (1990) also compared similar cooling rates. No significant difference in terms of post-thaw sperm motility was reported between these two cooling rates. In another study Anzar *et al.* (2010) used different cooling rates for buffalo bull spermatozoa and reported higher motion characteristics, acrosomal morphology and plasma membrane integrity at high freezing rates of -30^{o}C/ minute. However researchers prefer slow cooling rate of 0.2 to 0.4^{o}C/minute for the pre-freezing processing of the buffalo bull semen.

Slow cooling rate of diluted semen to 5^{o}C is considered beneficial (Ennen *et al*., 1976; Gilbert and Almquist, 1978) due to rapid penetration of glycerol in the cell membrane. Martin (1965) and De Leeuw *et al.* (1993) reported that glycerol can be added at any time during the cooling period. Investigators reported different equilibration durations. Short equilibration periods of 2 to 4 h is recommended by Singh *et al*., 1989; Del Sorbo *et al*., 1995) while other researchers preferred longer duration of about 6 h (Haranath *et al*., 1990; Televi *et al*., 1994). For cryopreservation of buffalo bull semen, it is generally believed that semen should be kept at 5^{o}C for not less than 2 h and not more than 6 h before freezing.

Semen is filled and sealed usually at 5^{o}C in the straws of 0.25 ml or 0.5 ml capacity (Cassou, 1964), Straws of 0.25 ml are commonly used because of their cost and storage space. Filled and sealed straws are placed in horizontal position 1 to 4 cm above liquid nitrogen gas for 10 to 20 minutes before plunging into liquid nitrogen gas at -196^{o}C.

Thawing rate and temperature

Thawing rate and temperature had a direct effect on the interior temperature of the semen. Different thawing temperature and time were used by the scientists. Mazur (1984) reported that rapid thawing of semen prevents formation of re-crystallization of water. Thawing of semen straws at 40^{o}C for 30 S was suggested by Dhami *et al*. (1994); Vale (1997). Slower thawing rates have also been tried by Kumar *et al*. (1993); Ramakrishnan and Ariff (1994); Fabbrocini *et al*. (1995) and reported various suitable times and temperatures: 37^{o}C for 30 S, 35^{o}C for 30 S and 39^{o}C for 30 S, respectively.

Thawing for shorter period may keep the internal temperature of the straw at level below

freezing while thawing for longer duration can also affect the acrosomal integrity. Some of the worker reported that buffalo bull semen quality can be enhanced using longer thawing time while Ziada *et al*. (1992) found no significant different when thawing was done at 35°C for 30 S or at 50°C for 15 S. El-Amrawi (1997) used different thawing procedure and got best fertility rates when semen was thawed at 35°C for 60 seconds.

Post-thaw spermatozoa appraisal

Different researches used different parameters to access the morphological status of the spermatozoa after thawing which include spermatozoa motility, viability, acrosomal/ DNA/plasma membrane integrity. Fabbrocini *et al*. (1996) studied the acrosomalintegrity using fluoresceinated lecithin, FITC-labeled Maclurapomifera Agglutinin (MPA) and suggested this technique for evaluating non lethal damage to spermatozoa.

Other protocols include analysis of enzymes that play role in fertility. Akhtar *et al.* (1990) reported significantly elevated hyaluronidase activity in semen after thawing. Previous studies conclude that after thawing, level of different enzymes increases significantly in extra-cellular medium as a result of leakage from spermatozoa. Therefore, enzyme leakage is another marker to evaluate the sperm freezability. The sperm motility and acrosomal integrity had negative correlation with enzyme like hyaluronidase amino transferaseand aspartate amino transferase, whereas, acid phosphatase (ACP), lactic dehydrogenase (LDH), alkaline phosphatase (AKP) had positive correlation. Kaker and Anand (1984) reported that glycerol concentration, cold shock, cooling and freezing rate influence on the release of glutamate oxaloacetate transaminase (GOT) and glutamic pyruvic transaminase (GPT) in seminal plasma. Dhami and Kodagali (1990) and Dhami and Sahni (1994) also measured post-thaw levels of GOT, GTP, LDH, ACP and AKP and recorded negative correlation of these enzymes with fertility.

Fertility evaluation

The changes in the morphology of spermatozoa are not reflected by the fertility rate. However, just laboratory aid can assess the severity of damage during cryopreservation and thawing. Fertility rate is the most suitable parameter to evaluate frozen thaw semen quality (Vale, 1997). Despite of all efforts, buffalo's hygienic conditions, estrus detection and time of insemination are also main contributor in poor fertility results (Vale, 1997). Buffalo rarely show behavioral sings, so estrus detection is a serious problem (Ohashi, 1994). Similar problems have also been reported by Danell *et al*. (1984); Drost *et al.* (1985). It is also believed that low conception rates in buffaloes using cryopreserved semen is due to its small uterus size as compared to cattle, so it is believed that during AI, semen is deposited in the uterine horn rather in its body. In contrast to this belief, Zicarelli *et al.* (1997) compared two semen deposit sites: cervical and cranial end of the horn, better fertility results were obtained when insemination was carried out at the cranial end of the horn. Pregnancy rate more than 50% using cryopreserved semen of buffalo bulls is believed to be a good result (Vale, 1997).

FUTURE DIRECTIONS

Future research for cryopreserved semen should emphasize on freezing protocols up-gradation for lower spermatozoa damage during cryopreservation. To achieve this goal, diluents

composition, freezing protocols along with the up gradation of presently used extenders must be focused for buffalo semen cryopreservation. Commercially available extenders i.e. Triladyl (Minitub Germany), Biociphos (IMV, France) and Laciphos (IMV, France), etc. should also be tried for cryopreservation of buffalo bull semen.

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