

CRYOPRESERVATION INDUCES CAPACITATION-LIKE CHANGES OF THE SWAMP BUFFALO SPERMATOZOA

D.J. Talukdar¹, K. Ahmed¹, S. Sinha¹, S. Deori^{2*}, G.C. Das¹ and Papori Talukdar³

ABSTRACT

A total of forty ejaculates were collected from eight buffalo bulls by artificial vagina method. Each ejaculate was splitted into two parts, one part was used for *in vitro* capacitation by TALP medium, and the rest was frozen in Tris-egg yolk-citrate-glycerol extender. The results revealed that the live acrosome reacted and total acrosome reacted frozen thawed spermatozoa differed significantly ($P<0.01$) from *in vitro* capacitated spermatozoa at different hours of incubation. The incidence of hyperactivated motility, total HOST-reacted spermatozoa, sperm membrane protein and cholesterol level of frozen thawed spermatozoa also differed significantly ($P<0.01$) from *in vitro* capacitated spermatozoa during different hours of incubation except at 0 h for hyperactivated motility, 6 h for total HOST-reacted spermatozoa, 1 h for sperm membrane protein level and at 2 h for cholesterol level. The study suggests that cryopreservation induces capacitation-like changes of the swamp buffalo spermatozoa in respect of acrosomal status, plasma membrane integrity, membrane protein and cholesterol levels.

Keywords: capacitation, cryopreservaion, spermatozoa, swamp buffalo

INTRODUCTION

Artificial insemination (AI) is useful for the improvement of milk productivity in buffaloes by the propagation of animals with high genetic potential (Ciptadi *et al.*, 2012). The best preservation technique to date of post-thaw survival is restricted to about 50% of the sperm population (Watson, 1995). The final cryopreservation goal of semen is not only to maintain the initial motility but also to maintain the necessary metabolism to produce viable sperm to survive in the female reproductive tract at the time of fertilization. The freezing and thawing process provokes morphological or biochemical cryogenic damage resulting in sperm dysfunction and changes in cell's membrane. The exposure of spermatozoa to low temperatures shortens their capacitation time, changing the membrane lipid architecture, membrane permeability and the reducing efficiency of enzymes extruding calcium ions. These changes resemble capacitation, and are likely to reduce long-term sperm viability and alter their motility. The term "cryocapacitation" have been introduced to emphasize the fact that cryopreservation procedures induce capacitation-like changes in spermatozoa (Watson, 1995; Cormier and Bailey, 2003). These cooling-related capacitation-like changes in spermatozoa, may affect the fertility of

¹College of Veterinary Science, Khanapara, Guwahati, Assam, India

²National Research Centre on Yak, Dirang, Arunachal Pradesh, India, *E-mail: sourabhd1@rediffmail.com

³National Dairy Research Institute, Karnal, Haryana, India

cryopreserved semen, by rendering the cells less stable in the female reproductive tract, after artificial insemination and therefore relatively short-lived. Such changes cannot easily be distinguished from true capacitation. Keeping in view, the study had been planned with the objective to study the functional characteristics of *in vitro* capacitated and frozen-thawed spermatozoa of swamp buffalo bull.

MATERIALS AND METHODS

A total of 40 semen samples were collected by artificial vagina method from each eight swamp buffalo bulls aged five to eight years maintained at College of Veterinary Science, Guwahati, Assam, India. Each ejaculate was evaluated for volume, mass activity, and initial motility immediately after collection. Samples having volume 1.0 ml or more, mass activity 3 or more and initial sperm motility 70% or more were divided into two parts, one part was used for *in vitro* capacitation by TALP medium (NaCl-92.9 mM; KCl-4 mM; NaHCO₃-25.9 mM; CaCl₂·2H₂O-10 mM; MgCl₂·6H₂O-0.5 mM; sodium lactate-7.6 mM; sodium pyruvate-1.3 mM; HEPES-20 mM; glucose-0.25%; heparin-200 µg/ml, BSA-0.6%, Penicillin G-40 IU/ml, Streptomycin sulphate-50 µg/ml and Deionised triple distilled water up to 1000 ml) at concentration of 6×10^9 spermatozoa/ml (Rogers and Yanangimachi, 1975) at 37°C with 5% CO₂ for 6 h and the rest was frozen with Tris-egg yolk-citrate extender [Tris (hydroxy methyl) amino methane-2.422 g; citric acid monohydrate-1.36 g; fructose-1.0 g; penicillin G sodium-1000 IU/ml; streptomycin sulphate-100 mg/ml; double distilled water up to 100ml] with 20% egg yolk and 6.4% glycerol to yield approximately 60 million motile

sperm/ml. The extended semen was equilibrated for 4 h at 5°C before filling in 0.25 ml French mini straws. After filling and sealing, the semen straws were placed in a rack at 4 cm above liquid nitrogen in the vapour phase for 8 min and finally plunged into liquid nitrogen container (-196°C) and stored. The frozen semen straws were thawed at 37°C for 30 seconds. Each sample of *in vitro* capacitated sperm was evaluated at one hour interval starting from 0 h and all the samples of frozen thawed and *in vitro* capacitated were evaluated for hyperactivated motility (Marquez and Susan, 2004), total HOST-reacted sperm (Jeyendran *et al.*, 1984), acrosomal status by using FITC labelled *Pisum sativum* agglutinin (Kaul *et al.*, 2001) and live intact acrosome using Eosin-Nigrosin-Giemsa staining technique (Tamuli and Watson, 1994), sperm membrane protein (Cheema *et al.*, 2011) and cholesterol level (Srivastava *et al.*, 2013) by using quality kit (Siemens Ltd., 589, Sayajipura, Ajwa Road, Vadodara-390 019, Gujarat, India) in a Systronics Spectrophotometer 106. The statistical analysis of the data was done using SAS Enterprise Guide 4.2 version.

RESULTS AND DISCUSSION

The results of analysis of variance (ANOVA) are presented in Table 1. ANOVA revealed highly significant effect of source (*in vitro* capacitated vs frozen thawed spermatozoa).

Before fertilizing of the oocyte, mammalian spermatozoa undergo the sequence of membrane alterations associated with accumulation of calcium ion and the increase of tyrosine phosphorylation resulting in sperm hyperactivation (Hewitt and England, 1998; Petrunkina *et al.*, 2003), which is characterized by high-amplitude and asymmetrical

Table 1. Functional integrity of *in vitro* capacitated and frozen thawed swamp buffalo spermatozoa (mean^a± se).

Stage Parameters	<i>In vitro</i> capacitation (hours)							Frozen Thawed
	0	1	2	3	4	5	6	
Hyperactivated Motility (%)	14.00 ^a ± 0.80	35.75 ^b ±1.12	52.87 ^c ±1.38	60.25 ^d ±1.05	74.50 ^e ±1.78	47.50 ^f ±1.11	41.25 ^g ±1.30	16.00 ^a ±0.76
Total HOST reacted Sperm (%)	82.55 ^a ± 1.00	78.02 ^b ±1.26	77.22 ^b ±1.12	75.55 ^{bc} ±1.21	71.70 ^{cd} ±1.49	69.25 ^d ±1.61	61.57 ^e ±2.20	62.20 ^e ±1.31
Live acrosome reacted sperm (%) (Eosin-Nigrosin-Giemsa stain)	8.62 ^a ±0.33	45.57 ^b ±2.97	52.42 ^{cd} ±2.49	50.92 ^{bd} ±2.39	56.92 ^c ±1.88	54.27 ^{cd} ±1.83	49.37 ^{bd} ±2.59	27.28 ^e ±0.81
Total acrosome reacted sperm (%) (FITC-PSA)	7.90 ^a ±0.59	43.50 ^b ±2.46	53.20 ^c ±1.44	68.00 ^d ±1.87	69.65 ^d ±1.39	68.47 ^d ±1.42	75.12 ^e ±1.24	38.72 ^f ±1.79
Sperm membrane Protein (mg/ 10 ⁹ sperm)	5.13 ^a ±0.12	3.92 ^b ±0.13	3.25 ^c ±0.12	2.64 ^d ±0.11	2.55 ^d ±0.13	1.82 ^e ±0.06	1.45 ^f ±0.08	3.95 ^b ±0.10
Cholesterol (µg/ 10 ⁸ sperm)	21.95 ^a ±0.44	19.08 ^b ±0.51	14.65 ^c ±0.51	12.94 ^d ±0.47	11.07 ^e ±0.59	8.45 ^f ±0.54	5.64 ^g ±0.46	14.74 ^c ±0.60

*40 observations.

Means bearing different superscripts in a row differ significantly.

flagellar beating that assists sperm in penetrating the oocyte zona pellucida (Marquez and Susan, 2004). Hyperactivation has been considered part of the capacitation process because sperm have been observed to hyperactivate while undergoing capacitation. During capacitation, several sperm proteins become phosphorylated on tyrosine residues and this phosphorylation has been demonstrated to be regulated by a cAMP pathway through activation of protein kinase A (PKA). Some of the proteins that become tyrosine phosphorylated during capacitation have been localized to the flagellum, and therefore it has been proposed that they are involved in hyperactivation (Si and Okuno, 1999). The incidence of hyperactivated motility of frozen thawed spermatozoa in the present study differed significantly ($P < 0.01$) with hyperactivated motility of *in vitro* capacitated spermatozoa at different hours of incubation and the incidence was slightly higher than 0 h and lower than 1 h which might be due to cryopreservation as cryopreserved spermatozoa have poor calcium efflux mechanisms and are less efficient in extruding calcium ions resulting in rapid accumulation of cytosolic calcium ion (Bailey *et al.*, 1994) and when these intracellular ion concentrations reach threshold levels, capacitation followed by acrosome reaction is triggered without the activation of zona pellucida receptors in the sperm plasma membrane or the associated signal transduction system (Bailey *et al.*, 2000).

The determination of the acrosome status in cryopreserved sperm is of the fundamental importance as cryopreservation directly damages sperm membrane, which could be followed by a loss of the acrosomal matrix contents. The incidence of total live acrosome reacted spermatozoa in the present observation differed significantly ($P < 0.01$) between *in vitro* capacitated spermatozoa at

different hours of incubation and frozen thawed spermatozoa. The incidence of live acrosome reacted spermatozoa in frozen thawed spermatozoa was higher than 0 h and lower than 1 h of incubation, which might be due to partial capacitation of buffalo spermatozoa occurs during freezing and thawing followed by equilibration (Watson, 1995). There is substantial evidence that cryopreservation promotes the premature capacitation of spermatozoa (Bailey *et al.*, 2000; Watson, 2000) and this cryocapacitation is frequently cited as one of the factors associated with the reduced longevity of cryopreserved spermatozoa in the female reproductive tract by a loss of the acrosomal matrix contents. Detection of the viability and stages of acrosomal exocytosis, either spontaneous or induced, was carried out using fluorescent probes. FITC-*Pisum sativum* lectin (FITC-PSA) was used to assess acrosomal status by staining glycoproteins in the acrosome of permeabilised spermatozoa. Fluorescein-conjugated plant lectins like fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) from the edible pea has been used as a selective acrosomal staining of the spermatozoa of human (Cross *et al.*, 1986), stallion (Casey *et al.*, 1993) and monkey (Cross *et al.*, 1989). As PSA binds to the acrosomal contents, the progress of the acrosome reaction is indicated by the intensity and distribution of fluorescence over the acrosomal region. The acrosome reaction in buffalo spermatozoa commences just anterior to the equatorial segment and proceeds in an arborizing fashion towards the apical ridge (Watson and Plummer, 1986). Therefore, as the reaction progresses, more acrosomal contents will be lost and, therefore, less fluorescence will be seen. Staining only in the equatorial segment is also characteristic of a cell that has only recently completed its acrosome

reaction whereas cells devoid of staining in this region have fully completed the acrosome reaction some time previously (Tesarik *et al.*, 1993). The total acrosome reacted (PSA-ve) spermatozoa in the present observation differed significantly ($P<0.01$) between frozen thawed spermatozoa and *in vitro* capacitated spermatozoa at different hours of incubation. The incidence in frozen thawed spermatozoa was slightly higher than in 0 h and lower than in 1 h of incubation. This might be due to cryocapacitation which could be followed by a loss of the acrosomal matrix contents (Cross *et al.*, 1986).

The plasma membrane integrity of sperm is of crucial importance for optimal sperm function and only a sperm with an intact plasma membrane can undergo a series of complex changes in the female reproductive tract and can acquire the ability to fertilize an oocyte (Yanagimachi, 1994). The mean percentage of total HOST reacted spermatozoa of frozen thawed semen in the present study differed significantly ($P<0.01$) with *in vitro* capacitated spermatozoa at different hours of incubation except 6 h where the values was in close proximity. This might be due to cryopreservation results partial capacitation that induced membrane phase changes, which are thought to result in lateral phase separation of membrane components and increased membrane permeability for solutes (Hammerstedt *et al.*, 1990). The disruption of plasma membrane integrity caused by disarrangement of lipids within the membrane during cryocapacitation may induce further cellular damage and consequently lead to irreversible damage to its integrity (Jeyendran *et al.*, 1984; Watson, 1995).

Capacitation is a post-testicular developmental and maturational process of mammalian spermatozoa occurring during their transit through the female reproductive tract with

modification of sperm surface proteins, added or removed and an array of proteins have been shown to undergo tyrosine phosphorylation in different species (Luconi *et al.*, 1996; Galantino *et al.*, 1997). During fertilization, mammalian sperm membrane proteins are also involved in the penetration of cumulus matrix, recognition of zona pellucida and fusion with the oocyte plasma membrane (Myles and Primakoff, 1997). In the present observations, there was leakage of proteins from the frozen thawed as well as capacitated spermatozoa but the leakage was significantly more in the latter as compared to the former. However, the leakage of proteins in the frozen thawed spermatozoa may be because of acrosomal damage and in capacitated, because of acrosomal damage as well as due to capacitation and acrosome reaction. As the mean sperm membrane protein level of spermatozoa in frozen thawed semen differed significantly ($P<0.01$) from *in vitro* capacitated spermatozoa at different hours of incubation except 1 h where the values was in close proximity. The similarity of sperm membrane protein level in frozen thawed and *in vitro* capacitated sperm at 1 h of incubation at 37°C in TALP medium in the present study indicated the initiation of capacitation of spermatozoa which corroborated the findings of earlier workers (Watson, 1995; Cromier and Bailey, 2003; Kadirvel *et al.*, 2009) who reported that the cryopreserved sperm are in a partially capacitated state. The alteration in the sperm membrane proteins might be due to sublethal damage which was occurred during cryopreservation leading to loss of sperm surface proteins (Lessard *et al.*, 2000), segregation of membrane proteins (De Leeuw *et al.*, 1990), inactivation of membrane-bound enzymes and decreased lateral protein diffusion within the membrane (Watson, 1995). The molecular mechanisms of capacitation are not

completely elucidated; however, recent studies have demonstrated involvement of numerous structural and biochemical modifications in spermatozoa, such as changes in membrane composition and membrane fluidity (Harrison *et al.*, 1996; Green and Watson, 2001), increased intracellular calcium (Visconti *et al.*, 1998), activation of ion channels (Florman *et al.*, 1998) and generation of reactive oxygen species (de Lamirande *et al.*, 1997). Various reports suggested an active participation of the sperm plasma membrane in the process of capacitation, mainly through the loss of cholesterol (Cross, 1998; Visconti *et al.*, 1999). The cholesterol efflux during *in vitro* capacitation increases the disorder of phospholipid packing, and results in increased bilayer permeability (Cross, 1998). Similar to physiological and *in vitro* capacitation, a significant reduction of cholesterol content after cryopreservation was observed in the present study. Our results are in agreement with Cerolini *et al.* (2001) and Kadirvel *et al.* (2009) who observed decreased free cholesterol content and increased phospholipids and triglycerol content after freezing-thawing of boar semen. Furthermore, sperm membranes are known to release phospholipids into surrounding medium during cold shock (Darin-Bennett *et al.*, 1973). The mechanism of loss of cholesterol during cryopreservation is not completely understood. However, most of the cholesterol loss is due to slow diffusion from cell and a net transfer of cholesterol from rat and bovine sperm to the medium has already been demonstrated (Ehrenwald *et al.*, 1988). Release of cholesterol from the membrane plane, a probable consequence of peroxidative damage to membrane lipids, could lead to the premature capacitation of cryopreserved bull spermatozoa (Cormier *et al.*, 1997). The loss of phospholipids in cryopreserved samples occurs at a more rapid rate

when compared to fresh samples and follows the pattern expected for lipid peroxidation (Alvarez and Storey, 1992). The present study observed that the mean cholesterol level of spermatozoa in frozen thawed semen differed significantly ($P < 0.01$) from *in vitro* capacitated spermatozoa at different hours of incubation except 2 h where the values were in close proximity. Therefore, cholesterol efflux may represent an integral part of the intrinsic regulatory property of sperm to undergo capacitation-like changes during cryopreservation. The reduction in cholesterol content which was similar in post thawed spermatozoa and *in vitro* capacitated spermatozoa after 2 h of incubation indicated the partial capacitation status of post thawed spermatozoa.

CONCLUSION

In conclusion, this study showed that cryopreservation induces capacitation-like changes of the Swamp buffalo spermatozoa in respect of acrosomal status, plasma membrane integrity, membrane protein and cholesterol levels.

ACKNOWLEDGEMENTS

The author expresses gratefulness to the Director of Post Graduate Studies, Assam Agricultural University, and to Dr. R.N. Goswami, the Dean, Faculty of Veterinary Science, Assam Agricultural University, Khanapara for providing necessary facilities needed for the study.

REFERENCES

- Alvarez, J.G. and B.T. Storey. 1992. Evidence for increased lipid peroxidation damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J. Androl.*, **13**: 232-241.
- 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.
- Bailey, J.L., L. Robertson and M.M. Buhr. 1994. Relationships among in vivo fertility, computer-analysed motility and in vitro Ca^{2+} flux in bovine spermatozoa. *Can. J. Anim. Sci.*, **74**: 53-58.
- Casey, P.J., R.B. Hillman, K.R. Robertson, A.I. Yudin, I.K. Liu and E.Z. Drobnis. 1993. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. *J. Androl.*, **14**: 289-297.
- Cerolini, S., A. Maldjan, F. Pizzi and T.M. Ghazzi. 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Reproduction*, **121**: 395-401.
- Cheema, R., A.K. Bansal, G.C. Bilaspuri and V.K. Gandotra. 2011. Correlation between the proteins and protein profile(s) of different regions of epididymis and their contents in goat buck. *Anim. Sci. Pap. Rep.*, **29**: 75-84.
- Ciptadi, G.M., A. Nasich, N. Budiarto and V.M.A. Nurgartiningasih. 2012. The estrus synchronization response following PGF 2α treatment in Indonesian Madura cattle with different body condition scores. *Pak. Vet. J.*, **32**: 624-626.
- Cormier, N. and J.L. Bailey. 2003. A differential mechanism is involved during heparin-and cryopreservation-induced capacitation. *Biol. Reprod.*, **69**: 177-185.
- Cormier, N., M.A. Sirard and J.L. Bailey. 1997. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *J. Androl.*, **18**: 461-468.
- Cross, N.L. 1998. Role of cholesterol in sperm capacitation. *Biol. Reprod.*, **59**: 7-11.
- Cross, N.L., P. Morales, M. Fukuda and E. Behboodi. 1989. Determining acrosomal status of the cynomolgus monkey (*Macaca jbscicularis*) sperm by fluorescence microscopy. *Am. J. Primatol.*, **17**: 157-163.
- Cross, N.L., P. Morales, J.W. Overstreet and F.W. Hanson. 1986. Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res.*, **15**: 213-226.
- Darin-Bennett, A., A. Poulos and I.G. White. 1973. The effects of cold shock and freeze-thawing on release of phospholipids by ram, bull and boar spermatozoa. *Aust. J. Biol. Sci.*, **26**: 1409-1420.
- De Lamirande, E., H. Jiang, A. Zini, H. Kodama and C. Gagnon. 1997. Reactive oxygen species and sperm physiology. *Rev. Reprod.*, **2**: 48-54.
- De Leeuw, F.E., H.C. Chen, B. Colenbrander and A.J. Verkleij. 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. *Cryobiology*, **27**: 171-183.
- Ehrenwald, E., J.E. Parks and R.H. Foote. 1988. Cholesterol efflux from bovine sperm. I. Induction of the acrosome reaction with lysophosphatidyl choline after reducing sperm cholesterol. *Gamete Res.*, **20**: 145-157.
- Florman, H.M., C. Arnoult, I.G. Kazam, C. Li and M.B. O'Toole. 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.
- Galantino-Homer, H.L., P.E. Visconti and G.S. Kopf. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by cyclic adenosine 3'5'-monophosphate-dependent pathway. *Biol. Reprod.*, **56**: 707-719.
- Green, C.E. and P.F. Watson. 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reproduction*, **122**: 889-898.
- Hammerstedt, R.H., J.K. Graham and J.P. Nolan. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. *J. Androl.*, **11**: 73-88.
- Harrison, R.A.P., P.J.C. Ashworth and N.G.A. Miller. 1996. Bicarbonate/CO₂, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.*, **45**: 378-391.
- Hewitt, D.A. and G.C. England. 1998. An investigation of capacitation and the acrosome reaction in dog spermatozoa using a dual fluorescent staining technique. *Anim. Reprod. Sci.*, **51**: 321-332.
- Jeyendran, R.S., H.H. Van der Ven, M. Perez-Pelaez, B.G. Carbo 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.
- Kadirvel, G., S. Kumar, A. Kumaresan and P. Kathiravan. 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.
- Kaul, G., G.S. Sharma, B. Singh and K.K. Gandhi. 2001. Capacitation and acrosomal reaction in buffalo bull spermatozoa assessed by chlortetracycline and *Pisum sativum* agglutinin fluorescence assay. *Theriogenology*, **55**: 1457-1468.
- Lessard, C., S. Parent, P. Leclerc, J.L. Bailey and R. Sullivan. 2000. Cryopreservation alters the level of the bull sperm surface protein P25b. *J. Androl.*, **21**: 700-707.
- Luconi, M., C. Krausz, G. Forti and E. Baldi. 1996. Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol. Reprod.*, **55**: 207-216.
- Marquez, B. and S.S. Susan. 2004. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol. Reprod.*, **70**: 1626-1633.
- Myles, D.G. and P. Primakoff. 1997. Why did the sperm cross the cumulus? To get to the oocyte. Functions of the sperm surface proteins PH-20 and fertilin in arriving at, and fusing with, the egg. *Biol. Reprod.*, **56**: 320-327.
- Petrunkina, A.M., K. Simon, A.R. Gunzel-Apel and E. Topfer-Petersen. 2003. Regulation of capacitation of canine spermatozoa during co-culture with heterologous oviductal epithelial cells. *Reprod. Domest. Anim.*, **38**: 455-463.
- Rogers, B.J. and R. Yanagimachi. 1975. Retardation of guinea-pig sperm acrosome reaction by glucose: The possible importance of pyruvate and lactate metabolism in capacitation and the acrosome reaction. *Biol. Reprod.*, **13**: 568-575.

- Si, Y. and M. Okuno. 1999. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol. Reprod.*, **61**: 240-246.
- Srivastava, N., S.K. Srivastava, S.K. Ghosh, K. Amit, P. Perumal and A. Jerome. 2013. Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa. *Asian Pac. J. Reprod.*, **2**: 126-131.
- Tamuli, M.K. and P.F. Watson. 1994. Use of a simple staining technique to distinguish acrosomal changes in the live sperm sub-population. *Anim. Reprod. Sci.*, **35**: 247-250.
- Tesarik, M.D., C. Mendoza and A. Carreras. 1993. Fast acrosome reaction measure: a highly sensitive method for evaluating stimulus-induced acrosome reaction. *Fertil. Steril.*, **59**: 424-430.
- Visconti, P.E., H. Galantino-Homer, G.D. Moore, J.L. Bailey, X.P. Ning, M. Fornes and G.S. Kopf. 1998. The molecular basis of sperm capacitation. *J. Androl.*, **19**: 242-248.
- Visconti, P.E., X. Ning, M.W. Fornes, J.G. Alvarez, P. Stein, S.A. Connors and G.S. Kopf. 1999. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Dev. Biol.*, **214**: 429-443.
- Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and assessment of their post-thawing function. *Reprod. Fertil. Dev.*, **7**: 871-891.
- Watson, P.F. 2000. The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.*, **60-61**: 481-492.
- Watson, P.F. and J.M. Plummer. 1986. Relationship between calcium binding sites and membrane fusion during the acrosome reaction induced by ionophore in ram spermatozoa. *J. Exp. Zool.*, **238**: 113-118.
- Yanagimachi, R. 1994. Mammalian fertilization, p. 189-317. *In The Physiology of Reproduction*. Raven Press, New York.