

Review Article

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Cryopreservation of Buffalo Bull Semen- Restriction and Expectation: A Review

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ABSTRACT

Semen cryopreservation is a widely used technique across the world for conservation and proliferation of genetically superior germplasm through artificial insemination. Artificial insemination (AI) is the most conclusive tool to increase genetic improvement, control of venereal diseases and improve herd performance and productivity. For achieving the mission, successful and effective cryopreservation of semen is essential. In recent years, increased demand of animal products due to very rapid growth of human population inspired the investigators to increase per animal milk production using various techniques of Biotechnology. The process of semen cryopreservation is stressful and around 40%-50% of sperm lose their motility and viability due to loss of structural and functional capabilities. One of the factors which affect post thaw survival of sperm is the freezing rate. In buffalo, poor fertility rate with cryopreserved semen is main obstacle in its proliferation. It is need of the time to spread the knowledge of various factors and components such as use of suitable extenders, additives, cryoprotectants, freezing and thawing protocols contributing in cryopreservation of semen to improve the fertility rate of buffalo.

Keywords

Buffalo, Buffer, Cryopreservation, Cryoprotectant, Extender, Semen Processing, Semen

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Introduction

The world buffalo population is appraised at 185.29 million, spread in some 42 countries, of which 179.75 million (97%) are in Asia (Buffalopedia, 2015, www.buffalopedia.cirb.res.in). India has 108.7 millions and they comprise approximately 56.7% of the total world buffalo population (DAHDF, Govt. of

India, 2015). Buffalo, a triple purpose animal, provides milk, meat and mechanical power to mankind. Due to its highly nutritious milk, leaner meat and best draught power for wet environments, buffalo offers immense potential for improvement of livelihood. Buffalo can efficiently convert low quality feed stuffs like straws and agro-industrial waste into human food, improve soil structure

through bio fertilizer and a financial asset which can be sold when needs arise (Pasha *et al.*, 2012).

The genetic improvement and disease control in domestic animals have primary importance in the success of agriculture and food industry. In this contribution, artificial insemination (AI) is perhaps the most conclusive tool for modern ways of breed improvement. Moreover, the quality of frozen–thawed semen is one of the most influential factors affecting the likelihood of conception (Saacke, 1984). Application of AI with frozen–thawed semen has been reported on a limited scale in buffalo, because of poor freezability and fertility of buffalo spermatozoa when compared with cattle spermatozoa (Kakar and Anand, 1981; Muer *et al.*, 1988; Raizada *et al.*, 1990; Singh and Pant 2000; Andrabi *et al.*, 2001, 2008; Ahmad *et al.*, 2003; Senatore *et al.*, 2004; Kumaresan *et al.*, 2005). In this process, each ejaculate collected from genetically superior male is used to inseminate females at a large scale and also controls sexually transmitted diseases.

Cryopreservation of spermatozoa

Cryopreservation is a non-physiological and complex method that involves a high level of adaptation of biological cells to the thermal and osmotic shocks that occur both during the dilution, cooling–freezing and during the thawing procedures (Watson *et al.*, 1992; Holt 2000a, b). Damage occurring during the freezing–thawing procedures affects mainly cellular membranes and nucleus (Blesbois 2007) and affects finally viability and fertility. The cryopreservation development protocols in dairy industry started in 1950s. Semen cryopreservation is a composite process which involves many stages: extension (dilution), cooling, freezing, storage and thawing. During each stages, sperm structure

and function are affected (Bailey *et al.*, 2003) resulting in reduced sperm motility (Tuli *et al.*, 1981), acrosomal damage and alteration in sperm membrane integrity (Rasul *et al.*, 2001). Sperm Cryopreservation exposes sperm to mechanical and anisotonic stresses (Hammerstedt *et al.*, 1990) that reduces cell survival and surviving sperm, thereby reducing cell longevity and fertility compared with fresh semen.

Cryopreservation generates sublethal sperm injury due to chemical, osmotic, thermal and mechanical stresses, which may result in loss of viability, motility, damage of deoxyribonucleic acid (DNA), destruction of acrosomal and plasma membrane (Numan Bucak *et al.*, 2007; Rasul *et al.*, 2001). Furthermore, changes in biochemical factors have been recognized during cryopreservation, including depletion of amino acids and lipoproteins, release of glutamic-oxaloacetic transaminase (GOT), decrease in phosphatase activity, decrease in loosely bound cholesterol, protein, inactivation of acrosin enzyme and hyaluronidase, prostaglandins diminution, increase in sodium, decrease in potassium content, reduction of ATP and ADP synthesis and decrease in acrosomal proteolytic activity (Barbas *et al.*, 2009). In fact sperm plasma membrane is a primary target for freezing or cold shock injury (Numan Bucak *et al.*, 2009). The extender used is an important factor in cryopreservation process. These mediums must have adequate pH and buffering capacity, appropriate osmolality and should protect spermatozoa from cryogenic lesion. Tris extender is an important medium that is often used during freezing of bulls, rams, bucks and buffaloes semen (Barbas *et al.*, 2009; Rasul *et al.*, 2001).

The production of Reactive Oxygen Species (ROS) is a normal physiological event in various organs. However, the over-production

of ROS can cause structural damage of sperm membranes (De Lamirande *et al.*, 1997). Reactive oxygen is responsible for sperm dysfunction due the lipid peroxidation of membranes (Arabi *et al.*, 2008). It has been documented that vitamin E is major antioxidant agent of sperm cells which is a potent scavenger of free radicals and is able to protect plasma membrane from damages mediated by ROS (Reactive Oxygen Species) and LPO (Lipid Peroxidation) (Yousef *et al.*, 2003; Gurel *et al.*, 2005 and Sinclair, 2000). It has also been established that presence of vitamin E is necessary for normal function of male reproductive system.

Sperm viability is depressed by as much as 50% during freezing (Watson 1995). To counteract destructive effects of ROS, seminal plasma has an antioxidant system that seems to be very relevant to protection of sperm (Alvarez and Storey, 1982). Furthermore, due to dilution of semen, anti-oxidant reserves of seminal plasma are depressed making spermatozoa more vulnerable to cryo insults. Therefore, it becomes all the more essential to incorporate anti-oxidants to semen extenders as protective agents. Assessing the structural and functional integrity of sperm membrane is of paramount importance in order to maintain optimum fertility potential of spermatozoa.

Cryodamage during freeze-thawing process to buffalo semen is higher than cattle spermatozoa due to unique physiology of buffalo spermatozoa and higher polyunsaturated phospholipids levels in plasma membrane (Nair *et al.*, 2006; Sreejith *et al.*, 2006; Andrabi, 2009). The fertility with frozen semen in buffaloes under field conditions is very poor and has been considered as 30% (Abhi, 1982; Chohan *et al.*, 1992; Anzar *et al.*, 2003; Andrabi, 2009). The reason for poor fertility rate in buffaloes under field conditions is poor post-thaw

characteristics of buffalo semen. As a matter of fact, the unique physiology of buffalo sperm requires buffalo specific semen extender to reduce the cryodamages. Improving the semen extender for the cryopreservation that ensures no or little damage to sperm motility, plasmalemma, acrosomal and chromatin integrity might enable to achieve a comparatively higher fertility rate with artificial insemination in buffalo under field conditions. It is well documented that motility, plasma membrane, acrosome and DNA integrity of buffalo bull spermatozoa significantly reduced after process of cryopreservation (Rasul *et al.*, 2001; Kadirvel *et al.*, 2009; Anzar *et al.*, 2010; Kumar *et al.*, 2011).

Cryopreservation of buffalo semen increases level of reactive oxygen species molecules that caused lipid peroxidation of bio-membrane system by reducing antioxidant potential of cryopreserved semen (Kadirvel *et al.*, 2009; Kumar *et al.*, 2011).

To control level of ROS and promote motility and survival of sperm, numerous antioxidants have proven beneficial in treating male infertility (Sinclair, 2000). Ascorbic acid and vitamin E is naturally occurring free radical scavenger and their presence also assist various other mechanisms in decreasing numerous disruptive free radical processes, including LPO (Bansal and Bilaspuri, 2009). Vitamin C and E are major antioxidants naturally present in mammalian semen against ROS to protect sperm from lipid peroxidation and to maintain its integrity (Bilodeau *et al.*, 2001; Gadea *et al.*, 2004; Bucak *et al.*, 2008; Andrabi, 2009; Akhter *et al.*, 2011). Concentration of antioxidant decreased during freeze-thawing process by dilution of semen with extender and excessive generation of ROS molecules (Andrabi, 2009; Kumar *et al.*, 2011).

Factors affecting freezability

It is found that buffalo spermatozoa are more susceptible to risks during freezing and thawing procedures than cattle spermatozoa, thus resulting in lower fertilizing capacity (Raizada *et al.*, 1990; Andrabi *et al.*, 2008). Moreover, there are specific biochemical factors that affect the capability of spermatozoa to prevent hazards caused by the cryogenic procedures. One of the many possible causes of lower freezability of buffalo bull semen compared to cattle bull can be due to the differences in the lipid ratio of the spermatozoa (Jain and Anand 1976; Tatham 2000; European Regional Focal Point on Animal Genetic Resources, 2003) due to presence of high phospholipids content found in buffalo sperm plasma membrane (Cheshmedjieva and Dimov, 1994). Therefore, there is a requirement to develop biochemically defined extenders and cryogenic protocols that are species specific, and may result in the improvement of viability and fertility of frozen thawed buffalo spermatozoa.

Diluter components

Buffer

A buffer solution is an aqueous solution consisting of a mixture of a weak acid and its conjugate base, or vice versa. Its pH changes very little when a small amount of strong acid or base is added to it. In various species considerable work has already been done on this aspect for diluter preparation. The composition of the diluter (extender) is decided on the basis of storage temperature of the semen, duration and need a suitable buffer for sperm survival during cryopreservation (Rasul *et al.*, 2000). Ideally, a buffer should have following features (i) Minimum salt effects/ buffer concentration (ii) pH between 6 to 8, favorably 7 (iii) Maximum water

solubility (iv) Minimum solubility in all other solvents (v) Minimum temperature effects (vi) Well behaved cations interaction (vii) Better ionic strengths and chemical stability (Bates 1961; Good *et al.*, 1966; Good and Izawa 1972; Keith and Morrison, 1981; Andrabi, 2009).

A most suitable buffering system for buffalo bull semen cryopreservation which should possess composition close to natural medium and help in maintaining fertility of the frozen semen. Beginning from the use of various organic buffers for the cryopreservation of the bull semen (Mughal *et al.*, 2017., Foote, 1970). In this consider, Matharoo and Singh (1980) evaluated citrate, Tris or citric acid as buffers for deep-freezing of buffalo spermatozoa. They found during freezing a minimal loss of post-thawed motility with Tris-based extender. However, Chinnaiya and Ganguli (1980) establish better post-thaw sperm motility with Tris-based extender than citrate or citric acid-based extenders and reported spermatozoa chilled (frozen) in citrate and Tris-based extender showed alike degree of acrosomal damage and similar recovery rates and Ahmad *et al.*, (1986) reported that Tris–citric acid based extender is fit for the freezing of buffalo bull spermatozoa regarding post-thaw motility and survivability while Dhama and Kodagali (1990) found that Tris-based extender improved the freezability of buffalo bull spermatozoa.

Identically, in buffalo bull spermatozoa, Singh *et al.*, (1990, 1991) found that minimal release of lactic dehydrogenase and sorbitol dehydrogenase during semen cryopreservation using Tris-based extender followed by citrate and citric acid extenders. Dhama *et al.*, (1994) reported that Tris-based extender provided higher post-thaw spermatozoa motility while Singh *et al.*, (2000) evaluated superior results with Tris-

based buffer as compared to Laiciphos and Biociphos. Rasul *et al.*, (2000) also found ameliorated semen motility rate using Tris citrate as compared to tri-sodium citrate, Tris-Tes or Tris-Hepes and also found that Tris citrate was suitable buffer for semen cryopreservation. Under other conditions, Oba *et al.*, (1994) and Chachur *et al.*, (1997) found nearest effects on semen motility rate, acrosomal integrity and post thaw motility using Tes which has equal value to Tris-based extender.

From these above mentioned results and studies, it is recommended that zwitterion buffers as a superior option for buffalo bull semen dilution or extension, particularly, Tris-citric acid may issue the most satisfactory buffering system to improve the post-thaw freezability and also fertility of buffalo spermatozoa. Graham *et al.*, (1972) reported that the pH of zwitterions buffers is nearest to the pKa (acid dissociation constant) and it is minimal affected by temperature.

Antibiotics

The viability or fertility of cryopreserved bovine spermatozoa is extremely affected either directly or indirectly by presence of bacteria in semen (Thibier and Guerin 2000; Morrell 2006). So, control of these bacteria by the addition of various antibiotics in the semen diluters to achieve maximum conception rate through artificial insemination (AI).

Sansone *et al.*, (2000) and Akhter *et al.*, (2008) were generally added benzyl penicillin 1000 IU/ml and streptomycin sulphate 1000 lg/ml alone or in combination as a freezing diluents in semen extender of buffalo bull. However, an inefficient combination of streptomycin and penicillin (SP) in buffalo bull semen to control of bacteriospermia by reason of SP was deleterious to post-thaw

quality of spermatozoa (Gangadhar *et al.*, 1986; Aleem *et al.*, 1990; Ali *et al.*, 1994; Amin *et al.*, 1999). Ahmed and Greesh (2001) reported that bacteria separated from buffalo bull semen were resistant to penicillin. However, the antibiotics of better choice to be added in semen extender for efficient cryopreservation of buffalo spermatozoa are gentamicin (500 lg/ml) or amikacin (500 lg/ml) or norfloxacin (200 lg/ml). Recently, a newly combination of gentamicin tylosin and lincospectin, GTLS has manifest extremely capacity to control bacteria and spermatozoal quality present in buffalo bull semen (Hasan *et al.*, 2001; Akhtar *et al.*, 2008). The evaluating of a broad range of antibiotics alone and in combinations is recommended to increase the quality of cryopreserved buffalo bull semen.

Cryoprotectant

Cryodamage has been attributed to ice crystal formation, membrane alteration, oxidative stress, osmotic changes, cryoprotectant toxicity and cold shock during cryopreservation (Watson and Martin, 1975). So, Optimum freezing and thawing rates, suitable cryoprotectant and composition of diluter (extender) are key factors for successful semen cryopreservation (Hammerstedt *et al.*, 1990; Curry *et al.*, 1994)

Semen cryopreservation causes deleterious effects regarding biochemical, functional and sperm ultra structural damage (Watson, 2000) causing in a depletion of membrane integrity, acrosome integrity and motility (Woelders *et al.*, 1997 and Celeghini *et al.*, 2007) and fertilizing ability (Purdy, 2006). During the cryopreservation, cryoinjuries are produced and can be lowered and significant enhancement can be obtained through using of cryoprotectants in the semen extender (Holt, 2000 and Johnson *et al.*, 2000). On a large scale, these cryoprotectants are

classified into two categories (permeable and non permeable). Permeable cryoprotectants include methanol, butanediol, glycerol, 1, 2-propenediol, ethylene glycol, propylene glycol and dimethylsulfoxide (DMSO). These cryoprotectants act as intra-cellularly and extra-cellularly have capability to move through the sperm plasma membrane and, reposition the membrane proteins, reduce formation of intracellular ice and thus protect the spermatozoa from damage (Holt, 2000). Non permeable cryoprotectants comprise mannose, raffinose egg yolk, amino acids, sucrose, xylose, fructose, trehalose, dextran, lactose, polyvinyl pyrrolidone (PVP), amides and fatless skimmed milk which do not pierce the sperm membrane (Aisen *et al.*, 2000). Cryoprotectants lower the freezing temperature and finally reduce extracellular ice formation (Kundu *et al.*, 2002). The optimum levels of glycerol and glycerolization are important key factor for the cryopreservation of buffalo semen. They also reported that post-thaw motility of spermatozoa was significantly better in a 5% glycerol extender, whereas the percentage of acrosomal integrity was higher in spermatozoa extended in 3% or 5% glycerol than in spermatozoa extended in 7% glycerol (Jainudeen and Das., 1982). Tris- and milk-based diluents have better level of glycerol as 6% and 9% glycerol for the sodium citrate diluent to acquire better post-thaw motility for buffalo spermatozoa (Kumar *et al.*, 1992) Glycerol is a small, poly-hydroxylated solute with a high solubility in water, and a low toxicity during short-term exposure to living cells. It can interact by hydrogen bonding with water and can permeate across the limiting plasma membrane of many different cell types (Fuller and Paynter, 2004). Ramakrishan and Ariff, (1994); Natri *et al.*, (1994) also reported that the reduction in glycerol below 5% decreased the post-thaw motility and acrosome integrity of spermatozoa in the extenders. Holt (2000b);

Medeiros *et al.*, (2002) investigated that the physiological role of glycerol as cryoprotectant takes place by lowering the freezing point of water, replacing intracellular water, binding with metallic ions and by reducing the electrolyte profile in the unfrozen portion. So, Glycerol (6 to 7%) is commonly used as cryoprotectant for buffalo bull semen. Abbas and Andrabi (2002) studied the effects of different concentrations of glycerol on post-thaw sperm quality and reported that the spermatozoa cryopreserved in 7% were significantly superior to those in other concentrations of glycerol as determined by post-thaw motility, plasma membrane integrity and survivability. Singh *et al.*, (2006) found that single step of glycerolization is more suitable for the cryopreservation of buffalo spermatozoa for post-thaw motility. Dimethyl sulfoxide (DMSO) is a rapid penetrating cryoprotectants having lower molecular weight than glycerol. Yu and Quinn (1994) evaluated that DMSO may inhibit harmful effects of hydroxyl radicals and these radicals appear during cell respiration and are detrimental to cell (Johnson and Nasr-Esfahani 1994). Rasul *et al.*, (2007) investigated the synergistic effects of dimethyl sulfoxide (DMSO) on cryoprotection ability of glycerol during cryopreservation of buffalo sperm in terms of motion characteristics, acrosome morphology and plasma membrane integrity using tris citric acid extender differing in glycerol and DMSO concentrations at various temperatures. DMSO in combination with glycerol is superior in providing cryoprotection in cattle bull (Snedeker and Gaunya 1970) rabbit and buck spermatozoa (Bamba and Adams 1990). During cryopreservation, DMSO has harmful toxic effect rather than the osmotic shock because of the lower molecular weight of DMSO, its penetrating ability into the cell is higher than glycerol (Rasul *et al.*, 2007). Glycerol is

useful for the sperm as its freezing point is much lower than water. Hence, it is suggested that a extender containing glycerol concentration of 5–7% may be suitable for the cryopreservation of buffalo bull spermatozoa. Under other conditions, development of less toxic and more powerful cryoprotectant could make an important contribution in improving post thaw quality of buffalo spermatozoa.

The other important component of semen extenders is egg yolk in the buffalo and most of the livestock species (Sansone *et al.*, 2000). Egg yolk containing low density lipoproteins (LDL) is highly responsible for sperm protection during cryo-preservation (Pace and Graham 1974; Watson 1976). LDL provides protection to sperm by stabilizing the membrane to adhere with sperm membrane. Sansone *et al.*, (2000); Andrabi *et al.*, (2008) reported that egg yolk which is commonly used at a concentration of 20% in semen extender, is mandatory for freezing of buffalo semen. The higher concentration of egg yolk in extender may have detrimental effects combined with toxicity of dead spermatozoa resulting in lower post-thaw sperm quality (Shannon 1972) probably due to the raised substrate available for hydrogen peroxide formation (Tosic and Walton 1950). Sahni and Mohan (1990) reported different levels of egg yolk in semen extender for cryopreservation of buffalo semen and concluded that the concentration of egg yolk in the extender could be reduced from 20% to 5% without any compromise in post-thaw motility of spermatozoa. Similarly Kumar *et al.*, (1994) reported that 5% egg yolk prove better than 10% and 20% while Singh *et al.*, (1999) concluded 10% concentration of egg yolk was superior for freezing of buffalo semen with regards to pre-freeze and post-thaw sperm motility and survivability of spermatozoa. Egg yolk containing lecithin and proteins preserve the lipoprotein sheath of spermatozoa (Kumar *et al.*, 1992). Sahni and

Mohan, (1990) found that egg yolk stimulates enzymes found in the spermatozoa which cause deamination of amino acids causing damage to spermatozoa during storage period. So, Egg yolk is an important and necessary component while diluting semen in the extenders in bovine and many species during cryopreservation protocols (Shannon and Curson, 1983; Priyadharsini *et al.*, 2011). The extender containing duck egg yolk improved freezability of buffalo bull spermatozoa as compared to other avian egg yolks (Andrabi *et al.*, 2008). Polyethylene glycol (PEG) is a non-permeable cryoprotectant and may decrease the process of ice nucleation during cryogenic process and protecting the cellular membrane. Cheshmedjieva *et al.*, (1996) found that the effect of addition of PEG 20 to egg yolk based freezing medium on the lipids in buffalo spermatozoa and concluded that incorporation of PEG 20 in semen extender preserved the lipids of frozen buffalo spermatozoa. In future, further studies on PEG 20 may be a better alternate for the cryopreservation of buffalo spermatozoa.

Sugars like as raffinose, trehalose lactose, sucrose and dextrans are non-permeable cryoprotectant and also added in semen extender (Nagase *et al.*, 1964). Sugars are not capable of diffusing across a plasma membrane and produce an osmotic pressure inducing cell dehydration and lower incidence of intracellular ice formation. These sugars also interact with reorganizing the membrane and phospholipids in the plasma membrane which results in surviving of sperm during cryopreservation process (Molinia *et al.*, 1994; Aisen *et al.*, 2002). Dhami and Sahni (1993) reported that the post-thaw quality of spermatozoa was superior with raffinose (1%) in Tris-based extender compared to other extenders in terms of release of lactate dehydrogenase. 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. It is feasible that extenders having ingredients of animal origin (egg yolk) can be the source of pathogens resulting in the contamination of semen (Bousseau *et al.*, 1998; Marco-Jimenez *et al.*, 2004; Ruigh *et al.*, 2006). In this regard, lecithin from non-animal source like soya needs to be trialed as a non-permeable cryoprotectant in extender for cryopreservation of buffalo spermatozoa.

Other additives

Attempts have been made to enhance the freezability of bubaline semen by adding different substance such as metabolic stimulants, detergents, antioxidants and chelating agents due to decrease the harmful effects of cryogenic procedures. Bhosrekar *et al.*, (1990) found that the effect of addition of caffeine or triethanolamine lauryl sulphate to Tris citric acid based extender and evaluated that the incorporation of the detergent upgraded the post thaw spermatozoa motility. Detergents have protective effects and may be exerted directly on the sperm membrane or emulsifying the egg yolk lipids which becomes immediately available to the plasma lemma during cryopreservation (Graham *et al.*, 1971; Arriola and Foote 1987; Buhr and Pettitt 1996). Singh *et al.*, (1996) found that incorporation of ascorbic acid (2.5 mM) in the buffalo bull semen extender yielded a significantly enhance the post-thaw motility and longevity. Although, Kolev (1997) reported that incorporation of 0.3 mg/ml of vitamin E in extender had exhibit better effects on acrosomal integrity, survivability and motility of cryopreserved buffalo bull spermatozoa and tocopherol (vitamin E) obstructs lipid peroxidation (LPO) in membranes, acting as a scavenger of lipid peroxy and alkoxy radicals, thus preventing oxidative damage in cryopreserved bovine

semen (Beconi *et al.*, 1991). Fabbrocini *et al.*, (2000) recommended that addition of 1.25 mM sodium pyruvate into the extender resulting in significantly improved post-thaw progressive motility and viability due to its antioxidant property during cryopreservation of buffalo semen. It is also reported that addition of oviductal proteins obtained from various stages of the oestrous cycle into extender affected the post-thaw semen characteristics due to lowered the LPO levels in buffalo spermatozoa during cryopreservation (Kumaresan *et al.*, 2006). Recently, Shukla and Misra (2007) studied that addition of Bradykinin (2 ng/ml) in Tris-based extender to improve post thaw buffalo semen characteristics during cryopreservation. Ijaz *et al.*, (2009) found that the Butylatedhydroxytoluene (BHT) at concentration 1.0 and 2.0 mM in tris citrate egg yolk extender provided better results for cryopreservation of buffalo bull semen. From the above cited studies, the incorporation of some additives in buffalo semen extender in terms of improvement in the quality of frozen– thawed buffalo spermatozoa.

Osmotic pressures

Semen dilution is a prime importance to maintain fertilizing potential of cryopreserved buffalo semen (Rasul *et al.*, 2000). In the extender the concentration of the solutes become high, the osmotic pressure is elevated. So, number of solute particles affects the osmotic pressure as well as freezing point of the solvents in a solution. When spermatozoa are exposed to hyperosmolal solution, more extra cellular ice crystals are formed (Watson, 1995) and vice versa. Polyhydric alcohols and water have high osmotic permeability coefficient which increases the shifting of these substance across the plasma membrane of the spermatozoa during cryopreservation (Noiles *et al.*, 1993). Spermatozoa either swell or

shrink under hypotonic/ hypertonic solution (DU *et al.*, 1994; Gilmore *et al.*, 1996). Any change in the osmotic pressure during cryopreservation causing damage of plasma membrane of spermatozoa. Hence, osmotic pressure plays a crucial role in semen cryopreservation and affects the frozen semen quality (Khan and Ijaz, 2008). The buffalo bull semen have been found different osmotic pressures, 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 above cited studies, it is concluded that buffalo bull semen has osmotic pressure lower than cattle semen.

Semen processing

Preliminary processing

The semen collection station is commonly located near the semen laboratory and it is commonly suggested that semen should be processed as early as possible after collection. No changes have been recognized in morphology, motility and survivability of spermatozoa, if the semen was processed within 1 h of collection (Fabbrocini *et al.*, 1995). Generally semen dilution, having all compulsory constituents, is done at 30°C to 37°C. Vale *et al.*, (1991) suggested the ejaculates should be kept for 10 to 15 minutes in its own plasma, after sometimes semen of buffaloes. To prevent agglutination immediately added the diluents very soon after collection may and also maintain the motility of spermatozoa. On the other words, there is delay in semen processing immediately after semen collection, it should be diluted with suitable extender and stored at 5°C (Televi *et al.*, 1994).

Semen dilution

In case of buffalo semen freezing, one step or two steps dilution methods are being used.

Del Sorbo *et al.*, (1994) correlated both methods (one and two steps) using tris-egg yolk-based extenders and reported that the two-step method provide superior results with long 6 h equilibration, while one-step dilution method required shorter period 2 to 4 h equilibration time before freezing. Fabbrocini *et al.*, (1995) reported that in two step dilutions method, addition of glycerol showed better sperm motility when glycerol was added 1 h before freezing. Del Sorbo *et al.*, (1995) also found two step semen dilution methods using sodium pyruvate (1.25 mM) with second dilution 1h prior to freezing the semen.

Cooling rates, equilibration time and freezing

After semen dilution, the semen is cooled at a temperature of 4 °C or 5 °C. Cooling is a necessary to spermatozoa to lowered metabolism. Diluted semen is cooled slowly to avoid cold shock. Cold shock is produced to impair function of membrane proteins that are necessary for structural integrity or ion metabolism of spermatozoa (Watson 2000). During freezing, buffalo spermatozoa are more susceptible to cryoinjury than cattle spermatozoa (Raizada *et al.*, 1990). These cryoinjury can be lowered by optimizing the cooling and freezing rates and using suitable diluting media (Kumar *et al.*, 1992). Talevi *et al.*, (1994) studied both slow and fast cooling protocols in buffalo bull semen and found the slow cooling (28°C to 5°C in 1 hour) and rapid cooling (28°C to 5°C in 15 minutes) protocols had no significant differences in post-thaw motility of spermatozoa. Mazur *et al.*, (1972); and Mazur, (1977) found that spermatozoa are exposed to high salt concentration and osmolality with changes in PH during slow cooling process, While during fast cooling, intracellular water may not pass out of membrane, resulting in intracellular ice crystals formation. Sahni and Mohan (1990)

reported that no significant difference on post-thaw sperm motility between these two cooling rates. In terms of other study, Sukhato *et al.*, (2001) found that cooling rates of 20 or 30°C / min yielded better progressive post thaw motility and fertility using the effects of three cooling rates (10, 20 or 30°C / min) from 4°C to each of either -40°C, - 80°C or - 120°C before plunging into liquid nitrogen. Anzar *et al.*, (2010) worked at different cooling rates for buffalo bull spermatozoa and reported higher motility, plasma membrane integrity and acrosomal morphology at high freezing rates of -30°C/ minute. The beneficial effects of slow cooling rate of diluted semen are considered at 5°C due to rapid penetration of glycerol in the cell membrane (Ennen *et al.*, 1976; Gilbert and Almquist, 1978). However investigators prefer slow cooling rate of 0.2 to 0.4 °C/minute for the pre-freezing processing of the buffalo bull semen.

Researchers found different equilibration durations during cryopreservation. Regarding equilibration, it is generally called the 'glycerol equilibration' period, during this stage; glycerol rapidly penetrates into the spermatozoa to establish equilibrium between its intracellular and extracellular concentration and other osmotically active extender components (Salamon and Maxwell, 2000). Singh *et al.*, (1989); and Del Sorbo *et al.*, (1995) recommended short equilibration periods of 2 to 4 h while another investigators favored longer duration of about 6 h (Haranath *et al.*, 1990; Televi *et al.*, 1994). It is commonly trusted that semen should be kept at 5 °C for not less than 2 h and not more than 6 h before freezing for cryopreservation of buffalo bull semen.

Cassou (1964) reported that semen is filled and sealed commonly at 5 °C in the straws of 0.25 ml or 0.5 ml capacity. Straws of 0.25 ml are generally 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°C.

Thawing rates and temperature

Different thawing temperature and time were used by the researchers. Mazur, (1984) found that fast thawing of semen prevents formation of recrystallization of water. Rao *et al.*, (1986) compared two thawing rates (37°C for 30 s and 75°C for 9 s) and found that the best value for post-thaw motility was observed for semen thawed at 37°C for 30 s. Dhami *et al.*, (1996) reported different thawing rates at 4 °C for 5 min, 40 °C for 1 min or 60 °C for 15 s and found that thawing of semen at 60 °C for 15 s yielded high post-thawing spermatozoal recovery and longevity. El-Amrawi (1997) worked at different thawing procedure and got best fertility rates when semen was thawed at 35°C for 60 seconds.

Future directions

Future research should highlight on freezing protocols improvement for lower spermatozoa damage during buffalo bull semen cryopreservation. To achieve this goal, diluents composition, freezing protocols along with the improvement 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. Therefore, a better understanding of the fundamental principle of cryopreservation of buffalo spermatozoa is necessary according to the specific requirements. Moreover, there is a need to develop biochemically defined extenders and cryogenic procedures that may result in improvement in viability and fertility of frozen thawed buffalo spermatozoa.

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