Wayamba Journal of Animal Science – ISSN: 2012-578X; P1815 - P1826, 2019
First Submitted May 02, 2019; Number 1549121012

ROLE OF CHOLESTEROL LOADED CYCLODEXTRIN (CLC) IN CRYOCAPACITATION OF SPERM – A REVIEW

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Artificial insemination using frozen semen is now the most widely employed technology nationwide, for improving the genetic potential of livestock. Freezing and thawing of semen can cause irreparable damage to sperm, thereby decreasing its life span and ability to interact with female tract. It also causes a leakage of enzymes due to membrane rupture and will reduce the ability to fertilize. The buffalo bull spermatozoa are more susceptible to freeze damage than cattle semen and also have low post-thaw motility. Cholesterol loaded cyclodextrin (CLC) has been reported to increase the membrane integrity, augment the strength of sperm binding to zona pellucida of oocytes, improve the rate of water permeability and decrease the osmotic stress. However, the effect of CLC on freezability has not documented much by experimental studies. Its role in protection of membrane architecture and reducing cryocapacitation in buffalo spermatozoa needs to be documented so that improvement can be made in cryopresrvation buffalo semen employed for A.I. Considering the above facts the present review elucidates the effect of CLC on freezability of buffalo spermatozoa. The review highlights the fact that addition of CLC has led to significant improvement in the post-thaw semen quality parameters like progressive motility, livability and acrosomal integrity of spermatozoa.

Keywords: Artificial insemination, Buffalo, Cholesterol loaded cyclodextrin, Freezability, Semen

The world buffalo population is 185.29 million, out of that India possesses more than half i.e. 105.3 million buffaloes (FAO, 2013). Indian buffalo *(Bubalus bubalis)* has significant contribution to national health and economy in the form of milk, meat, manure and draught power (Agnihotri, 1992). Cryopreservation techniques have made semen accessible to poor farmers regardless of time. Large scale dissemination of A.I technology primarily depends on the efficiency of long term semen preservation techniques. Freezing and thawing of semen causes irreparable damage to sperm, which will decrease the life span of sperm, its ability to interact with female tract and causes leakage of enzyme and a reduction in fertilizing ability. Post-thaw survival of sperm population is approximately 50% even by using the best preservation techniques (Raizada *et al.,* 1990).

Buffalo spermatozoa are more susceptible to damages during freezing than that of cattle spermatozoa and average post-thaw motility of buffalo spermatozoa was generally reported lower than cattle spermatozoa (Raizada *et al,* 1990; Andrabi *et al*., 2008). Also Fertility rate in buffalo was poor when inseminated with frozen semen than cattle semen (Haranath *et al*., 1990 and Chohan *et al*., 1992). The fertility of frozen thawed buffalo spermatozoa has been reported to be lower due to the sub lethal damages even though the mechanisms for reduced fertility are largely unknown. There are specific biochemical factors that affect the ability of spermatozoa to prevent damages caused by the cryogenic procedures. One of the many possible causes of lower freezability of buffalo bull semen compared to cattle bull could be due to the differences in the lipid

ratio of the spermatozoa (Tatham, 2000). Sperm sensitivity to cold shock damage is determined by membrane phospholipid composition as well as the membrane cholesterol to phospholipid ratio (Holt, 2000). Buffalo sperm possess high cholesterol to phospholipids ratio, about 0.44±0.05 (Kumar, 2012) as compared to cattle spermatozoa in which the range is between 0.42-0.45 (Darin-Bennett and White, 1977).

Sperm membranes are composed of lipids and proteins with the lipids arranged in a bilayer with hydrophilic lipid head groups oriented to the membrane exterior and hydrophobic fatty acyl chains in the membrane interior. Phospholipid and cholesterol are two predominant lipids present in most of cell membrane. The protein-lipids association is not random, in sperm and the lipid environment around a protein influences functional properties of the aggregate unit (Hammerstedt *et al*., 1990; Rajoriya *et al*., 2014). At body temperature, the membrane is in fluid state, in which phospholipids and proteins move laterally within the membrane (Amann and Pickett, 1987). During cooling of membrane the phospholipids undergo a phase transition from a liquid state to crystalline-gel state. In this state, the phospholipid acyl chains straighten and lengthen, resulting in a more ordered and packed membrane, which restrict the lipid and protein movement (Amann and Pickett, 1987; Hammerstedt *et al*., 1990). Both ratio of cholesterol to phospholipid and the amount of polyunsaturated fatty acyl chains composing the phospholipids determine the overall fluidity of a membrane (Amann and Pickett, 1987).

Cholesterol has multiple effects on membrane like stabilizing the membrane, reducing membrane permeability, facilitating morphological membrane characteristics, enabling cell-to-cell interactions, influencing the membrane phase transition, providing suitable microenvironments for membrane-associated proteins and serving as a membrane antioxidant (Crockett, 1998). In model membranes, increasing the ratio of cholesterol to phospholipid broadens the phase transition, reduces membrane leakage and membrane phase transition (Drobnis *et al*., 1993). Therefore, treating sperm with cholesterol before cryopreservation could reduce the sensitivity of sperm membranes to cooling damage, by eliminating or at least minimizing the lateral phase separation of the lipids (Watson, 1981). Cholesterol content of sperm membranes can be modified using cholesterol loaded cyclodextrin (Purdy and Graham, 2004; Moore *et al*., 2005).

Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch and they possess an external hydrophilic face and an internal hydrophobic core (Dobziuk, 2006) that can encapsulate hydrophobic compounds such as cholesterol. The diameter of cyclodextrin is about 15 A^o and the diameter of high density lipoprotein (HDL) particles ranges from 70 to 120 A^o (Yancy *et al*., 1996). So cyclodextrin is capable of accessing the cholesterol domains on the plasma membrane more efficiently due to its small size and unlike HDL particles, cyclodextrin can diffuse readily through the media and filter through barriers, such as the spermatozoa gycocalyx. Since cholesterol efflux from the sperm membranes plays an important role in sperm capacitation, it is possible that increasing sperm cholesterol content, using CLC technology, may reduce premature sperm capacitation thereby increasing the lifespan of a cryopreserved sperm cell, in addition to increasing the number of sperm that survive cryopreservation. Cholesterol also decreases the capacitation like changes (cryocapacitation) that occurs when sperm are frozen. Cholesterol loaded cyclodextrin has been used in several species like bull, ram, stallion, boar and donkey's semen cryopreservation with varying degree of success. Addition of CLC on membrane architecture has been thoroughly studied and such studies reported that addition of CLC at the dose rate of 2.0 mg/120×10^6spermtaozoa has significant protecting effect on sperm plasma-membrane (Kumar, 2012). A significant improvement in the post-thaw motility has also been reported at this dose rate of CLC

addition. There is a further scope of protection of membrane architecture and reducing cryocapacitation by addition of higher dose rate of CLC before cryopreservation.

Cryopreservation induces damage to all sperm compartments (Bailey *et al*., 2000). This damage is responsible for the loss of sperm motility, viability, acrosomal integrity and of course the fertilizing capacity of frozen-thawed sperm (Holt, 2000). Sperm cryopreservation is a multifactorial problem, where the diluents, the protocols, the species, breed and the individual sires within each breed are merely some of the many parameters that need to be included in the overall evaluation for success. Each of these parameters includes a variety of sub factors and each influence the other parameters. So testing all factors at the same time to optimize a freezing protocol is technically impossible, therefore, a few factors have been used to solve this problem.

Physico-morphological attributes

a.) Mass Activity

Mass activity of semen is one of the most common seminal parameter used for assessing its quality due to its simplicity (Shukla and Mishra, 2005). Mass activity is significantly and positively correlated with spermatozoa concentration, initial motility and live spermatozoa count. Mass activity reported in buffalo bull semen was 3.88±0.84 by Kumar (2012).

b.) Initial Progressive Motility

Mammalian spermatozoa develop the capacity for motility during their epididymal transit. On reaching cauda epididymis spermatozoa are essentially mature and they are capable of fertilizing eggs. Maturation of spermatozoa during their epididymal transit involves physico-biochemical and morphological alterations, resulting into cytophysiological changes to achieve capacity for motility and fertility. Bovine spermatozoa are stored in cauda epididymis in a quiescent state, *i.e.* spermatozoa in neat cauda semen have flagellating tails but no progressive motility. Individual motility depends on the factors like age of the bull, temperature, frequency of collections and sexual excitement before collection (Misra *et al*., 1994). The individual motility reported in buffalo bull by different workers as 83.60±0.13% (Singh *et al*., 1992), 77.92±0.33% (Shukla and Mishra, 2005) and 84.19±0.65 % (Kumar, 2012).

c.) Sperm Concentration

Sperm concentrations in buffalo bull semen have been reported from 908.45±20.17 million/ml (Kumar, 2012) to 1046.64±34.21 million/ml (Shukla and Mishra, 2005). Difference in spermatozoa concentration may be due to managemental practices such as restraint before collection, frequency of semen collection.

d.) Live Sperm Percentage

Estimation of per cent live spermatozoa in a semen sample has direct and positive correlations with fertility of a bull. Various workers reported live spermatozoa per cent of buffalo bull semen as 89.07±0.44% (Shukla and Misra, 2005) and 88.13±0.64% (Kumar, 2012).

EFFECT OF CHOLESTEROL LOADED CYCLODEXTRIN (CLC) ON SEMEN CRYOPRESERVATION:

Method of cholesterol addition in semen

Cholesterol is a hydrophobic molecule and is not soluble in aqueous semen diluents. Attempts have been made to solubilize cholesterol by incorporating it into liposomes and adding these liposome-cholesterol complexes to sperm. Results have been variable using this technique (from no response to moderate improvement after cooling at 4°C or after cryopreservation) in various species (Parks *et al*, 1981; Ollero *et al*., 1996 and Wilhelm *et al*., 1996). Recently, cyclodextrins have been used to insert or remove cholesterol from cell membranes. Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch, and they possess an external hydrophilic face and an internal hydrophobic core (Dobziuk, 2006) that can encapsulate hydrophobic compounds, such as cholesterol.

These molecules have a high affinity for sterols *in vitro* and are very efficient in stimulating the removal of cholesterol from the membranes of many types of cells (Christian *et al*., 1997), including spermatozoa (Companyo *et al*., 2007). In addition, if they are pre-loaded with

cholesterol, they can insert cholesterol into cell membranes (Navratil *et al*., 2003). These molecules more efficiently transfer cholesterol than phospholipid acceptors (Zidovetzki and Levitan, 2007). Because of their hydrophilic face, cyclodextrins can solubilize hydrophobic molecules. However, adding methyl or hydroxypropyl residues to the cyclodextrins enhances both their solubility in water and their ability to dissolve hydrophobic compounds (Yancey *et al*., 1996). Therefore, methyl- β cyclodextrin and 2-hydroxypropyl- β-cyclodextrin accept cholesterol from mouse L-cell fibroblasts more efficiently than β-cyclodextrin (Yancey *et al*., 1996).

Treatment of sperm with cyclodextrins pre-loaded with cholesterol (CLC)

In general treating sperm from different species with cyclodextrins pre-loaded with cholesterol prior to cryopreservation, improves sperm quality after freezing-thawing. Cyclodextrins can be loaded with cholesterol in a number of ways including adding both ingredients to an aqueous diluent followed by sonication (Zeng and Terada, 2000; Galantino-Homer *et al*., 2006 and Movassaghi *et al*., 2009) or by diluting them in a mixture of methanol: chloroform followed by desiccation of the solution to obtain the crystals (Purdy and Graham, 2004). Methyl- β-cyclodextrin (MBC) is most commonly used chemical to treat sperm with cholesterol prior to cryopreservation, although 2-hydroxypropyl- β-cyclodextrin is also effective for treating ram sperm (Moce *et al*., 2010).

Sperm are treated with CLC at concentrations between 1 and 2 mg CLC/120 x 10^6 sperm, although in some species CLC concentrations as high as 5 mg CLC/120 x 10^6 sperm have been reported (Purdy and Graham, 2004; Moce and Graham, 2005; Moce *et al*., 2010; Moore *et al*., 2005 and Kumar, 2012). Sperm treated with CLC at room temperature for 15 minute is sufficient for cholesterol transfer into the spermatozoa membrane, although incubations for longer times and/or at higher temperatures have been described, with time as high as 3 hours being reported. (De Graaf *et al*., 2007; Moce and Graham, 2006; Purdy and Graham, 2004; Zeng and Terada, 2000). It is important to treat sperm with CLC in diluents void of egg yolk and skim milk because these components interfere with cholesterol transfer into the sperm (Combes *et al*., 2000 and Purdy and Graham, 2004).

Effect of CLC treatment on sperm quality after cryopreservation

Treating sperm with CLC increases the sperm cholesterol content 2-3 fold in bull, trout, ram and stallion sperm (Purdy and Graham, 2004; Muller *et al*., 2008 and Moce *et al*., 2010), and this additional cholesterol would raise the cholesterol: phospholipid ratios of these sperm to cholesterol: phospholipid ratios (>0.8) that are similar to sperm that are not sensitive to cold shock. Treating sperm with CLC prior to cryopreservation improves sperm cryosurvival rates. Motility and viability of CLC treated sperms was found to be increased by 10-20 percentage points in most studies. Kumar (2012) reported 0.42±0.067 C:P ratio and 62.78±0.59 individual progressive motility at post-thaw stage. The benefit of CLC treatment on cryosurvival rates is different for sperm from different species, lines within a species, different males and for sperm treated in different ways (*i.e.* sex-sorted sperm). It appears to have no effect on sperm from species whose sperm are not sensitive to cold shock, such as rabbit and rainbow trout sperm. This lack of beneficial effect might be expected for rabbit sperm, as rabbit sperm normally possess high membrane cholesterol: phospholipid ratio 0.88 (Watson, 1981) and is unlikely that CLC treatment could increase the cholesterol content of these sperm significantly.

Muller *et al.* (2008) suggested that cholesterol treatment may modify some domains of the sperm plasma membrane, but not membrane domains important for sperm motility or fertilization, particularly if cholesterol incorporates into different membrane domains differently, depending on the lipid composition or the degree of saturation of the fatty acids from each specific membrane domain. Even for sperm that are sensitive to cold-shock damage, the effectiveness of adding cholesterol to the

sperm may depend upon other treatments to the sperm.

Sperm from stallions that normally do not cryopreserve well (classified as 'bad freezers') benefited more from CLC treatment than sperm from stallions that normally cryopreserve well (Moore *et al*., 2005). Similarly, CLC treatment increased the cryosurvival rates for sperm from lines of mice that did not freeze well (B6C3F1; B6SJLF/J), while it did little for sperm from mouse lines that cryopreserve well (ICR; B6D2F1; Loomis and Graham, 2008). When cholesterol content of the sperm from the different mouse strains was determined, the sperm from strains that did not freeze well contained less cholesterol than sperm that survived cryopreservation well (Loomis and Graham, 2008). The beneficial effects observed after cryopreservation are because of increasing cell cholesterol content and not to the cyclodextrins, because treating sperm with cyclodextrins alone prior to cryopreservation would remove cholesterol from cells (Companyo *et al*., 2007) which tends to decrease cryosurvival rates of sperms (Moce *et al*., 2010).

Again, sperm from all species do not react the same, because rabbit and rainbow trout sperm treated with cyclodextrin alone do not show depressed cryosurvival rates, but exhibit similar cryosurvival rates to untreated, control, sperm after thawing (Moce and Graham, 2005; Muller *et al*., 2008). The manners in which boar sperm react to CLC treatment is more complicated. Boar sperm membranes have low C:P (0.26) and are extremely sensitive to cold shock as well as changes in osmotic conditions (Watson, 1981). However, Zeng and Terada (2000, 2001) reported that further depleting cholesterol from boar sperm enhanced sperm quality after thawing. Conversely, others have reported that sperm treated with cyclodextrin alone exhibited higher percentages of acrosome-reacted sperm after thawing than did control sperm (Mao *et al*., 2005). In addition, others reported that increasing cholesterol content of boar sperm prior to freezing improves sperm quality after thawing (Torres *et al*., 2009). The response of boar sperm to treatment with cyclodextrins alone or treatment with CLC is highly variable, making it difficult to evaluate whether adding cholesterol or removing cholesterol improves boar cryosurvival rate (Blanch *et al*., 2008; Blanch *et al*., 2009).The reason for this lack of response to CLC treatment for cold-shock sensitive sperm is not known. Perhaps this lack of response is owing to differences in the specific phospholipid and fatty acid composition of boar sperm, which is different from sperm from other species (Watson, 1981). The cholesterol, itself, can be replaced with some cholesterol-like molecules (such as cholestanol or cholesteryl- pelargonate) and these molecules along with CLC produce similar beneficial cryosurvival results as cholesterol. However, not all of the cholesterol-conjugates were found to be effective in CLC treated cryopreservation (Amorim *et al*., 2009 and Moraes *et al*., 2010).

Sperm longevity

Ram sperm treated with CLC prior to cryopreservation not only exhibit higher cryosurvival rates immediately after thawing than control sperm, but these sperm maintain higher percentages of motile sperm after incubation at 38.5°C for three hours (Moce *et al*., 2010). These results seem promising, considering that maintaining higher percentages of motile sperm during incubation reflects a greater likelihood of the sperm to survive in the female reproductive tract, undergo capacitation and fertilize an oocyte (Fiser *et al*., 1991). Again, results obtained with boar sperm are controversial. Although addition of 10 mM cholesterol to an extender containing 20 mM of 2-hydroxypropyl-p-cyclodextrin resulted in higher percentages of total motile sperm after 2 h of incubation at 37°C compared to the control samples, it was lower than that obtained for sperm treated with cyclodextrin alone (Zeng and Terada, 2000). These results contradict other reports which show plasma membrane integrity being similar for CTC or cyclodextrin treated and control sperm, during 3 h incubation at 38°C (Blanch and Moce, 2007). Kumar, 2012 reported pre-freeze and post-thaw livability 83.25±0.28 and 74.16±0.59 after CLC treatment.

Capacitation Status by Chlortetracycline (CTC) Assay

An indirect test of capacitation status, using the antibiotic chlortetracycline (CTC) has provided a new insight into the functional status of spermatozoa (Fraser, 1995). With CTC as a fluorescent probe, spermatozoa show various fluorescent patterns, which have been correlated with predominantly, incapacitated or acrosome reacted sperm populations. Cooled and frozen-thawed spermatozoa apparently become more "capacitated" as determined by this test. Indeed, the antibiotic chlortetracycline accumulates and fluorescens in membrane compartments in which high concentrations of calcium ions exist next to hydrophobic sites (Tsien, 1989). It has been reported that CTC can be used as a fluorescent probe to visualize the course of capacitation and acrosome reaction in spermatozoa in mouse and bull (Fraser, 1995). A flow-cytrometric assay for capacitation has also been developed based on CTC-fluorescence (Maxwell and Johnson, 1997). CTC staining is currently an assay of choice because it distinguishes free stages of sperm activation, non capacitated acrosome -intact, and capacitated acrosome reacted. However, a clear understanding of how CTC interacts with the sperm surface at the molecular level is lacking and unfortunately, the evaluation of CTC staining is performed on fixed sperm cells.

Assessment of acrosomal status by Fluorescent isothiocynate-*Pisum sativum* (FITC-PSA)

The integrity of sperm membranes can be assessed also by using FITC-PSA (Maxwell and Johnson, 1997), which is reported to bind specifically to acrosomal contents (Cross *et al* 1986). Graham *et al*. (1990) established that fluorescently labelled PSA could used in flow cytometry to assess percentage of cells with or without intact acrosomes based on comparisons with napthol yellow/erythrosin B, and an assessment of percentage of cells with intact acrosome when acrosome reaction was induced with lysophosphatidylcholine. In order to detect changes taking place specifically in live sperm population, Maxwell and Johnson, (1997) stained spermatozoa with PI, and PI-positive cells were excluded from estimate of acrosome intact (low) and reacted (high FITC fluorescence). The proportion of acrosome reacted spermatozoa estimated by FITC-PSA fluorescence tended to be higher than CTC method but as yet acrosome status has not been confirmed in these spermatozoa by microscopy.

Intracellular calcium

Although importance of calcium to sperm function has been appreciated for many years (Yanagimachi, 1994), it is only in last decade that research has been able to directly assay Ca^{2+}, in mammalian spermatozoa. This was result of development and use of a variety of membrane permeable ion-selective fluorescent calcium probes that measure intracellular concentration of free (i.e., unbound) calcium (Thomas *et al*., 2006). Bailey *et al*, (1994) described changes in intra and extra cellular Ca^{2+} ion concentration by spectrofluorometer.

The mean Ca^{2+} of whole sperm population has been most commonly measured using fluorescence spectrometry, but these assays cannot detect variation in Ca^{2+} in different sperm subpopulations (Brewis *et al*., 1997). Assays based on measurement of individual cells using, fluorescence microscopy have recently been reported (Tesarik *et al*., 1996). Although it is technically difficult and time consuming, advances in low-level light detector systems and image processing have resulting in this approach becoming more widely employed (Florman *et al*., 1998).

Kadirvel *et al*. (2009) reported mean percentage of sperm cells with high and low intracellular calcium in fresh and frozen thawed spermatozoa and there was no significant difference between bulls. In fresh semen, significantly ($p< 0.01$) higher proportion of live sperm had low intracellular calcium (11.72±0.54) than that of frozen-thawed semen (43.68±2.65). There was significant increase in proportion of live sperm with high intracellular calcium after freezing–thawing.

Membrane fluidity

An increase in both plasma membrane phospholipid scrambling and phospholipid

disorder during capacitation is associated with enhanced plasma membrane fluidity. Increased fluidity is important for several events downstream of capacitation, including acrosomal exocytosis and sperm-oocyte fusion.

Many biological and biophysical factors may affect ability of sperm cells to prevent damage caused by freezing-thawing procedure (Holt, 2000) and factors involved in resistance of the plasma membrane to thermal and osmotic changes appear to be essential. These factors include membrane permeability, lipid composition and fluidity. Interspecies variations in spermatozoon membrane permeability have been shown to occur in birds under different osmotic conditions (Blanco *et al*., 2000). Membrane fluidity may be assessed by measuring florescence polarization anisotrophy of sperm membranes 1,6-diphenyl-l,3,5-hexatriene (DPH), a fluorescent dye inserted in lipid fraction of plasma membranes, and it has been shown to be a reliable predictor of sperm ability to resist freezing thawing damage in humans (Giraud *et al*., 2000).

Membrane fluidity has previously been measured in spermatozoa of many mammalian species (Giraud *et al*., 2000). It has been suggested that intra species differences in membrane fluidity are linked to lipid composition, and membrane fluidity has been shown in human to be related to restoration of membrane viability and motility after freezing. Kadirvel *et al*. (2009) reported significant ($p< 0.01$) increase in proportion of live sperm with high membrane fluidity in frozen-thawed semen than that of fresh semen. In fresh semen, only 25.67±1.23% of live spermatozoa showed high membrane fluidity, while 53.62±2.86% of live spermatozoa showed high membrane fluidity in frozen-thawed semen.

Protein tyrosine phosphorylation

Protein tyrosin phosphorylation increases in spermatozoa during capacitation in a number of species including mice, human, cattle, pigs, hamsters and cats (Leclerc *et al*., 1997; Kalab *el al*., 1988; Pukazenthi *et al*., 1998; Si and Okuno, 1999). Protein tyrosine phosphorylation appears to be a necessary pre requisite for a spermatozoan to fertilize an egg (Urner *et al*., 2001). In human spermatozoa, A-kinase anchorin protein (AKAPS) which is localized to fibrous sheath AKA P82, its precursor pro-AKA P82, and FSP95 are most prominent tyrosine phosphorylated proteins during capacitation (Carrera *et al*., 1996), indicating that affinity for regulatory subunit of protein kinase A or other molecules is regulated by their phosphorylation status. An 86kDa calcium binding antityrosine phosphorylation-regulated protein was also found to be localized in the principal piece of human spermatozoa (Naaby-Hansen *et al*., 2002).

In various species including humans (Luconi *et al*., 1996), cattle (Galantino-Homer *et al*., 1997) and swine (Flesch *et al*., 1999), a clear association between sperm capacitation and protein tyrosine phosphorylation has been established. Interestingly, such changes in the tyrosine phosphorylation of proteins have also been observed in frozen-thawed bull (Bailey *et al*., 2000) and boar (Tardif *et al*., 2001) spermatozoa, suggesting that cryopreservation could also induce protein tyrosine phosphorylation.

REFERENCES

1. Agnihotri, M.K. 1992. Current status and future prospects of buffalo meat production in India. *Buffalo J*. **8(2)**: 95-102.
2. Amann, R.P. and Pickett, B.W. 1987. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. Equine Vet. Sci. 7: 145-173.
3. Amorim, E.A.M., Graham, J.K., Spirizzi, B., Meyers, M. and Torres, C.A.A. 2009. Effect of cholesterol or cholesteryl conjugates on the cryosurvival of bull sperm. Cryobiology. 58: 210-214.
4. Andrabi, S.M.H., Ansari, M.S., Ullah, N., Anwar, M., Mehmood, A. and Akhter, S. 2008. Anti fertilization action of cholesterol. Arch. Androl. 5: 249-254.

5. Bailey, J.L., Bilodeau, J.F. and Cormier, N. 2000. Sperm cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl. 21: 1-7.
6. Bailey, J.L., Robertson, L. and Buhr, M.M. 1994. Calcium regulation, computerized motility parameters and the fertility of bovine spermatozoa. Can. J. Anim. Sci. 74: 53-58.
7. Blanch, E. and Moce, E. 2007. Perdida de viabilidad de los espermatozoides de verraco a lo largo del proceso de crioconservacio´ n. In: AIDA-ITEA (ed.), XII Jornadas sobre Produccio´ n Animal, Zaragoza, Spain. ITEA 28 (Tomo I), pp. 21-23.
8. Blanch, E., Toma´s C., Moce´ M.L., Viudes de Castro, M.P., Vicente J.S. and Moce´ E. 2008. Effect of cholesterol-loaded cyclodextrins and two diluents on boar sperm cryosurvival. In: Go´mez E(ed.), 1st Joint International Meeting of AERA-BAS, Gijo´ n, Spain. Reprod. Domest. Anim., 43: 71-72 (Abstract P59).
9. Blanch, E., Toma´s C., Moce´ M.L., Viudes de Castro, M.P., Vicente J.S. and Moce´ E. 2009. Influencia de la adicio´ n de colesterol al semen de verraco y el tiempo de almacenamiento a 16-C sobre la calidad del semen congelado. In: Asociacio´ n Interprofesional para el Desarrollo Agrario-AIDA (ed.), XIII Jornadas sobre Produccio´ n Animal, Zaragoza, Spain. AIDA, Tomo II, pp. 729-731.
10. Blanco-Rodriguez, J. and Martinez-Garcia, C. 2000. Apoptosis is physiologically restricted to a specialized cytoplasmic compartment in rat spermatids. Biol. Reprod. 61: 1541-1547.
11. Brewis, I.A. and Moore, H.D.M. 1997. Molecular mechanisms of gamete recognition and fusion at fertilization. Hum. Reprod. Natl. Suppl. JBFS. 2: 156-165.
12. Carrera, A., Moos, J., Ning., Gerton, G.L., Tesarik, J., Kopf, G.S. and Moss, S.B. 1996. Regulation of protein tyrosin phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: identification of a kinase anchor protein as major substrates for tyrosin phosphorylation. Dev. Biol. 180: 284-296.
13. Chohan, K.R., Iqbal, J., Asghar, A.A. and Chaudhary, M.A. 1992. Fertility of liquid and frozen semen in Nili-Ravi buffaloes. Pak. Vet. J. 12: 4-5.
14. Christian, A.E., Haynes, M.P., Phillips, M.C. and Rothblat, G.H. 1997. Use of cyclodextrins for manipulating cellular cholesterol content. J. Lipid Res. 38: 2264-2272.
15. Combes, G.B., Varner, D.D., Schroeder, F., Burghardt, R.C. and Blanchard, T.L. 2000. Effect of cholesterol on the motility and plasma membrane integrity of frozen equine spermatozoa after thawing. J. Reprod. Fertil. Suppl. 56: 127-132.
16. Companyo, M., Iborra, A., Villaverde, J., Martı´nez, P. and Morros, A. 2007. Membrane fluidity changes in goat sperm induced by cholesterol depletion using beta-cyclodextrin. Biochim. Biophys. Acta. 1768: 2246-2255.
17. Crockett, E.L. 1998. Cholesterol function in plasma membranes from ectotherms: membrane-specific roles in adaptation to temperature. Am. Zool. 38: 291-304.
18. Cross, N.L., Morales, P., Overstreet, J.W. and Hanson, F.W. 1986. Two simple methods for detecting acrosome-reacted human sperm. Gamete Res. 15: 213-226.

19. Darin-Bennett, A. and White, I.G. 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. Cryobiology. 14: 466-470.
20. De Graaf, S.P., Evans, G., Gillan, L., Guerra, M.M., Maxwell, W.M. and O'Brien, J.K. 2007. The influence of antioxidant, cholesterol and seminal plasma on the in vitro quality of sorted and nonsorted ram spermatozoa. Theriogenology, 67: 217-227.
21. Dobziuk, H. 2006. Molecules with holes– cyclodextrins. In: Dodziuk H (ed.), Cyclodextrins and their complexes. Wiley-VCH Verlag GmbH&Co, Weinheim, Germany, pp. 1-30.
22. Drobnis, E.Z., Crowe, L.M., Berger, T., Anchordoguy, T.J., Overstreet, J.W. and Crowe, J.H. 1993. Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. J. Exp. Zool. 265: 432-437.
23. FAOSTAT (2013) http://appsfao.org
24. Fiser, P.S., Hansen, C., Underhill, H. and Marcus, G.J. 1991. New thermal stress test to assess the viability of cryopreserved boar sperm. Cryobiology, 28: 454-459.
25. Flesch, F.M., Colenbrander, B., van Gold, L.M. and Gadella, B.M. 1999. Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. Biochem. Biophys. Res. Commun., 262: 787-792.
26. Florman, H.M., Arnoult, C., Kazam, I.G., Li, C. and O'Toole, M.B. 1998. A perspective on the control of mammalian fertilization by egg activated ion channels in sperm: a tale of two channels. Biol. Reprod., 59: 12-16.
27. Fraser, L.R. 1995. Ionic control of sperm function. Reprod. Fertil. Dev., 7: 905-925.
28. Gadella, B.M. and Harrison, R.A. 2002. Capacitation induces cyclic adenosine 3'5''-monophosphate dependent but apoptosisunrelated exposure of amino phospholipids at the apical head plasma membrane of boar sperm cells. Biol. Reprod., 67: 340-350.
29. Galantino-Homer, H.L., Viscont, P.E. and Kopf, G.S. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. Biol. Reprod., 56: 707-719.
30. Galantino-Homer, H.L., Zeng, W.X., Megee, S.O., Dallmeyer, M., Voelkl, D. and Dobrinski, I. 2006. Effects of 2-hydroxypropylbeta-cyclodextrin and cholesterol on porcine sperm viability and capacitation status following cold shock or incubation. Mol. Reprod. Dev., 73: 638-650.
31. Giraud, M.N., Motta, C., Boucher, D. and Grizard, G. 2000. Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. Human Reprod., 15: 2160-2164.
32. Graham, J.K., Kune, E and Hamerstedt, R.H. 1990. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flowcytrometry. Biol. Reprod., 43: 55-64.
33. Hammerstedt, R.H., Graham, J.K. and Nolan, J.P. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J. Androl., 11: 73-88.
34. Haranath, G.B., Suryaprakasam, T.B., Rao, A.V.N. and Somasekharam, G. 1990. Freezability of semen and fertility of frozen semen packaged in mini and medium French straws: a note. In: Acharya, R.M., Lokeshwar, R.R., Kumar, A.T. (eds) Proceedings of 2nd World Buffalo Cong, New Delhi, India. International Buffalo Federation, Roma, Italy, pp. 87-88.

35. Holt, W.V. 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology, 53: 47-58.
36. Kadirvel, G., Kumar, S., Kumaresan, A. and Kathiravan, P. 2009. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. Anim. Reprod. Sci., 116: 244–253.
37. Kalab, P., Peknicova, J., Geussova, G. and Moss, J. 1988. Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. Molecular Reprod. and Develop., 51: 304-314.
38. Kumar, A. 2012. Studies on effect of cholesterol loaded cyclodextrin on freezability and in vitro fertility of buffalo Spermatozoa. Ph.D. Thesis submitted to Deemed University, IVRI, Izatnagar, U.P., India.
39. Leclerc, P. deLamiraude, E. and Gaghon, C. 1997. Regulation of protein tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic Biol.Med., 22: 643-656.
40. Loomis, P.R. and Graham, J.K. 2008. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim. Reprod. Sci., 105: 119-128.
41. Luconi, M., Krausz, C., Forti, G. and Baldi, E. 1996. Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosin kinase activity during capacitation of human spermatozoa. Biol. Reprod., 55: 1135-1142.
42. Mao, J., Wu, G.M., Prather, R.S., Smith, M.F., Cantley, T., Rieke, A., Didion, B.A. and Day, B.N. 2005. Effect of methyl-β-cyclodextrin treatment of pig spermatozoa on in vitro fertilization and embryo development in the absence or presence of caffeine. Theriogenology, 64: 1913-1927.
43. Maxwell, W.M. and Johnson, L.A. 1997. Chlorotetracycline analysis of boar spermatozoa after incubation; flow cytrometric sorting, cooling, or cryopreservation. Mol. Reprod. Dev., 46: 408-418.
44. Misra, A.K., Patel, S.H., Joshi, B.V., Jaiswal, R.S. and Trivedi, K.R. 1994. Buffalo semen characters and its freezability under Indian conditions, pp. 495-497. In: Proceedings of 4th World Buffalo Congress. Sao Paulo, Brazil
45. Moce, E. and Graham, J.K. 2005. Effect of egg yolk, cooling⁄ thawing rates and cholesterol on cryosurvival of rabbit sperm. In: The American Society of Andrology (ed.), 30th Annual Meeting of the American Society of Andrology, Seattle, WA, USA. J Androl Suppl March–April, 79, abstract 121.
46. Moce, E. and Graham, J.K. 2006. Cholesterol-loaded cyclodextrins added to fresh bull ejaculates improve sperm cryosurvival. J. Anim. Sci., 84: 826-833.
47. Moce, E., Purdy, P.H. and Graham, J.K. 2010. Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. Anim. Reprod. Sci., 118: 236-247.
48. Moore, A.I., Squires, E.L. and Graham, J.K. 2005. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology, 51: 241-249.
49. Moraes, E.A., Graham, J.K., Torres, C.A., Meyers, M. and Spizziri, B. 2010. Delivering cholesterol or cholestanol to bull sperm membranes improves cryosurvival. Anim. Reprod. Sci., 118: 148-154.

50. Movassaghi, S., Saki, G., Javadnia, F., Panahi, M., Mahmoudi, M. and Rhim, F. 2009. Effects of methyl-beta-cyclodextrin and cholesterol on cryosurvival of spermatozoa from C57BL/6 mouse. Pak. J. Biol. Sci., 12: 19-25.
51. Muller, K., Muller, P., Pincemy, G., Kurz, A. and Labbe, C. 2008. Characterization of sperm plasma membrane properties after cholesterol modification: consequences for cryopreservation of rainbow trout spermatozoa. Biol. Reprod., 78: 390-399.
52. Naaby-Hansen, S., Mandal, A., Wolkowicz, M.J., Sen, B., Westbrook, V.A., Shetty, J., Coonrod, S.A., Klotz, K.L., Kim, Y.H., Bush, L.A., Flickinger, C.J. and Herr, J.C. 2002. CABYR, a novel calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein involved in capacitation. Dev. Biol., 242: 236-254.
53. Navratil, A.M., Bliss, S.P., Berghorn, K.A., Haughian, J.M., Farmerie, T.A., Graham, J.K., Clay, C.M. and Roberson, M.S. 2003. Constitutive localization of the gonadotropin-releasing hormone (GnRH) receptor to low density membrane microdomains is necessary for GnRH signaling to ERK. J. Biol. Chem., 278: 31593-31602.
54. Ollero, M., Muino-Blanco, T. Lo., pez-Perez, M.J. and Cebrian-Perez, J.A. 1996. Surface changes associated with ram sperm cryopreservation revealed by counter-current distribution in an aqueous two-phase system: effect of different cryoprotectants. J. Chromatogr. B. Biomed. Sci. Appl., 680: 157-164.
55. Parks, J.E., Meachan, T.N. and Saacke, R.G. 1981. Cholesterol and phospholipids of bovine spermatozoa. II. Effect of liposomes prepared from egg phosphatidylcholine and cholesterol on sperm cholesterol, phospholipids, and viability at 40C and 370C. Biol. Reprod., 24: 399-404.
56. Pukazhenthi, B.S., Wildt, D.E., Ottinger, M.A. and Howard, J. 1998. Inhibition of domestic cat spermatozoa acrosome reaction and zona pellucid penetration by tyrosin kinase inhibitors. Molecular Reprod. And Develop., 49: 48-57.
57. Purdy, P.H. and Graham, J.K. 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology, 48: 36-45.
58. Rajoriya, J.S., Prasad, J.K., Ghosh, S.K., Ramteke, S.S., Barik, N.C., Das, G.K., Pande, M. 2014. Cholesterol loaded cyclodextrin increases freezability of Buffalo bull (Bubalus bubalis) spermatozoa by increasing cholesterol to phospholipid ratio. Vet. World, 7(9): 702-706.
59. Raizada, B.C., Sattar, A. and Pandey, M.D. 1990. A comparative study of freezing buffalo semen in two dilutors. In: Acharya R.M., Lokeshwar R.R., Kumar A.T. (eds), Proceedings of 2nd World Buffalo Cong, New Delhi, India. International Buffalo Federation, Roma, Italy, pp. 66-74.
60. Shukla, M.K. and Mishra, A.K. 2005. Correlation between seminal characteristics in Murrah bulls. Indian J. Anim. Sci., 75(3): 263-266.
61. Si, Y. and Okuno, M. 1999. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. Biol. Reprod., 61: 240-246.
62. Singh, M., Singh, G.D. and Pant, H.C. 1992. A study on semen quality of Murrah buffalo bulls under temperate climate. Indian Vet. Med. J., 16: 194-197.
63. Tardif, S., Dube, C., Chevalier, S. and Baily, J.L. 2001. Capacitation is associated with tyrosine phosphorylationand tyrosine

kinase-like activity of pig sperm proteins. Biol. Reprod., 65: 784-792.
64. Tatham, B. 2000. Increasing Buffalo Production; Using Reproduction Technology. Report Rur. Indust. Res Corp. Dev., Kingston, ACT, Australia.
65. Tesarik, J., Carrears, A. and Mendoza, C. 1996. Single cell analysis of tyrosine kinase dependent and independent Ca2+ fluxes in progesterone induced acrosome reaction. Mol. Hum. Reprod., 2: 225-232.
66. Thomas, A.D., Meyers, S.A. and Ball, B.A. 2006. Capacitation-like changes in equine spermatozoa following cryopreservation. Theriogenology, 65: 1531-1550.
67. Torres, C.A.A., Moraes, E.A., Graham, J.K. and Romualdo, P.L. 2009. The effect of adding cholesterol-loaded cyclodextrin on boar sperm cryosurvival. In: Fazleabas A.T., Hunt P.A., Plant T.M., 42nd Annual Meeting of the Society for the Study of Reproduction (SSR) ‘‘Science for the Public Good’’, Pittsburgh, PA, USA. Biol. Reprod. Special Issue, 81: 455.
68. Tsien, R.Y. 1989. Fluorescent indicators of ion concentrations. In: D.L. Taylor and Y.L. Wang (Editors), Fluorescence Microscopy of Living cells in Culture. Part B. Quantitative Fluorescence Microscopy-Imaging and Spectroscopy. Method in Cell Biology, Volum 30, Chapter 5, Academic Press New York, pp. 127-156.
69. Urner, F., Leppens-Luisier, G. and Sakkas, D. 2001. Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. Biol. Reprod., 64: 1350-1357.
70. Watson, P.F. 1981. The effects of cold shock on sperm cell membranes. In: Morris G.J., Clark A (eds), Effects of Low Temperatures on Biological Membranes. Academic Press, London, pp. 189-218.
71. Wilhelm, K.H., Graham, J.K. and Squires, E.L. 1996. Effects of phosphatidylserine and cholesterol liposomes on the viability, motility, and acrosomal integrity of stallion spermatozoa prior to and after cryopreservation. Cryobiology, 33: 320-329.
72. Yanagimachi, R. 1994. Mammalian fertilization. In: Physiology of Reproduction. 2nd Ed. E. Knobil and J.D. Neill, eds. Raven press, New York. pp. A189-A317.
73. Yancey, P.G., Rodrigueza, W.V., Kilsdonk, E.P.C., Stoudt, G.W., Johnson, W.J., Phillips, M.C. and Rothblat, G.H. 1996. Cellular cholesterol efflux mediated by cyclodextrins. Demonstration of kinetic pools and mechanism of efflux. J. Biol. Chem., 271: 16026-16034.
74. Zeng, W. and Terada, T. 2000. Freezability of boar spermatozoa is improved by exposure to 2-hydroxypropyl-beta-cyclodextrin. Reprod. Fertil. Dev., 12: 223-228.
75. Zeng, W. X. and Terada T. 2001. Effects of methyl-beta-cyclodextrin on cryosurvival of boar spermatozoa. J. Androl., 22: 111-118.
76. Zidovetzki, R. and Levitan, I. 2007. Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochim. Biophys. Acta., 1768: 1311–1324.

