CRYOPRESERVATION OF BUFFALO (*BUBALUS BUBALIS*) SEMEN: CURRENT STATUS AND FUTURE PROSPECTIVE

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ABSTRACT

Most progress in improving reproductive efficiency of buffalo can be made by accurate estimation of the fertility of males and their selective use. Because of the major impact of individual males on multiple pregnancies and the ability to estimate fertility of males, more emphasis is placed on evaluating males. Spermatozoa were the first mammalian cells to be cryopreserved successfully due to the serendipitous discovery of the cryoprotective effect of glycerol. The development of cryopreservation protocols for the bull to be used for AI in the dairy industry began in the 1950s. Cryopreservation of semen has permitted the rapid expansion of reproductive technology such as artificial insemination. Advances in the reproductive biotechnology particularly cryopreservation of semen paved the way for better freezing of buffalo semen which was earlier considered as problematic. However, cryopreservation induces damage to all sperm compartments. Moreover, there is also variable degree of morphological, physiological and biochemical alterations in remaining population of live spermatozoa making them unsuitable for optimum fertility. Till today, there is no single objective test to evaluate the fertility of bull. The development of fertility markers to identify bulls of high breeding values at young age represents a remarkable way for achieving genetic gain in dairy farming. This review summarizes the developmental path of semen cryopreservation in buffalo along with the recent development in this field and critical gap therein.

Keywords: *Bubalus bubalis*, buffaloes, cryopreservation, semen, prospect

INTRODUCTION

The world buffalo population is estimated 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

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population (DAHDF, Govt. of India, 2015). Due to the importance of buffalo for the production of milk, meat and leather, it has received significant attention regarding the improvement of breeding practices particularly semen cryopreservation and artificial insemination. Advances in the reproductive biotechnology particularly cryopreservation of semen paved the way for better freezing of buffalo semen (Sansone G *et al*., 2000; Singh *et al*., 2013a) which was earlier considered as problematic. Cryopreservation of semen, permitting the rapid expansion of artificial insemination, has inherent limitations (Singh *et al*., 2014). The fertility of frozen semen remains poorer compared to fresh semen and is recorded to be 33% in buffalo (Chohan *et al*., 1992). It is generally accepted that up to 50% of sperm viability is lost during cryopreservation (Watson *et al*., 1995) which might be a possible mechanism involved in lower fertility of frozen semen (Anzar *et al*., 2002). Cryopreservation induces detrimental changes to all sperm compartments (Bailey *et al*., 2000; Medeiros *et al*., 2002). During cryopreservation, sperm cells experience both intracellular and extracellular stresses resulting in membrane perturbations, membrane lipid/ protein reorganization and osmotic changes across the membrane (Mazur *et al*., 1984). Moreover, variable degree of morphological, physiological and biochemical alterations in live spermatozoa following storage at ultra-low temperatures makes these germ cells unsuitable for optimum fertility (Holt, 2000).

Buffalo spermatozoa due to its unique cell membrane composition are more susceptible to cryodamage during freezing than cattle spermatozoa (Raizada *et al*., 1990). However, the buffalo semen cryopreservation still faces paucity of information about physiological mechanisms involved in the sperm cell damage. This review summarizes the recent advances in buffalo semen cryopreservation technology and critical gap therein.

Evaluation of semen quality parameters (SQP) of buffalo semen

Semen samples are collected from trained buffalo bulls by using an artificial vagina maintained at temperature between 40°C and 42°C and the pressure within the AV should be adjusted to suit individual bulls. Intact or castrated male buffalo is used as a dummy to avoid the disease transmission. Sperm concentration is increased by allowing two or three false mounts before actual collection. The semen is collected early in the morning, before feeding, within a week there are two collections per bull and each collection consists of two ejaculates taken within a minimum interval of 30 minutes. The semen quality parameters (SQP) generally examined are: volume, colour, consistency, concentration, mass activity, individual progressive motility, viability, acrosomal and membrane integrity, and percentage of abnormal spermatozoa. Till date there is no single test which can be relied upon for accurate semen quality. Moreover, the correlation between fresh semen quality and post-thaw semen quality is highly variable. Den Daas (1992) reported that the relationship between the semen characteristics and fertility has often been inconsistent. For assessment of semen quality whether at fresh or post-thaw stage, temperature of laboratory and other semen handling equipment should be between 33°C to 37°C.

Volume

The volume of the buffalo semen is measured immediately after collection as ejaculates is collected in graduated tubes having conical bottom. It varies, depending on breed, age,

season and stimulus provided to the bull. Volume of the ejaculates varies from 1 to 6 ml (low during summer month) although exceptional bulls can give up to 11 ml semen (Misra *et al*., 1994; Shukla and Mishra, 2005; Ravimurugan *et al*., 2008).

Colour and consistency

The colour of semen is milky white to creamy whereas consistency varies from milky white to thick creamy which varies with the concentration of spermatozoa. Any departure from above should be recorded as abnormal ejaculates and that should be discarded (Dhami and Kodagali, 1990). Kumar *et al*. (1993a) found significant differences in viscosity of ''static'' and ''motile'' ejaculates, but not between different bulls. Static ejaculates are one in which mass motility is zero with watery consistency but sometime revives after dilution with extender and incubation at 37°C.

Mass activity/ Mass motility

Mass activity of semen is one of the most common seminal parameter used for assessing its quality at the time of collection due to its simplicity (Mishra and Tyagi, 2006).Mass activity is assessed under microscope at 10X without cover slip. Mass motility is significantly and positively correlated with the spermatozoa concentration, initial motility and live spermatozoa count (Patel *et al*., 1989). Mass motility reported in buffalo bull semen were 3.50±0.10 by Patil (1981); 2.53±0.24 by Vyawanare *et al.* (1989) and 3.62±0.04 by Shukla and Misra (2005). Variation in mass activity is reported to be due to climatic factors (Bhosrekar *et al*., 1994; Javed *et al*., 2000), frequency of semen collection (Sayed and Oloufa, 1957) and age of bulls (Collins *et al*., 1951).

Individual progressive motility

Mammalian sperm develop the capacity for motility during their epididymal transit (Bedford, 1975). Individual progressive motility is assessed by microscopic observation of the percentage of motile spermatozoa and most commonly this is the single most test to see the quality of semen at fresh or post-thaw stage. A small drop of semen is placed on a dry slide maintained at 37°C, and examined at a magnification of 40X. The advent of computer-aided semen analysis CASA has enabled to objective assessment of the sperm motility. The CASA system can evaluate parameters like total sperm motility (TM), progressive motility (PM), straight line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP) speed, direction and the beat cross frequency of sperm cells. The individual progressive motility reported in buffalo bull is 72% to 83% (Shukla and Mishra, 2005). Singh *et al*. (2014) reported individual progressive motility in freezable (81.15±1.26) and non-freezable (80.69±1.90) semen of buffalo estimated by Semen Quality Analyser (SQA-Vb).

Spermatozoa concentration

Most routine measurements of concentration are made by haemocytometer, spectrophotometer, photometer or by automated machine like SQA-Vb. The mean spermatozoa concentration (millions/ml) in freezable (487.74±57.02) and non-freezable (438.23±57.97) semen was reported by Singh *et al*. (2014). Whereas other workers reported as 1316.00±44.00 million/ml (Bedi *et al*., 1984); 1123.5±60 million/ml (Ratan, 1990) and 1046.64±34.21 (Shukla and Misra, 2005) in buffalo semen. The difference in spermatozoa concentration might be due to managemental practices such as restraint before collections, frequency of semen collection,

seasonal variations, and age of bulls and methods of spermatozoa concentration estimation (Tomar *et al*., 1966; Singh *et al*., 2012).

Spermatozoa viability

The percentage of live spermatozoa determines the quality of the ejaculate. Ejaculates with more than 30% initial dead spermatozoa may not be suitable for freezing and generally discarded. Differential staining techniques using Eosin-Nigrosin stain have been used for determination of live and dead spermatozoa (Lasley and Bogart, 1942). Now-a-day's fluorescent dyes like propidium iodide (PI) and fluorescein-labeled *Pisum sativum* agglutinin (FITC-PSA), PI and Sybergreen-14, Hoechst 3342 are used to analyze sperm viability as these dyes are nucleophillic and bind with DNA (Garner and Jhonson, 1995; Singh *et al*., 2015). The live percent of spermatozoa depends on the factors like age of the bulls (Javed *et al*., 2000), temperature (Chandra *et al*., 1999), frequency of semen collections, sexual excitement before collection (Badaway *et al*., 1973).

Abnormal spermatozoa

Abnormal spermatozoa are detected by staining methods (Eosin-Nigrosin stain) and are usually classified as head, middle-piece and tail abnormalities (Kumar *et al*., 1993a). In semen of Nili-buffaloes, most abnormalities are found on sperm heads (2.1%) while middlepiece abnormalities were less than 1% and abnormal tails varied from 3.92% to 5.7 %. Occurrence of cytoplasmic droplet is less than 1% (Saeed *et al*., 1990). Similar proportions of abnormalities were observed in semen of Murrah buffaloes (Kumar *et al*., 1993a). The latter suggested that semen showing over 15 to 20% abnormal spermatozoa should be examined for their fertility. Sperm abnormalities were found to be higher during summer followed by rainy and winter seasons (Bhakat *et al*., 2015). Manik and Mudgal (1984); Bhosrekar *et al*. (1991) reported higher abnormality during winter season.

Acrosomal and membrane integrity

The acrosomal integrity of mammalian spermatozoa is prerequisite for capacitation, normal acrosome reaction and successful fertilization *in vivo*. The enzymes localized in the acrosome determine the sperm penetrating and fertilizing capacity. The most commonly used staining method to detect acrosomal changes, based on staining intensity and contrast of background is Giemsa stain (Watson, 1975). Flourescent dye, Fluorescine Isothiocynate (FITC) conjugated with *Pisum sativum* agglutinin (PSA) or *Peanut* agglutinin (PNA) now-a-days is used to assess the intactness of acrosome. Singh *et al*. (2014) reported per cent acrosomal intactness of spermatozoa between freezable (84.63±1.14) and non-freezable (84.04±1.19) semen at fresh stage.

Hypo-osmotic swelling test (HOST) is used (Jeyendran *et al*., 1984) to evaluate the functional integrity of the sperm membrane. The swelling pattern and swelling of sperm tail is observed by counting 200 cells for each sample at 400× magnification under a phase contrast microscope. The principal is based on the fact that volume of sperm cell increase due to osmotic uptake of water, cells become more spherical without a significant increase in its area then the flexor motor apparatus of the sperm tail is forced by cell membrane to bend or coils (Prasad *et al*., 1999; Singh *et al*., 2013b).

Seminal plasma protein

Most of the proteins of seminal plasma are secretory products of the seminal vesicle (Aumuller *et al*., 1988; Chandonnet *et al*., 1990; Dostalova *et al*., 1995). Seminal plasma proteins influence

various functions of the sperm such as capacitation, acrosome reaction, motility, DNA integrity and interaction with the oocyte (Moura *et al*., 2007). The seminal plasma proteins are significantly and positively correlated with sperm concentration and sperm motility (Saxena and Tripathi, 1979), morphology and fertility (Pangawkar *et al*., 1988). Proteins are also known to be of great importance for the motility and the survival of spermatozoa during storage (Singh *et al*., 2014). Proteins, designated as BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa, are secreted by the bovine seminal vesicles and are dependent on androgen level for their synthesis (Aumuller *et al*., 1988). It has been established that PDC-109 (BSP-A1/A2) in seminal plasma is beneficial during fertilization in female reproductive tract by inducing capacitation, zona binding and acrosome reaction (Therien *et al*., 1998), hyper-activation (Sanchez-Leungo *et al*., 2004) and by forming oviduct sperm reservoir (Gwathmey *et al*., 2003). Level of PDC-109 was significantly higher in non-freezable as compared to freezable semen of buffalo bull indicating its specific deleterious effect in cryodamage (Singh *et al*., 2014). The electrophoretic pattern of seminal plasma on polyacrylammide gel SDS-PAGE may be used to identify the normal physiology of the reproductive glands, and thereby to select the donor bulls whose semen could be used for artificial insemination. The level of HBPs in freezable (1.15±0.043 mg/ml) was found significantly (P<0.01) lower than non-freezable (2.18±0.06 mg/ml) samples. Similarly, the level of PDC-109 in freezable (0.532±0.026 mg/ml) was found significantly (P<0.01) lower than non-freezable (1.22±0.52 mg/ml) samples in buffalo seminal plasma (Singh *et al*., 2013a; Singh *et al*., 2014).

Biochemical characteristics of semen

The semen is provided with a defense of natural antioxidant systems which consist of enzymes like Catalase (CAT), Superoxide Dismutase (SOD), reduced Glutathione (GSH) , Glutathione Peroxidase (GSH-Px) (Gadela *et al*., 2004), and non-enzymatic antioxidant system like (vitamin C, E, cysteine, glutathione). These safeguard the sperm from ROS mediated cryo-injuries (Garg *et al*., 2008). The SOD activity in fresh spermatozoa is varied from 14.34 to 29.34 IU/10^9 spermatozoa. The Catalase activity was varied from 0 to 3.8 IU/10^9 spermatozoa with mean value of (0.80±0.01). The GSHPx activity level was varied from 3.80 to 13.20 nmol/NADPH oxidized per min/10^9 sperm in fresh semen. Similarly, the GSH activity is 23.46±0.87 nmol/10^9 sperm in fresh semen (Kadirvel *et al*., 2014). Zinc concentration in spermatozoa and seminal plasma is one of the regulating mechanisms of cations between the intra and extra cellular compartment, and hence it may affect the sperm metabolism and motility (Ahmed and El Tohamy, 1997). Vijayasaraswathy *et al*. (2014) demonstrated the presence of an array of proteases and APA in buffalo semen and the activities of which changed during cryopreservation. The leakage of the specific protease activity and changes in the proteases and APA might be attributed to reduced motility and fertility of cryopreserved semen in these species. Shukla *et al*. (2009) reported the averages of the calcium, chloride, inorganic phosphorus, potassium, magnesium, sodium, bicarbonate, total cholesterol, citric acid, and GOT in the seminal plasma 44.95+0.96 (mg %), 366.73+53.69 (mg %), 6.82+1.63 (mg %), 98.18+11.67 (mg %), 6.61+0.49 (mg %), 106.46+11.92 (mg %), 9.49+1.26 (milliequivalents per liter), 53.67 8.72 (mg %), 499.09+18.46 (mg %) and 87.45+13.52

(Units ml^{-1}).

Other tests

The sperm chromatin stability assay (SCSA) is the commonly used assay to determine the stability of sperm chromatin (Martínez-Pastor *et al*., 2010). For the SCSA, the sperm are treated with an acid, which may result in the exposure of single-stranded DNA, indicating instable chromatin, whereas stable DNA remains double-stranded. When the added dye acridin-orange binds to single-stranded DNA, the sperm fluoresces red (>630 nm) when it binds to double-stranded DNA, green light (530±30 nm) is emitted (Martínez-Pastor *et al*., 2010). Another DNA assay used with fluorescence or light microscopy and flow cytometry is the terminal transferase dUTP nick end labeling (TUNEL) assay that enables the detection of different degrees of DNA denaturation (Martínez-Pastor *et al*., 2010). The changes in membrane fluidity are measured with the lipid dye merocyanin 540 which is attached to a fluorescent probe like YO-PRO-1 or Hoechst 33342 (Hossain MS *et al*., 2011). Hoechst 33342 binds specifically to DNA and is able to detect all sperm regardless of their membrane status. Changes in the intracellular calcium level that occur during capacitation is assessed with chlortetracycline, Indo-1 AM or fluo-3 AM (Graham and Mocé, 2005). Apoptotic like changes that are probably caused by cryopreservation is detected by apoptotic markers like Annexin V, YO- PRO -1 iodide and others (Martínez-Pastor *et al*., 2010). Oxidative stress and lipid oxidation is assessed by measuring reactive oxygen species (ROS) or superoxide anion levels by specific fluorescence dyes as 2′,7′-dichlorodihydrofluorescin or dihydroethidium, respectively.

Factors affecting quality of buffalo semen

Wide variations exist in properties of semen among different bulls as well as different ejaculates of the same bull. In buffalo, lesser volumes of semen with its seasonal influence, poor freezability and static or zero ejaculates are major limiting factors in wider application of cryopreservation technology. A number of physical and chemical changes occur during the collection and processing of the semen in the laboratory, which alter properties of bull semen. Among different seasons hot-dry and hot humid seasons reported to be unfavorable for production as well as reproduction. The season affects the animal directly through macro and micro climatic factors, like temperature, humidity, rainfall and photoperiod. Indirectly it acts by affecting the vegetation, forage quality and soil-plant-animal interaction. The magnitude of variations differs from breeds, location, prevailing climatic conditions, feeding and general management (Mandal *et al*., 2000). Luteinizing hormone responsible for spermatogenesis is inhibited by increasing level of plasma corticosteroids due to heat stress. During summer, thyroxin secretion declines leading to reduced intake of feed by the animal, subsequent metabolism and is responsible for reduction in sperm production. Due to extreme heat stress bulls get physically exhausted and their reduced libido might result in higher reaction time (Mandal *et al*., 2000). Koonjaenak *et al*. (2007) reported that, climatic changes did not seem to largely affect sperm output or viability in swamp buffalo bulls in Thailand. Although the proportions of tail abnormalities were affected by season, they were always below what is considered unacceptable for AI bull sires. Some research workers had reported ill effects of heat stress (Bhosrekar *et al*., 1992), while others observed (Bhat *et al*., 2004) similar findings during the winter season; whereas such

effects had been reported in the spring season by Sengupta *et al*. (1963). Ravimurugan *et al*. (2003) reported the best quality of semen during monsoon season in Murrah buffalo bulls.

Another influencing factor is the age of the buffalo bull. Semen of best quality, with regard to sperm morphology, was observed in 3 to 5 years old Murrah buffalo (Kumar *et al*., 1993a). Saeed (1988) reported the best quality semen at 3 to 4 years of age. He concluded that the age of the bull and season of the year significantly affect semen characteristics but that variations in these parameters do exist even in the same season and at same age in different localities.

The problems of spermatozoa sensitivity to processing and freezing that differ significantly among ejaculates of the same bull still needs to be elucidated (Andrabi, 2009). Also, quality as well as freezability of spermatozoa is influenced by seminal plasma (Jobima *et al*., 2005) containing several proteins many of which are secretory products of epididymes and seminal vesicles (Chandonnet *et al*., 1990; Singh *et al*., 2014). The addition or removal of SP protein(s) during epididymal maturation and at ejaculation of spermatozoa have an important role in the maintenance of plasma membrane stability (Desnoyers and Manjunath, 1992), motility (Henricks *et al*., 1998; Sanchez-Luengo *et al*., 2004), capacitation (Therien *et al*., 1998), sperm-egg interaction and fertilization (Yanagimachi, 1994). PDC-109 is known to increase sperm motility (Sanchez-Luengo *et al*., 2004), capacitation and acrosome reaction (Therien *et al*., 1998) and helps in formation of oviductal sperm reservoir (Gwathmey *et al*., 2003). However, continuous exposure of sperm to PDC-109 protein, as in modulated semen or during protracted storage causes an efflux of cholesterol and phospholipid from the plasma membrane (Singh *et al*., 2014). This renders sperm very sensitive to storage in liquid or frozen states (Manjunath *et al*., 2007).

Cryopreservation of semen extender

Semen extenders composition is an important factor influencing freezability of semen. The role of extenders is to protect spermatozoa from cold shock and agglutination; maintain stable pH and osmotic pressure (Garner *et al*., 2001; Chen *et al*., 1993). Extenders containing tris, sodium citrate, egg yolk, glycerol, sugar etc in different proportions have been reported (Arthur *et al*., 1983; Samad, 1985). Extenders are based on a particular buffer, which provides the best results for a given species (Hopkins and Evans, 1989). Buffers play an important role in stabilizing the pH of extenders due to the products of metabolism in stored sperm. Carbohydrates (sugars) are necessary to provide the energy required for sperm glycolysis, egg-yolk based fractions provide phospholipids necessary to promote membrane stabilization at lower temperature and limit premature acrosomal membrane activation. Since the discovery of egg yolk as a protectant for cryopreservation of spermatozoa, it has been widely used to cryopreserve sperm of a variety of species (Phillips and Lardy, 1940; Watson, 1995). The protective agent in the egg yolk is believed to be the low-density lipoprotein (LDL) fraction. The osmotic pressure of 285 to 295 mOsm/kg of extender is considered to be most suitable for most of the spermatozoa characteristics for the cryopreservation of buffalo bull semen (Mughal *et al*., 2013).

In various studies tris-fructose-egg yolk, citrate-egg yolk and lactose-egg yolk diluents did not differ in their ability to protect spermatozoa against freeze-thawing damages (Chinnaiya and Ganguli, 1990; Raizada *et al*., 1990; Dhami *et al*.,

1994). However, the tris-based diluent yielded higher post-thaw motility compared with the other two media (54.1%, 46.5% and 41.6%) respectively. Both tris- and lactose-based media were superior to the milk diluent as determined by post-thaw motility (Akhtar *et al*., 1990). In other in vitro comparisons of diluents, based on post-thaw motility of spermatozoa, milk was better than tris (Galli *et al*., 1993) or both these media performed better than the citrate-based extender Kumar *et al*. (1992a). Tes Hydroxymethyl aminoethan is another buffer used for freezing buffalo semen, and proved to be of equal value to tris- and glycine-based diluents (Oba *et al*., 1994; Chachur *et al*., 1997).

Due to various limitations like wide variability of egg yolk composition (Forouzanfar *et al*., 2010), risk of microbial contamination (Aires *et al*., 2003), presence of high-density lipoproteins (Pace and Graham, 1974), steroid hormones (Lipar *et al*., 1999), and variability in composition of egg yolk from batch to batch there is demand for replacement of egg yolk in extenders. Now-a-days there are numerous synthetic defined media are available in market for semen cryopreservation. The alternative to egg yolk-based extender (EY) can be soya lecithin based extender (SL) and liposome-based extender (LP).The composition of many commercial extenders such as Laicipfos 478, Biociphos PLUS, TRYLADIL Konzentrat, BULLXcell, AndroMed and Bioxcell is not revealed, and they are presented to the consumer only by indicating the methods of their preparation (Kumar *et al*., 2015). In nutshell, tris-fructose-citric egg yolk extender has still been widely recommended for routine use to cryopreserve buffalo bull semen. However, the freezability and fertility rates with cryopreserved buffalo semen are lower using tris citric egg yolk extender compared to fertility rates using cryopreserved semen in cattle (Akhter *et al*., 2010).

Cryoprotectant

For sperm to survive freezing, they need to be extended in a diluents that contains substance that protect them against cold shock but also cryoprotectant, such as glycerol (Polge *et al*., 1949). Cryoprotecive agents are classified as those that enter the ell (permeating e.g. glyceeol and DMSO) or that are retained in the extracellular fluid (non-permeating e.g. disaccharides or proteins). The most effective way of protecting sperm against the detrimental effects of cooling is by the inclusion of egg yolk or milk in the diluents. The lecithin and lipoprotein contents in egg yolk contribute to the preservation of the lipoprotein sheath of the sperm cell (Kumar *et al*., 1992a). However, in addition to its protective action against cold shock, the egg yolk also stimulates the enzyme system of spermatozoa. Little attention has been paid to the level of egg yolk necessary for freezing buffalo semen, and the majority of workers are using concentrations around 20%. It should be noted, however, that as the yolk concentration is increased in the diluent, the pH of the medium decreases and tends towards the acidic side. This may also be the reason for the depressing effect of higher amounts of yolk on motility of thawed spermatozoa. The toxic effect of egg yolk may be combined with toxicity of dead spermatozoa (Shannon, 1972). Sahni and Mohan (1990) examined various levels of egg yolk in a tris-glycerol-based freezing diluent and found that egg yolk beyond 5% did not show any significant improvement in post-thaw motility. This indicated the scope of reducing the yolk level from 20% to 5% without adversely affecting the freezability of buffalo semen.

Glycerol is generally added to the extender

for freezing buffalo semen in concentrations from 6% to 7%, but attempts have been made to reduce the amount, or substitute it with other cryoprotective substances. Reduction of glycerol to 3% or 2%, and concentrations above 7% decreased the post-thaw motility of spermatozoa in the diluents tested (Ramakrishnan and Ariff, 1994; Nastri *et al*., 1994). Glycerol stabilizes lipid membranes by hydrogen bonding with the polar head groups of membrane lipids, which is especially important under severely dehydrated conditions. In addition, these substances may affect the mechanical properties of the unfrozen fraction, especially its viscosity and glass-forming tendency. Glycerol is a freezing point depressing compound which helps prevent ice and solute damage to the cells during freeze-thaw process. Glycerol at the final recommended level is slightly toxic to the sperm if added in one step, so the glycerolated extender is mixed gradually with the semen over 1 h. After the final addition, the semen is ready for packaging. Kumar *et al*. (1992b) found that the protective effect of sugars depend on the type of diluent used for freezing. In milk diluent, fructose and sucrose gave the best protection, as judged by post-thaw motility results, due probably to the synergistic action of lactose present in milk. Some protection to spermatozoa was given by the sugars at 2% concentration in tris diluent, and none in citrate-based medium. The high molecular weight sugars may play a cryoprotective role by altering the permeability of the cell membrane and by maintaining the electrolyte balance. However, the best post-thaw motilities is never higher than 30% when sugar is used as the sole cryoprotectant.

Antibiotics and additives

In addition to the infective agents transmittable by reproduction, as reported by the World Organization for Animal Health (OIE, 1996), there are several other pathogens and saprophytic bacteria that may be able to contaminate bull semen. The bacterial contamination of semen can occur at any time during the production of the insemination doses, but the main contamination coincides with collection. The reproductive tract is not a sterile environment, since several types of bacteria have been found in healthy males. Accordingly, semen is frequently contaminated by bacteria that survive the storage temperature of -196^{o}C in liquid nitrogen and therefore acquire a level of resistance to antibiotics (Ronald and Prabhakar, 2001). Antibiotics are added to most semen diluents as a prophylactic measure against the transmission of pathogenic bacteria and to reduce the load of non-pathogenic organisms that contaminate the semen (Noakes *et al*., 2009). Penicillin 1000 IU per ml and streptomycin sulphate 1.0 mg/liter in combination are commonly added to freezing diluents. Aleem *et al*. (1990) found that a combination of penicillin and neomycin is more effective than the combination of penicillin and streptomycin currently used. Concern over the potential transmission of Mycoplasma and Ureaplasma species in buffalo semen has led to the incorporation of lincomycin and spectinomycin into semen diluents in an efforts to control these organisms.

Dhami and Sahni (1993); Dhami *et al*. (1994) examined the effects of raffinose (1%) L-cysteine (0.1%) and EDTA (0.1%) in the generally used freezing media (tris-, milk- citrate-based diluents) for buffalo semen. The beneficial effects of raffinose on post-thaw motility and the fertility rate were observed only in the citrate buffer which did not contain other sugar. L-cysteine and EDTA improved the fertility rate, compared to raffinose or the control extenders without additive. This was attributed to significantly better progressive

motility of spermatozoa when chelating agents cysteine and EDTA were present in the diluent. Citric-whey extender supplemented with 0.1% and 0.2% cysteine increased post-thaw sperm motility, particularly at the higher (0.2%) concentration (Singh *et al*., 1990). Sodium pyruvate, an energy source that can be readily used by the cells, had a beneficial effect on post-thaw sperm survival only when added 1 h before freezing to citrate-yolk-glycerol diluent (Del Sorbo *et al*., 1995). Caffeine was found to stimulate motility of buffalo spermatozoa (El-Menoufy *et al*., 1985). Incubation of spermatozoa with CLC prior to processing improves frozen-thaw semen quality in bull (Purdy and Graham, 2004), equine (Moore *et al*., 2005), ram (Moce *et al*., 2010) and buffalo (Kumar, 2012; Rajoriya *et al*., 2016).

Processing of semen for cryopreservation

The breeding farms are generally located near the semen laboratory and it is generally recommended that semen should be processed as early as possible after collection. It is a general practices in most of the semen laboratory to mix the semen of all the bulls after mixing in diluents. No changes have been observed in motility, morphology or freezability, of spermatozoa, if the semen was processed within 1 h of collection (Fabbrocini *et al*., 1995). Vale *et al*. (1991) recommended keeping the ejaculate in its own plasma for 10 to 15 minutes, although semen of some buffaloes may show agglutination. In this case, addition of the diluent immediately after collection could prevent the semen from irreversibly agglutinating and also maintain the motility of spermatozoa. It has been observed that in case of static ejaculate, after addition of diluents it regained its motility.

Now a day semen is diluted in single step in diluents which is already premixed with final concentration of glycerol to a final concentration of about 100 to 150×10^6 sperm/ml. When the two-step method is adapted, the second diluent portion has a higher cryoprotectant concentration than the first portion. Del Sorbo *et al*. (1994) examined both methods using tris-egg yolk-based extenders, and found that the two-step method gave better results with long 6 h equilibration, while the extenders used for one-step dilution required shorter 2 to 4 h equilibration time before freezing. After dilution of semen, it is packaged in straws and then kept at 4ºC for four hours for equilibration. There is no agreement among investigators regarding the duration of equilibration. Some suggested short 2 to 4 h periods (Singh *et al*., 1990; Dhami and Sahni, 1994; Del Sorbo *et al*., 1995), while others recommended longer duration of about 6 h equilibration (Rao *et al*., 1990; Dhami and Kodagali, 1990). It is a general belief that buffalo semen should stand at 5°C for not less than 2 h and no longer than 6 h before freezing.

Freezing and thawing

After equilibration, the semen packaged in mini straws (0.25 ml) or midi straw (0.5 ml) is frozen in liquid nitrogen vapour. Mini straws are generally used due to their better freezability, cost effectiveness and saving of storage space. Haranath *et al*. (1990) found an improvement in conception rate for semen frozen in mini straws as compared to that frozen in medium straws (52.7% vs. 50.4%). Freezing in liquid nitrogen vapour is practical and can be done by using a simple isotherm box. The straws are suspended in horizontal position 4 cm above liquid nitrogen for 10 minutes, after which they are immersed into liquid nitrogen at 140°C. The advances in semen cryopreservation industry has leads to development of some of the finest technology, one of them is programmable

freezer which cryopreserve the semen from 4°C to -196°C in a step wise manner at controlled rate which eliminates the error of manual system. In this system the rack along with the straws was transferred to Biological Cell Freezer for automated freezing. The freezing in Biological Cell Freezer was carried 5°C per minute for 4 to -10°C; 40°C per minute for -10 to -100°C and 20°C per minute for -100 to -140°C. Straws are then plunged into liquid nitrogen (-196°C) for storage until analysis (Singh *et al*., 2015).

Thawing of the semen is recommended at 37°C for 30 seconds after 24 h of freezing. To avoid recrystallization, rapid warming rates are commonly used. Dhami *et al*. (1994); Vale (1997) suggested thawing of straws in a water bath at 40°C for 30 seconds. Somewhat slower thawing rates were used by Kumar *et al*. (1993b) i.e. 37°C for 30 seconds and Fabbrocini *et al*. (1995) 39°C for 30 seconds. Although some workers state that the quality of buffalo semen frozen in straws can be improved by using a longer thawing time, Ziada *et al*. (1992) found no difference between thawing at 35°C for 30 seconds and at 50°C for 15 seconds.

Fertility of cryopreserved buffalo semen

Post thaw motility of 40% and above is generally accepted for further use in artificial insemination and below it is discarded (Singh *et al*., 2014). It is generally accepted that approximately 50% of buffalo spermatozoa are damaged during semen freezing process (Watson, 2000), which affects spermatozoa motility, acrosomal and chromatin integrity (Rasul *et al*., 2001; Mahmood and Ijaz, 2006; Khan and Ijaz, 2008) resulting in low conception rate. There has been a tremendous progress in the field of semen cryopreservation, but the conception rate to cryopreserved semen is around 33% (Chohan *et al*., 1992; Bhosrekar *et al*., 2001). The lower conception rate may be due to acrosomal damage (Akhtar and Chaudry, 1989), reduced post thaw motility (Tuli *et al*., 1981; Rasul *et al*., 2001) and altered membrane integrity (Rasul *et al*., 2001) of buffalo spermatozoa following cryopreservation. Loss in percentage motility, reduced plasma membrane integrity, decreased viable sperm count, and increased percentage of apoptotic sperm in buffalo due to freezing and thawing were reported by Khan *et al*. (2009).

A lower conception rate (33.0%) has been reported in buffaloes using cryopreserved semen (Bhosrekar *et al*., 2001). Buffalo spermatozoa are more susceptible to hazards during freezing–thawing than that of cattle spermatozoa and hence, the fertility rate of frozen-thawed buffalo spermatozoa is poorer than that of cattle spermatozoa (Chohan *et al*., 1992; Dhami *et al*., 1994). Several mechanisms have been attributed to the reduced fertility of cryopreserved semen, however, cryopreservation-induced capacitation-like changes in frozen thawed spermatozoa gained momentum recently (Watson, 2000; Cormier and Bailey, 2003; Thomas *et al*., 2006, Singh *et al*., 2014). Kadirval *et al*. (2009) established the relationship among capacitation status, cholesterol level, membrane fluidity and intracellular calcium in frozen-thawed buffalo spermatozoa.

The quality of post-thawed spermatozoa is affected by oxidants like reactive oxygen species (ROS) (Alvarez and Storey, 1992). These substances are also produced physiologically in living cells during respiration (Bamba and Cran, 1992) as well as by abnormal or dead sperm and phagocytic cells of both the ejaculate and the female reproductive tract (Killian, 1989). Reactive oxygen species decrease the sperm quality and render it incapable of fertilizing the oocyte (Mammoto, 1996). They have deleterious effects

on sperm DNA and postfertilization development of male pronuclei (Aitken, 1993). Moreover, ROS were shown to inhibit the motility, capacitation, and acrosome reaction in sperm and that the inhibitory effects are mediated mainly by lipid peroxidation of spermatozoal membrane (Aitken, 1995). Spermatozoa contain high concentrations of polyunsaturated fatty acids, which are susceptible to lipid peroxidation due to oxidative stress induced by freezing and thawing. Moreover, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing (Alvarez and Storey, 1992).

CONCLUSION AND DISCUSSION

Artificial insemination is the most important single technique devised for genetic improvement of animals. It is impossible to control semen quality during natural service; there is always a risk of spreading infectious, diseases. Cryopreservation is the only method through which spermatozoa from genetically superior elite bulls can be preserved at -196^{o}C in the liquid nitrogen. The frozen semen can safely and easily be transported to the remotest parts of the world. However, cryopreservation induces damage to all sperm compartments. Moreover there is also variable degree of morphological, physiological and biochemical alterations in remaining population of live spermatozoa making them unsuitable for optimum fertility. Even with the best available preservation technique, post thaw survival of sperm population is approximately 50%. Sperm cryopreservation is a multi-factorial problem, where the diluents, the protocol, the species, breed and individual sires within each breed are some of the many parameters that need to be included in the evaluation for the success of cryopreservation. Till date there is no single objective test to evaluate the fertility of bull. The search is now on for finding the molecular markers of fertility. The development of such markers to identify bulls of high breeding values represents a remarkable way for achieving genetic gain in dairy farming.

REFERENCES

Aires, V.A., K.D. Hinsch, F. Mueller-Sxhloesser, K. Bogner, S. Mueller Schloesser and E. Hinsch. 2003. *In-vitro* and *in-vivo* comparison of egg yolk based and soybean lecithin based extenders for cryopreservation of bovine semen. *Theriogenology*, **60**: 269-279.

Ahmed, W.M. and M.M. El Tohamy. 1997. Zinc profile in blood and semen ofbreeding buffalo bull with particular emphasis on age variation and semencharacteristics. *In Proceeding of 5th World Buffalo Congress*, Caserta, Italy. **1**: 825-828.

Aitken, R.J. 1995. Free radicals, lipid peroxidation and sperm function. *Reprod. Fert. Develop*., **7**: 659-668.

Aitken, R.J., D. Harkiss and D. Buckingham. 1993. Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J. Reprod. Fertil.*, **98**: 257-265.

Akhtar, T. and R.A. Chaudhry. 1989. Effect of different extender on extracellular release of hyaluronidase from buffalo bull semen. *Buffalo J*., **2**: 137-142.

Akhtar, T., R.A. Chaudhary, I.H. Khan and T.M. Khan. 1990. Extracellular release of hyaluronidase and acrosin from buffalo bull spermatozoa extended in different extenders. *Recent Adv. Buffalo Res.*, **3**: 75-79.

Akhter, S., M.S. Ansari, B.A. Rakha, S.M.H. Andrabi, S. Iqbal and N. Ullah. 2010. Cryopreservation of buffalo (*Bubalus bubalis*) semen in Bioxcell extender. *Theriogenology*, **74**: 951-955.

Aleem, M., R.A. Chaudhry, N.U. Khan, A.R. Rizvi and R. Ahmed. 1990. Occurrence of pathogenic bacteria in buffalo semen. *Buffalo J*., **6**: 93-98.

Alvarez, J.G. and B.T. Storey. 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J. Androl*., **13**: 232-241.

Andrabi, S.M.H. 2009. Factors affecting the quality ofcryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod. Domest. Anim*., **44**: 552-569.

Anzar, M., L. He, M.M. Buhr, T.G. Kroetsch and K.P. Pauls. 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biol. Reprod*., **66**: 354-360.

Arthur, G.H., D.F. Noakes and H. Pearson, 1983. *Veterinary Reproducti on and Obstetrics, 6th ed*. Bailliere Tindall, London. p. 569-575.

Aumuller, G., M. Vesper, J. Seitz, M. Kemme and K.H. Scheit. 1988. Binding of a major secretory protein from bull sseminal vesicles to bovine spermatozoa. *Cell Tissue Res*., **252**: 377-384.

Badaway, A.M., A.M. Yaseen, A.S. Elbashary and M.A. Ibrahim. 1973. Effect of sexual preparation on some characteristics of semen of buffalo and cattle bulls. *Alexandria. J. Agri. Res*., **21**: 185-191.

Bailey, J.L., J.F. Bilodeau and N. Cormier. 2000. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. *J. Androl*., **21**: 1-7.

Bamba, K. and D.G. Cran. 1992. Effect of treatment with butylated hydroxytoluene on the susceptibility of boar spermatozoa to cold stress and dilution. *J. Reprod. Fertil*., **95**: 69-77.

Bedford, J.M. 1975. Maturation, transport and fate of spermatozoa in the epididymis. *In* Hamilton, D.W. and R.O. Greep (ed.) *Handbook of Physiology*, American Physiological Society. Washington DC, USA. p. 303-317.

Bedi, K.S., R.K. Biswas and Y.G. Dugwekar. 1984. Fructolysis in semen and its relation to breeding performance in buffalo bulls. *Indian J. Anim. Reprod*., **4**: 60-63.

Bhakat, M., T.K. Mohanty, A.K. Gupta, S. Prasad, A.K. Chakravarty and H.M. Khan. 2015. Effect of season on semen quality parameters in murrah buffalo bulls. *Buffalo Bull.*, **34**(1): 100-112.

Bhat, V., T.G. Honnappa and B.M. Dubey. 2004. Seasonal effects on seminal attributes in Murrah bulls under Bangalore agroclimatic conditions. *Indian J. Anim. Reprod*., **25**(1): 23-24.

Bhosrekar, M.R., R.S. Rane and R.C. Mazokari. 2001. Fertility of Murrah buffalo bulls in Bhilawadi area of Sangli district. *In Proceedings of the 17th Annual Convention and National Seminar on Fertility Management of Farm Animals Under Adverse Agro-climatic Conditions*, Jodhpur, India.

Bhosrekar, M.R., S.P. Mokashi, J.R. Purohit, S.B. Gokhale and B.R. Mangurkar. 1994. Effect of glycerolization and deep freezing on the levels and release of enzymes in buffalo

semen in relation to initial semen attributes, p. 465-467. *In Proceedings of the 4th International Buffalo Congress*, Sao Paulo, Brazil.

Bhosrekar, M.R., S. Mokashi, J.R. Purohit, S.B. Gokhale and B.R. Mangurkar. 1991. Studies on the effect of deep freezing and seasons on the leakage of aspertate amino transferase into extracellular medium and sperm morphology of Murrah buffalo bulls. *Anim. Reprod. Sci*., **26**: 219-226.

Bhosrekar, M.R., J.R. Purohit, S.B. Gokhale and B.R. Mangurkar. 1992. Effect of seasons on production performanceof surti buffalo bulls. *Indian J. Anim. Sci*., **62**(5): 443-447.

Buffalopedia. 2015, www.buffalopedia.cirb.res.in

Chachur, M.G.M., E. Oba and C.I.M. Gonzales. 1997. Equilibrium time influence on motility, vigour and membrane integrity of thawed buffalo semen using triladyl, glycine-egg yolk and tes extenders, p. 846-849. *In Proceedings of the 5th World Buffalo Congress*, Caserta, Italy.

Chandonnet, L., K.D. Roberts, A. Chapdelaine and P. Manjunath. 1990. Identification of heparin-binding proteins in bovine seminal plasma. *Mol. Reprod. Dev*., **26**: 313-318.

Chandra, M., V.K. Srivastava and A.K. Shukla. 1999. Study of effect of temperature on semen quantity (volume) and quality (motility) using fuzzy approach. *Buffalo J*., **15**: 105-113.

Chen, Y., R.H. Foote and C.C. Brockett. 1993. Effect of sucrose, trehalose, hypotaurine, taurine and blood serum on survival of frozen bll sperm. *Cryobiology*, **30**: 423-431.

Chinnaiya, G.P. and N.C. Ganguli. 1990. Effect of age and season on the initial attributes and preservability of buffalo bull semen. *In* Acharya, R.M., R.R. Lokeshwar and S. Kumar (eds.) *Recent Advances in Buffalo Research*, **3**: 80-86.

Chohan, K.R., J. Iqbal, A.A. Asghar and M.A. Chaudhary. 1992. Fertility of liquid and frozen semen in Nili-Ravi buffaloes. *Pak. Vet. J*., **12**: 4-5.

Collins, W.J., R.W. Braton and C.R. Henderson. 1951. The relationship of semen production to sexual exitment of dairy bulls. *J. Dairy Sci*., **34**: 224.

Cormier, A. and J.L. Bailey. 2003. Differential mechanism is involved during heparin and cryopreservation induced capacitation of bovine spermatozoa. *Biol. Reprod*., **6**: 177-185.

Del Sorbo, C., G. Fasano, A. Fabbrocini, L.S. Lavadera and G. Sansone. 1995. Piruvato quale substrato energetico in extenders crioprotettivi. Effetti sulla motilita allo scongelamento di spermatozoi bufalini `*Bubalus bubalis*. *In Proceedings of the 7th* Meeting Nazionale ‘‘Studio sulla efficienza riproduttiva degli animali di interesse zootecnico’’, Bergamo, Italy. **1**: 585-588.

Del Sorbo, C., G. Fasano, A. Fabbrocini, M.J.F. Nastri and G. Sansone. 1994. Studio preliminare su effetti di Ž. vari agenti crioprotettivi sulla motilita di spermatozoi bufalini B. bubalis allo scongelamento. *In Proceedings of the 6th Meeting Nazionale “Studio della efficienza riproduttiva deli animali di interesse zootecnico”*. Bergamo, Italy. **1**: 63-67.

Den Daas, N. 1992. Laboratory assessment of semen characteristics. *Anim. Reprod. Sci*., **28**: 87-94.

Desnoyers, L. and P. Manjunath. 1992. Major proteins of bovine seminal plasma exhibit

novel interactions with phospholipid. *J. Biol. Chem*., **267**: 10149- 10155.

Dhami, A.J. and K.L. Sahni. 1993. Effect of extenders, additives and deep freezing on the leakage of transaminases from Murrah buffalo spermatozoa. *Buffalo J*., **1:** 55-64.

Dhami, A.J. and K.L. Sahni. 1994. Effects of various cooling from 30°C to 5°C, equilibration and diluent treatments on freezability, post-thaw thermoresistance, enzyme leakage and fertility of bubaline spermatozoa. *Buffalo J*., **2**: 147-159.

Dhami, A.J. and S.B. Kodagali. 1990. Freezability, enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. *Theriogenology*, **34**(5): 853-863.

Dhami, A.J., V.R. Jani, G. Mohan and K.L. Sahni. 1994. Effect of extenders and additives on freezability, post-thaw thermoresistence and fertility of frozen Murrah buffalo semen under tropical climate. *Buffalo J*., **10**: 35-45.

Dostalova, Z., J.J. Calvete, L. Sanz and E. Topfer-Petersen. 1995. Boar spermadhesin AWN-1. Oligosaccaride and zona binding characteristics. *Eur. J. Biochem*., **230**: 329-336.

El-Menoufy, A.A., A.A. Seida, S.M. El Fattouh and M.M. Abou Ahmed. 1985. Effect of caffeine on metabolic activity of ejaculated and epididymal spermatozoa of buffalo (*Bubalus bubalis*). *In Proceedings of the 1st World Buffalo Congress*, Cairo, Egypt. **1**: 1078-1087.

Fabbrocini, A., C. Del Sorbo, G. Fasano, M.R. Martusciello and G. Sansone. 1995. Effect of cryoprotectant addition mode on freezed-thawed *Bubalus bubalis* semen. *In Proceedings of the 30th Symposium "Reproduction and Animal Breeding: Advance and Strategy"*, Milano, Italy. 1: 373-374.

Forouzanfar, M., M. Sharafi, S.M. Hosseini, S. Ostadhosseini, M. Hajian, L. Hosseini, P. Abedi, N. Nili, H.R. Rahmani and M.H. Nasr-Esfahani. 2010. *In vitro* comparison of egg yolk based and soybean lecithin based extenders for cryopreservation of ram semen. *Theriogenology*, **73**: 480-487.

Gadea, J., E. Selles, M.A. Marco, P. Coy, C. Matas, R. Romar and S. Ruiz. 2004. Decrease in glutathione content in boar sperm after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*, **62**: 690-701.

Garg, A., A. Kumaresan and M. R. Ansari. 2008. Effect of hydrogen peroxide on fresh and cryopreserved buffalo sperm function during incubation at 37°C *in-vitro*. *Reproduction in Domestic Animals*, **10**: 1439-1445.

Galli, A., V. Bornaghi, D. Balduzzi, L. Buttazzoni and R. Aleandri. 1993. Sexual behaviour and semen quality relating to italian buffalo. *In Proceedings of the 3rd World Buffalo Congress*, Varna, Bulgaria. **1**: 562-570.

Garner, D., A. Thomas and C. Gravance. 2001 Seminal plasma addition attenuates the dilution effect in bovine sperm. *Theriogenology*, **56**: 31-40.

Garner, D.L. and L.A. Johnson. 1995. Viability assessment of mammalian sperm using SYBR- 14 and propidium iodide. *Biol. Reprod.*, **53**: 276-284.

Government of India. 2012. *19th Livestock census*. Department of Animal Husbandry, Dairying and Fisheries, Government of India, India.

Graham, J.K. and E. Mocé. 2005. Fertility

evaluation of frozen/thawed semen. *Theriogenology*, **64**: 492-504.

Gwathmey, T.M., G.G. Ignotz and S.S. Suarez. 2003. PDC-109 (BSP A1/A2) promotes bull sperm binding to oviductal epithelium *in vitro* and may be involved in forming the oviductal sperm reservoir. *Biol. Reprod.*, **69**: 809-815.

Haranath, G.B., T.B. Suryaprakasam, A.V.N. Rao and G. Somasekharam. 1990. Freezability of semen and fertility of frozen semen packaged in mini and medium French straws: A note. *In* Acharya, R.M., R.R. Lokeshwar and S. Kumar (eds.) *Recent Advances in Buffalo Research*, 3: 87-88.

Henricks, D., A. Kouba, B. Lackey, W. Boone and S. Gray. 1998. Identification of insulin-like growth factor I in bovine seminal plasma and its receptor on spermatozoa: influence on sperm motility. *Biol. Reprod*., **59**: 330-337.

Holt, W.V. 2000. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology*, **53**: 47-58.

Hopkins, S.S. and L.E. Evans. 1989. Artificial Insemination. p. 354-388. *In* MacDonald, L.E. and M.H. Pineda (ed.) *Veterinary Endocrinology and Reproduction*, 4th ed. Philadelphia, Lea and Feibeger, USA.

Hossain, M.S., A. Johannisson, M. Wallgren, S. Nagy, A.P. Siqueira and H. Rodriguez-Martinez. 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J. Androl*., **13**: 406-419.

Javed, M.T., A. Khan and R. Kausar. 2000. Effect of age and season on some semen parameters of Nili-Ravi buffalo (*Bubalus bubalis*) bulls. *Vet. Arhiv*., **70**: 83-94.

Jeyendran, R.S., H.H. Vander Ven, M. Perez-Pelaez, B.G. Crabo and L.J.D. Zaneveld. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil*., **70**: 219-228.

Jobim, M.I.M., E.R. Oberst, C.G. Salbego, V.B. Wald, A.P. Horn and R.C. Mattos. 2005. BSP A1/A2-like proteins in ram seminal plasma. *Theriogenology*, **63**: 2053-2062.

Kadirvela, G., S. Kumar and A. Kumaresana. 2009. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. *Animal Reproduction Science*, **114:** 125-134.

Kadirvel, G., S. Kumar, S.K. Ghosh and P. Perumal. 2104. Activity of antioxidative enzymes in fresh and frozen thawed buffalo (*Bubalus bubalis*) spermatozoa in relation to lipid peroxidation and semen quality. *Asian Pacific Journal of Reproduction*, **3**(3): 210-217.

Khan, D.R., N. Ahmad, M. Anzar and A.A. Channa. 2009. Apoptosis in fresh and cryopreserved buffalo sperm. *Theriogenology*, **71**: 872-876.

Khan, M.I.R. and A. Ijaz. 2008. Effect of osmotic pressure on motility, plasma membrane integrity and viability in fresh and frozen-thawed buffalo spermatozoa. *Animal*, **2**: 548-553.

Killian, G., T. Honadel, T. McNutt, M. Henault, C. Wegner and D. Dunlap. 1989. Evaluation of butylated hydroxytoluene as a cryopreservative added to whole or skim

milk diluent for bull semen. *J. Dairy Sci*., **72**: 1291-1295.

Koonjaenak, S., V. Chanatinart, S. Aiumlamai, T. Pinyopumimintr and H. Rodriguez-Martinez. 2007. Seasonal variation in semen quality of swamp buffalo bulls (*Bubalus bubalis*) in Thailand. *Asian J. Androl*., **9**(1): 92-101.

Kumar, A. 2012. *Studies on effect of cholesterol loaded cyclodextrins on freezability and in vitro fertility of buffalo spermatozoa*. Ph.D. Thesis, Deemed University, Indian Veterinary Research Institute, Izanagar, Bareilly, Uttar Pradesh, India.

Kumar, P., M. Saini, D. Kumar, A.K. Balhara, S.P. Yadav, P. Singh and P.S. Yadav. 2015. Liposome-based semen extender is suitable alternative to egg-yolk based extender for cryopreservation of buffalo (*Bubalus bubalis*) semen. *Animal Reproduction Science*, **159**: 38-45.

Kumar, S. 1989. *Studies on the effect of various levels of certain cryprotectants on deep freezing of buffalo semen*. Ph.D. Dissertation, Indian Veterinary Research Institute, Izatna-gar, India.

Kumar, S., K.L. Sahni and G. Mohan. 1992b. Effect of different sugars as sole cryoprotectant in freezing of buffalo semen. *Buffalo J*., **l3**: 305-309.

Kumar, S., K.L. Sahni and G.S. Bistha. 1993a. Cytomorphological characteristics of motile and static semen of buffalo bulls. *Buffalo J*., **2**: 117-127.

Kumar, S., K.L. Sahni and G. Mohan. 1992a. Effect of different levels of glycerol and yolk on freezing and storage of buffalo semen in milk, tris and sodium citrate buffers. *Buffalo J*., **2**: 151-156.

Lasley, J.F. and R. Bogart. 1942. A comparative study of epididymal and ejaculated spermatozoa of boar. *J. Anim. Sci*., **3**: 360-370.

Lipar, J.I., E.D. Ketterson, Jr.V. Nolon and J.M. Costo. 1999. Egg yolk layers very in the concentration of steroid hormones in two avian species. *Gen. Comp. Endocrinol*., **115**: 220-227.

Mahmood, S.A. and A. Ijaz. 2006. Effect of cold shock on frozen-thawed spermatozoa of buffalo and cow bulls, p. 701-708. *In Proceedings of the 5th Asian Buffalo Congress*, Nanningh, China.

Mammoto, A., N. Masumoto, M. Tahara, Y. Ikebuchi, M. Ohmichi and K. Tasaka. 1996. Reactive oxygen species block sperm-egg fusion via oxidation of sperm sulfhydryl proteins in mice. *Biol. Reprod*., **55**: 1063-1068.

Mandal, D.K., P.K. Nagpaul and A.K. Gupta. 2000. Seasonal variation in seminal attributes and sexual behaviour of Murrah buffalo bulls. *Indian J. Dairy Sci*., **53**: 278-283.

Manik, R.S. and V.D. Mudgal. 1984. Monthly and seasonal variation in physico-chemical and biochemical attributes of buffalo semen. *World Rev. Anim. Prod*., **4**: 45-50.

Manjunath, P., A. Bergeron, J. Lefebvre and J. Fan. 2007. Seminal plasma proteins: functions and interaction with protective agents during semen preservation. *Soc. Reprod. Fertil. Suppl*., **65**: 217-228.

Martínez-Pastor, F., M. Mata-Campuzano, M. Álvarez-Rodríguez, M. Álvarez, L. Anel and P. De Paz. 2010. Probes and techniques for sperm evaluation by flow cytometry. *Reprod. Domest. Anim*., **45**(2): 67-78.

Mazur, P. 1984. Freezing of living cells: mechanisms

and implications. *Am. J. Physiol*., **247**: C125-C142.

Medeiros, C.M.O., F. Forell, A.T.D. Oliveira and J.L. Rodrigues. 2002. Current status of sperm cryopreservation: why is it better. *Theriogenology*, **57**: 327-344.

Mishra, A.K. and S. Tyagi. 2006. Recent trends in the semen collection, evaluation and cryopreservation, p. 95-105. *National Seminar on Artificial Insemination: Acceptability, Impact, Constraints and Solutions*, New Delhi, India.

Misra, A.K., S.H. Patel, B.V. Joshi, R.S. Jaswal and K.R. Trivedi. 1994. Buffalo semen characteristics and its freezability under Indian conditions. *In Proceedings of 4th International Buffalo Congress*, Sao Paulo, Brazil. **3**: 495-497.

Mughal, H., A. Ijaz, M.S. Yousaf, H. Rehman, M. Aleem, H. Zaneb and F. Wadood. 2013. Assessment of optimal osmotic pressure of citrate egg yolk extender for cryopreservation of buffalo bull (*Bubalus bubalis*) semen. *J. Anim. Plant Sci.*, **23**(4): 964-968.

Nastri, M.J.F., C. Del Sorbo, A. Fabbrocini, G. Fasano and G. Sansone. 1994. Performances motility in cooled and freeze thawed *B. bubalis* spermatozoa at different osmotic pressures. *In Proceedings of 7th International Symposium on Spermatology*, Cairns, Australia. **1**: 99-100.

Noakes, D., E.T.J. Parkinson and G.C.W. England. 2009. Veterinary Reproduction and Obstetrics. *Arthur's Veterinary Reproduction and Obstetrics,* 8th ed. Saunders, Philadelphia, USA.O.I.E. 1996. *OIE Manual of Standards for Diagnostic Testes and Vaccines*, 3rd ed. Office International des Epizooties.

Oba, E., E.J. Fuck, S.D. Bicudo, F.O. Papa and O.M. Ohashi. 1994. Preliminary study on different mediums for deep freezing of buffalo semen. *In Proceedings of the 4th International Buffalo Congress*, Sao Paulo, Brazil. **3**: 579-581.

Pace, M.M. and E.F. Graham. 1974. Components in egg yolk which protect bovine spermatozoa during freezing. *J. Anim. Sci*., **39**: 1144-1149.

Pangawkar, D.R., R.D. Sharma and R. Sharma. 1988. Phosphatase and transaminase activity in seminal plasma of bulls in relation to freezability of semen. *Theriogenology*, **29**: 1393-1399.

Raizada, B.C., A. Sattar and M.D. Pandey. 1990. A comparative study of freezing buffalo semen in two diluters. *In* Acharya, R.M., R.R. Lokeshwar and S. Kumar (eds.) *Recent Advances in Buffalo Research*, **3**: 66-74.

Rajoriya, J.S., J.K. Prasad, S.S. Ramteke, P. Perumal, S.K. Ghosh, M. Singh, M. Pande and N. Srivastava. 2016. Enriching membrane cholesterol improves stability and cryosurvival of buffalo spermatozoa. *Anim. Reprod. Sci.*, **164**: 72-81.

Ramakrishnan, P. and M.O. Ariff. 1994. Effect of glycerol level and cooling rate on post-thaw semen quality of Malaysian swamp buffalo. *In Proceedings of the 4th International Buffalo Congress*, Sao Paulo, Brazil. **3**: 540-542.

Rao, A.V.N., G.B. Haranath and G. Somasekharam. 1990. Effect of equilibration and thawing rates on survival and acrosomal maintenance of buffalo spermatozoa in straws. *In* Acharya, R.M., R.R. Lokeshwar and S. Kumar (eds.) *Recent Advances in Buffalo*

Research, **3**: 97-99.

Rasul, Z., N. Ahmad and M. Anzar. 2001. Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. *J. Androl*., **22**: 278-283.

Rattan, P.J.S. 1990. Physico-chemical constituents of buffalo semen, p. 26-30. *In Proceedings of the 2nd World Buffalo Congress*, Indian Society of Buffalo Development and Indian Council of Agricultural Research, India.

Ravimurgan, T., K.S. Raman and P. Thangaraju. 2003. Seasonal influences on the semen production traits of Murrah buffalo bulls. *Indian Buffalo J*., **1**(2): 85-87.

Ronald, B.S.M. and T.G. Prabhakar. 2001. Bacterial analysis of semen and their antibiogram. *Indian. J. Anim. Sci*., **71**: 829-831.

Saeed, A. 1988. *Studies on morphology of buffalo bull semen of different age groups*. Thesis, University of Agriculture. Faisalabad, Pakistan.

Saeed, A., R.A. Chaudhry, I.H. Khan and N.U. Khan. 1990. Morphology of semen buffalo bulls of different age groups. *In* Acharya, R.M., R.R. Lokeshwar and S. Kumar (eds.) *Recent Advances in Buffalo Research*, **3**: 17-19.

Sahni, K.L. and G. Mohan. 1990. Effect of various levels of yolk on viability of buffalo semen at 37, 5 and 196 degrees C. *In* Acharya, R.M., R.R. Lokeshwar and S. Kumar (eds.) *In Proceedings of the 2nd World Buffalo Congress,* New Delhi, India.

Samad, S.A. 1985. Diluters of the buffalo semen, p. 34-46. *In Processing and use of Buffalo Semen*. National Animal Science Research Institute, Islamabad, Pakistan.

Sanchez-Luengo, S., G. Aumuler, M.M. Albrecht, P.C. Sen, K. Rohm and B. Wilhelm. 2004. Interaction of PDC-109, the major secretory protein from bull seminal vesicles, with bovine sperm membrane Calcium ion ATPase. *J. Androl*., **25**(2): 234-244.

Sansone, G., M.J. Nastri and A. Fabbrocini. 2000. Storage of buffalo (*Bubalus bubalis*) semen. *Anim Reprod Sci*., **62**: 55-76.

Saxena, V.B. and S.S. Tripathi. 1979. Semen characteristics and preservability in Red Dane bulls. *Indian J. Anim. Sci*., **49**(11): 878-883.

Sengupta, B.P., M.S. Mishra and A. Roy. 1963. Climatic environment and reproductive behaviours of buffaloes. 1. Effect of different seasons on various seminal attributes. *Indian J. Dairy Sci*., **16**: 150-165.

Shannon, P. 1972. The effect of egg yolk level and dose rate of semen diluted in caprogen, p. 1440-1442. *In Proceedings of 7th International Congress on Animal Reproduction and Artificial Insemination*, Munich, Germany.

Shukla, M.K. and A.K. Misra. 2005. Effect of antioxidants alpha tocopherol, ascorbic acid and n-propyl gallate on Murrah semen cryopreservation. *Buffalo J*., **21**: 27-38.

Shukla, M.K., A.K. Misra and H.P. Gupta. 2009. Studies on some biochemical constituents of Murrah buffalo (*Bubalus bubalis*) seminal plasma. *Buffalo Bull*., **128**(3): 123-126.

Singh, J., G.R. Pangawkar, R.K. Biswas, A.K. Srivastava and R.D. Sharma. 1990. Studies on lactic dehydrogenase and sorbitol dehydrogenase release in relation to deep freezing of buffalo semen in certain extenders. *Theriogenology*, **34**: 371-378.

Singh, M. 2012. Isolation and purification of PDC-109 protein from buffalo seminal plasma

and its relation with semen freezability. M.V.Sc. Thesis, Deemed University, IVRI, Uttar Pradesh, India.

Singh, M., N.C. Barik, S.K. Ghosh, J.K. Prasad, J.S. Rajoriya, Y.K. Soni, A. Kumar, J.K. Chaudhary and N. Srivastava. 2015. Egg yolk powder an alternative to fresh-egg yolk for buffalo semen cryopreservation. *Indian J. Anim. Sci*., **85**(1): 40-42.

Singh, M., S.K. Ghosh, J.K. Prasad, A. Kumar, R. P. Tripathi, S.K. Bhure and N. Srivastava. 2014. Sminal PDC-109 protein *vis-à-vis* cholesterol content and freezability of buffalo spermatozoa. *Animal Reproduction Science*, **144**: 22-29.

Singh, M., S.K. Ghosh, J.K. Prasad, A. Kumar, S.S. Ramteke and S.K. Bhure. 2013a. Heparin binding proteins of buffalo bulls seminal plasma and their relationship with semen freezability. *Indian J. Anim. Sci.*, **83**(7): 700-704.

Singh, M., S.S. Ramteke, S.K. Ghosh, J.K. Prasad and J. Rajoriya. 2013b. Efficacy of egg yolk from three avian species on semen freezability of Tharparkar bull. *Indian J. Anim. Reprod*., **34**(2): 25-28.

Thérien, I., R. Moreau and P. Manjunath. 1998. Major proteins of bovine seminal plasma and highdensity lipoprotein induce cholesterol efflux from epididymal sperm. *Biol Reprod*., **59**: 768-76.

Thomas, A.D., S.A. Meyers and B.A. Ball. 2006. Capacitation-like changes in equine spermatozoa following cryopreservation, *Theriogenology*, **65**: 1531-155.

Tomar, N.S., B.S. Mishra and C.B. Johri. 1966. Seasonal variation in reaction time and semen production in Hariana and Murrah bulls. *Indian J. Dairy Sci*., **19**: 87-93.

Tuli, R.K., M. Singh and J.S. Matharoo. 1981. Fertility trial under field conditions with frozen buffalo bull semen using Tris yolk glycerol extender. *Indian J. Dairy Sci*., **34**: 456-458.

Vale, W.G. 1997. Sperm cryopreservation. *Bubalus Bubalis*, **1**: 129-140.

Vale, W.G., O.M. Ohashi, H.F.L. Ribeiro and J.S. Sousa. 1991. Semen freezing and artificial insemination in water buffalo in the amazon valley. *Buffalo J*., **72**: 137.

Vijayasaraswathy, S.G., B.C. Divyashree and S.C. Roy. 2014. Cryogenic changes in proteases and antiprotease activities of buffalo (*Bubalus bubalis*) and cattle (*Bos taurus*) semen. *Theriogenology*, **81**: 396-402.

Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thaw function. *Reprod. Fertil. Dev*., **7**: 871-891.

Watson, P.F. 2000. The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci*., **60-61**: 481-482.

Yanagimachi, R. 1994. Mammalian fertilization. p. A189-A317. *In* Knobil, E. and J.D. Neill (eds.) *Physiology of Reproduction*, 2nd ed. Raven Press, New York, USA.

Ziada, M., G. Abdel-Malak and M.S. Abdou. 1992. Effect of glycerol concentration and thawing regimes on the viability of frozen buffulo spermatozoa. Proceeding of the 4th International Congress of Egypt, Cairo, Egypt. *Society of Animal Production Fertilization*, **2**: 59-67.