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Lipid peroxidation status and plasma membrane integrity of frozen thawed buffalo semen treated with heparin binding protein

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Abstract
The experiment was conducted to study the sperm lipid peroxidation status and plasma membrane integrity of frozen thawed buffalo semen treated with heparin binding protein (HBP). Buffalo semen straws from 10 bulls were procured from Central Frozen Semen Production and Training Institute, Hesseraghatta, Banglore-560088. The frozen straws were thawed at 37 ºC for 30 seconds and emptied into a 15mL sterile plastic centrifuge tube containing 1mL capacitation medium (control), addition of 25µg/mL (treatment I), 50µg/mL (treatment II) and 100µg/mL (treatment III) of HBP. The contents were incubated at 37 ºC for 2 hours. The sperm LPO status was measured by production of malondialdehyde (MDA) level. The MDA levels were significantly (P<0.05) low in HBP treatment I (1.34 µmol/ml ± 0.08) and II (1.23 µmol/ml ± 0.06) and III (1.21 µmol/ml ± 0.03) when compared to control (1.85 µMol/mL ± 0.02). MDA levels did not differ significantly among different treatments. The sperm functional membrane integrity was studied by hypo-osmotic swelling test (HOST). The functional membrane integrity of spermatozoa in HBP treatment I, II III and control were 59.20% ± 0.90, 53.50% ± 0.71,52.00% ± 0.47and 51.50% ± 0.73, respectively. Significantly (P<0.05), higher percentages of spermatozoa were lost their plasma membrane integrity in HBP treatments as compared to control. It is evident from this study that supplementation of HBP in capacitation medium reduces sperm lipid peroxidation and destabilizes plasma membrane integrity.

Keywords: Heparin binding protein, lipid peroxidation, plasma membrane integrity, spermatozoa, buffalo semen

Introduction
Indian buffaloes are an important source of milk supply today and yield nearly three times as much milk as cows. In order to achieve the maximum production from these animals, it is necessary that maximum number of females to be served with the semen from bulls of superior genetic constitution. The fertility in bovines is directly correlated with the quality of spermatozoa used for fertilization [1]. Though artificial insemination (AI) is already used as a tool for the production of successful next generation calves in cattle, it is not so powerful tool in case of buffaloes. Reduced efficiency of AI in buffaloes has necessitated to find out the factors that contribute to the infertility. Several studies have been conducted in the past decades in search of exact potential fertility markers in bulls [2].

The influence of seminal proteins in male reproduction has drawn attention because many studies proved that their expression is associated with fertility scores in dairy cattle [3], beef cattle [4] and horses [5]. Studies have proved correlations between non return rates of bulls and seminal plasma proteins [6, 7, 8]. Some of these proteins are bound to the sperm surface during ejaculation and thus protein-coating layers are formed [9]. In the female reproductive tract, seminal plasma proteins bound to the sperm surface most probably participate first in the formation of the oviductal sperm reservoir [10, 11]; second, in the control of sperm capacitation by the intensive action of negative (decapacitation factors) and positive regulatory (capacitation-stimulating factors) factors, and finally in central fertilization events such as sperm-zona pellucida interaction and sperm-egg fusion [12, 13].

A number of seminal plasma proteins have been investigated which act as molecular markers of fertility in different species [14]. Proteins such as bovine seminal plasma proteins (BSP A1, A2 and A3),heparin binding proteins (HBPs),osteopontin (OPN), fertility associated antigen (FAA), prostaglandin D synthase, phospholipase A2, sperm adhesion Z13, clusterin (CLU)

and heat shock proteins (HSPs) have been reported as markers of fertility [14]. HBP is one of seminal plasma proteins and has been identified in bovine seminal plasma which are shown to bind with sperm membrane and affect a series of events that contribute to normal fertility, such as sperm capacitation [15], formation of the oviduct reservoir [16] and binding to the oocyte [17].

HBPs have been associated with removal of cholesterol from sperm membrane during capacitation [18]. Cholesterol is believed to limit protein insertion into the phospholipid bilayer, to restrict lateral motility of membrane components and to modulate the activity of membrane proteins by changing their conformation [19]. The ability of spermatozoa to transport compounds selectively across its membrane is essential for maintenance of sperm motility, induction of acrosome reaction and other events associated with fertilization and binding of the spermatozoa to the oocyte surface [20]. The integrity of sperm plasma membrane for successful cryopreservation is important and it is well known that rearrangement of sperm plasma membrane occurs during freezing and thawing [21].

Cholesterol is recognized to have a stabilizing effect on membranes [22] and changes in cholesterol level in the plasma membrane over the middle piece and principal piece increases the lateral mobility of membrane components [23]. Exposure of sperm for 4 h to BSP leads to about 25% cholesterol efflux [24]. Cholesterol efflux is expected to provoke reorganization or destabilization of the membrane [25].Spermatozoa itself produce small amounts of ROS that are essential for many of physiological processes i.e. capacitation, hyperactivation and sperm oocyte fusion [26]. But, excessive levels can negatively impact the quality of spermatozoa and impair the fertilizing capacity [27]. Addition of H_2O_2 causes significant increase in LPO levels in frozen thawed bull spermatozoa and supplementation of 28to 30 kDa HBP helped in controlling the oxidative stress of sperm cells induced by H_2O_2 [28].

Fresh spermatozoa with higher cholesterol content are expected to resist destabilization of membrane following cryopreservation. Since membrane integrity is altered with cryopreservation, spermatozoa with more cholesterol content will resist membrane damage [29]. Hence, the study was carried out to determine the effects of HBP on lipid peroxidation status and plasma membrane integrity of frozen thawed buffalo semen.

Materials and Methods

Materials

All the plasticware used for this study were purchased from Tarson, India. All the glassware used in this study were purchased from Borosil, India. All chemicals used in this study were procured from Sigma-Aldrich chemicals Co., USA.

Methods

Capacitation stock solution

Capacitation stock solution / Sperm TALP was prepared as detailed below.

Components	For 1000 mL (in mM)
Sodium chloride (NaCl)	114
Potassium chloride (KCl)	3.2
Calcium chloride ($CaCl_2.2H_2O$)	2.0
Magnesium chloride ($MgCl_2.6H_2O$)	0.5
Sodium lactate (60% syrup)	1.86 µl/mL
Sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$)	0.34
Phenol red	10µg/mL
Pyruvate stock solution	
Sodium pyruvate	22mg/10 mL capacitation medium
Heparin stock solution	
Heparin	5mg/10mL capacitation medium
Gentamicin stock solution	
Gentamicin	50mg/mL in saline

All the stock solutions were filter sterilized (0.2 µm) and stored at 4˚C until use.

Capacitation working solution

Components	For 10 mL
Capacitation stock solution	9.5 mL
Pyruvate stock solution	0.1 mL
Heparin stock solution	0.4 mL
Gentamicin stock solution	10 µL
Bovine serum albumin (Fatty acid free)	60 mg

The working solution was prepared freshly, filter sterilized (0.2 µm) and pre-warmed at 37 ˚C for 30 minutes before use. pH and osmolality of the stock and working medium were maintained at 7.6-7.8 and 280-300 mOsm, respectively.

Sperm Preparation

Frozen semen straws from ten buffalo bulls were procured from Central Frozen Semen Production and Training Institute, Hesseraghatta, Banglore-560088. The straws were collected in liquid nitrogen (LN_2 at -196 ºC) container, transported and stored in the semen bank of Madras Veterinary College, Chennai- 600 007. The frozen straws were thawed at 37 ºC for 30 seconds and emptied into 15 mL sterile plastic centrifuge tube containing 1 mL of capacitation medium and treated as below.

Experimental groups and Method of treatment

Experimental groups	Method of treatment
Control	Capacitation medium alone (sperm TALP)
Treatment I	Capacitation medium + Heparin binding protein (HBP-25µg/ml)
Treatment II	Capacitation medium +Heparin binding protein (HBP-50µg/ml)
Treatment III	Capacitation medium +Heparin binding protein (HBP-100µg/ml)

The contents were incubated at 37 ºC for 2 hours. After incubation, sperm lipid peroxidation status and plasma membrane integrity were assessed.

Evaluation of Sperm LPO Status

Lipid peroxidation level of spermatozoa was estimated in semen samples by measuring MDA production using TBA with slight modifications in sperm concentration and incubation time. The semen was thawed and washed twice in Tris buffer by centrifugation at 500 g for 5 minutes. Then, the sperm pellet was resuspended in 1 mL of PBS (pH 7.2) or a variable volume of PBS to obtain a sperm concentration of 30×106/mL. Lipid peroxidation level was measured in spermatozoa after the addition of 2 mL of TBA-TCA reagent (15% w/v TCA and 0.375% w/vTBA in 0.25N HCl) to 1 mL of sperm suspension. The mixture was kept in a boiling water bath for 45 minutes. After cooling, the suspension was centrifuged at 500 g for 15 minutes. The supernatant was separated and absorbance measured at 535 nm under UV spectrophotometer (Cecil CE 2021, 2000 series). The MDA concentration was determined by the specific absorbance coefficient (1.56×105/molcm-3) [30].

$$\text{MDA } (\mu\text{Mol/mL}) = \frac{\text{OD x } 10^6 \times \text{total volume (3mL)}}{1.56 \text{ x } 10^5 \times \text{test volume (1mL)}} = \frac{\text{OD} \times 30}{1.56}$$

Evaluation of Sperm Functional Membrane Integrity

100µl semen from each group was taken in a separate Eppendorff tube and 500µl distilled water was added and mixed properly. This solution was kept at 37 ºC for 1 hour. After 1 hour incubation, one drop of the well mixed sample was placed on a clean grease free glass slide and covered with cover slip. Spermatozoa were observed under 40X. Spermatozoa with intact functional membrane showed coiled tail and those without intact functional membrane showed straight tail. A minimum of 200 spermatozoa were observed [31].

Statistical Analysis

The statistical analysis was carried out by IBM, SPSS version 20.0 for windows. The percentage value of variables was converted into Arsine value before performing one way analysis of variance (ANOVA).

Results

Effect of HBP on Sperm LPO Status

The sperm LPO status was measured by production of malondialdehyde (MDA) level. MDA levels in frozen thawed capacitated buffalo semen samples of control and HBP treatments are presented in Table 1. The MDA level in control was 1.85 µMol/mL ± 0.02 and in HBP treatment I, II and III were 1.34 µMol/mL ± 0.08, 1.23 µMol/mL ± 0.06 and III (1.21 µMol/mL ± 0.03), respectively. MDA levels were significantly (P<0.05) low in HBP treatments when compared to control. Among HBP treatments, insignificant difference in MDA levels was observed.

Effect of HBP on Sperm Functional Membrane Integrity

The sperm functional membrane integrity was studied by hypo-osmotic swelling test (HOST). Table 2 shows that 40.80% ±0.90 spermatozoa in control had lost functional membrane integrity and in HBP treatment I, II and III, 46.50% ± 0.71, 48.00% ± 0.47and 48.50% ± 0.73 spermatozoa, respectively had lost functional membrane integrity. Significantly (P<0.05), higher percentages of spermatozoa lost their plasma membrane integrity in HBP treatments as compared to control. But, difference was not significant among HBP treatments. However, 59.20% ± 0.90 spermatozoa in control had intact functional membrane and in HBP treatment I, II and III, 53.50% ± 0.71, 52.00% ± 0.47and 51.50% ± 0.73 spermatozoa, respectively had intact functional membrane. Significantly (P<0.05), higher percentages of spermatozoa retained their plasma membrane integrity in control as compared to HBP treatments. Again, insignificant difference was noticed among HBP treatments.

Discussion

Effect of HBP on Sperm LPO Status

In this study, significant decrease in the level of MDA on treatment with HBP was observed. This result showed that HBP had a protective effect on spermatozoa by preventing oxidative stress and thereby reducing LPO.

The MDA values obtained for frozen buffalo bull sperm in the present study were in accordance with reported the values ranging between 0.34 ± 0.18 and 4.95 ± 0.31µMol/ mL in frozen thawed bovine semen [32] and lesser than the reported values of 8.00 ± 0.31 µM/ml at immediate post thaw and 9.36 ± 0.36 µM/ml at 120 min post thaw incubation in buffalo frozen semen [33]. The elevated level of MDA concentration in buffalo sperm in comparison with bull sperm might be due to the fact that the sperm membrane of buffalo is rich in poly unsaturated fatty acids [34].

The resumption of metabolic activity of the sperm cells after freeze-thawing leads to generation of excessive ROS. Activation of an aromatic amino acid oxidase enzyme from dead sperm cells might be an additional source of ROS in frozen thawed semen samples [35].Fertility associated proteins helped in combating oxidative stress of sperm cells in bulls. Exogenous addition of protein reduces MDA level because the seminal proteins might counteract the lipid peroxidation on dose dependent manner [36].

Seminal proteins added alone or with other compounds showed a protective effect and accounted for an increase in the sperm enzyme activity levels not only in the fresh sample but also after freezing and thawing. Oxidative stress to the sperm cells increased during incubation of frozen thawed semen samples and the addition of fertility associated proteins of sperm membrane helps in controlling the level of lipid peroxides in frozen thawed semen samples [37]. Hence, addition of HBP to the capacitation medium would have reduced LPO and generation of ROS in this study.

Effect of HBP on Sperm Functional Membrane Integrity

In this study, significant influence was noticed on functional membrane integrity of spermatozoa by supplementing with HBP. Higher percentages of spermatozoa in HBP treatments lost the functional membrane integrity.

Loss of functional membrane integrity is due interaction between HBP and heparin which leads to structural and biochemical modifications in spermatozoa, such as changes in membrane composition and fluidity [38, 39], increased intracellular [40], cytoplasmic alkalinization [41, 42], activation of ion channels [43, 44] and generation of reactive oxygen species [45, 46] which are essential events in sperm capacitation for subsequent acrosome reaction and successful fertilization.

Table 1: Effect of HBP Supplementation on Sperm LPO Status of Frozen Thawed Buffalo Semen

Groups	Malondialdehyde (µMol/ml±SE)
Control	1.85 [a] ± 0.02
Treatment I (HBP-25µg/mL)	1.34[b] ± 0.08
Treatment II (HBP-50µg/mL)	1.23 [b] ± 0.06
Treatment III (HBP-100µg/mL)	1.21 [b] ± 0.03

Mean with different superscripts (a and b) in a column are significantly different (P<0.05) between groups.
Data are presented as mean % ± SE

Table 2: Effect of HBP supplementation on sperm functional membrane integrity of frozen thawed buffalo semen

Groups	Functional membrane intact spermatozoa (% ± SE)	Functional membrane non intact spermatozoa (% ± SE)
Control	59.20 [a] ± 0.90	40.80 [a] ± 0.90
Treatment I (HBP-25µg/mL)	53.50 [b] ± 0.71	46.50 [b] ± 0.71
Treatment II (HBP-50µg/mL)	52.00 [b] ± 0.47	48.00[b] ± 0.47
Treatment III (HBP-100µg/mL)	51.50 [b] ± 0.73	48.50[b] ± 0.73

Mean with different superscripts (a and b) in a column are significantly different (P<0.05) between groups.
Data are presented as mean % ± SE

Conclusion

This study suggested that supplementation of HBP in capacitation medium reduces sperm lipid peroxidation and destabilizes plasma membrane integrity for subsequent sperm capacitation, acrosome reaction and successful fertilization.

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References

1. Garner DL, Thomas CA, Joerg HW, Dejarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 1997; 57:1401-1406.
2. Mermillod P, Qussaid B, Cognie Y. Aspects of follicular and oocyte maturation that effect development potential of embryos. J Reprod Fertil. 1999; 54:449-461.
3. Cancel AM, Chapman DA, Killian GJ. Osteopontin is the 55-kilodalton fertility associated protein in Holstein bull seminal plasma. Biol. Reprod. 1997; 57:1293-1301.
4. Parent S, Lefievre L, Brindle Y, Sullivan R. Bull subfertility is associated with low levels of a sperm membrane antigen. Mol. Reprod. Dev. 1999; 52:57-65.
5. Brandon CI, Heusner GL, Caudle AB, Fayrer RAH. Two dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. Theriogenology. 1999; 52:863-873.
6. Killian GJ, Chapman DA, Rogowski LA. Fertility associated proteins in Holstein bull seminal plasma. Biol. Reprod. 1993; 49:1202-1207.
7. Gerena RL, Irikura D, Urade Y, Eguchi N, Chapman DA, Killian GJ. Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. Biol. Reprod. 1998; 58:826-833.
8. McCauley TC, Zhang HM, Bellin ME, Ax RX. Identification of a heparin-binding protein in bovine seminal fluid as tissue inhibitor of metalloproteinases-2. Mol. Reprod. Dev. 2001; 58:336-341.
9. Varilova T, Semenkova H, Horak P, Madera M, Pacakova V, Ticha M *et al*. Affinity liquid chromatography and capillary electrophoresis of seminal plasma proteins. J Sep. Sci. 2006; 29:1110-1115
10. Evans JP, Kopf GS. Molecular mechanisms of sperm–egg interactions and egg activation. Andrologia. 1998; 30:297-307.
11. Jansen S, Ekhlasi-Hundrieser M, Topfer-Peterse E. Sperm adhesion molecules: structure and function, Cells Tissues Organs. 2001;168:82-92
12. Primakoff P, Myles DG. Penetration, adhesion, and fusion in mammalian sperm–egg interaction. Science. 2002; 296:2183-2185.
13. Yi YJ, Manandhar G, Oko RJ, Breed WG, Sutovsky P. Mechanism of sperm–zona pellucida penetration during mammalian fertilization: 26S proteasome as a candidate egg coat lysine. Reprod. Fertil. Suppl. 2007; 63:385-408
14. Jonakova V, Jonak J, Ticha M. Proteomics of male seminal plasma. In: Zhihua J, Troy LO (Ed.). Reproductive genomics in domestic animals, Wiley-Blackwell, Iowa. 2010, 339-366.
15. Therien I, Bleu G, Manjunath P. Phophatidyl choline binding proteins of bovine seminal plasma modulates capacitation of spermatozoa by heparin. Biol. Reprod., 1995; 52:1372-1379.
16. Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30- kDa share functional roles in storing sperm in the oviduct. Biol. Reprod. 2006; 75:501-507.
17. Talevi R, Gualtieri R. Molecules involved in sperm–oviduct adhesion and release. Theriogenology. 2010; 73:796-801.
18. Rodriguez-Martinez HE. Sperm function in cattle and pigs: morphological and functional aspects.Archiv. Fur. Tierzucht. 2001; 44:102-13.
19. Yeagle DL. Lipid regulation of cell membrane structure and function. Faseb J. 1983; 3:1833-1842.
20. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneweld LJ. Development of assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod. Fertil. 1984; 70:219-228.
21. Russel LD, Montag B, Hunt W, Peterson RN. Properties of boar sperm plasma membranes (PM): proteins released by washing and differential solubility in salts, detergents and sensitivity to surface radiolabelling. Gamete Res.

1985; 11:237-252.
22. Yeagle DL. Cholesterol and the cell membrane. Biochim. Biophys. Acta. 1985; 822:267-287.
23. Lin Y, Kan FWK. Regionalization and redistribution of membrane phospholipids and cholesterol in mouse spermatozoa during *in vitro* capacitation. Biol. Reprod. 1996; 55:1133-1146.
24. Moreau R, Manjunath P. Characteristics of the cholesterol efflux induced by novel seminal phospholipid binding proteins. Biochim. Biophys. Acta. 2000; 1487:24-32.
25. Manjunath P, Therien I. Role of seminal plasma phospholipid binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod. Immunol. 2002; 53:109-119.
26. Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its rolein the aetiology of male infertility and genetic disease. Reprod. Biomed. 2003; 7:65-70.
27. Tvrda E, Knazicka Z, Bardos L, Massanyi P, Lukac N. Impact of oxidative stress on male fertility. A review. Acta Vet. Hung. 2011; 59:465-484.
28. Karunakaran M, Devanathan TG, Kulasekar K, Sridevi P, Jawahar T, Loganatahsamy K *et al.* Effect of fertility associated protein on oxidative Stress of bovine sperm cells. Ind. J Anim. Reprod. 2012; 33:43-47.
29. Srivastava N, Srivastava SK, Ghosh SK, Kumar A, Perumal P, Jerome A. Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa. Asian Pac. J Reprod. 2013; 2:126-131.
30. Agarwal A, Prabakaran SA. Mechanism, measurement and prevention of oxidative stress in male reproductive physiology. Ind. J Exp. Biol. 2005; 43:963-974.
31. Ferrusola CO, Fernandez LG, Garcia BM, Sandoval CS, Rodriguez AM, Martinez HR *et al*. Effect of cryopreservation on nitric oxide production by stallion spermatozoa. Biol. Reprod. 2009; 81:1106-1111.
32. Beorlegui N, Cetica P, Trinchero G, Cordoba M, Beconi S. Comparative study of functional and biochemical parameters in frozen bovine sperm. Androl. 1997; 29(1):37-42.
33. Selvaraju S, Ravindra JP, Ghosh JJ, Gupta PSP, Suresh KP. Evaluation of sperm functional attributes in relation to *in vitro* sperm zona pellucida binding ability and cleavage rate in assessing frozen thawed buffalo (Bubalus bubalis) semen quality. Anim. Reprod. Sci. 2008; 106:311-321.
34. Nair SJ, Brar AS, Ahuja CS, Sangha SPS, Chaudhary KC. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. Reprod. Sci. 2006; 96(1):21-29.
35. Upreti GC, Hall EL, Koppens D, Oliver JE, Vishwanath R. Studies on the measurement of phospholipase A_2 (PLA_2) and PLA_2 inhibitor activities in ram semen. Anim. Reprod. Sci. 1999; 56:107-121.
36. Schoneck C, Braun J, Einspanier R. Sperm viability is influenced *in vitro* by the bovine seminal protein aSFP: effects on motility, mitochondrial activity and lipid peroxidation. Theriogenology. 1996; 45:633-642.
37. Marti E, Perez-Pe R, Colas C, Muino-Blanco T, Cebrian-Perez JA. Study of apoptosis-related markers in ram spermatozoa. Anim. Reprod. Sci. 2008; 106:113-132.
38. Langlais J, Roberts KD. A molecular membrane model of sperm capacitation and acrosome reaction of mammalian spermatozoa. Gamete Res. 1985; 12:183-224.
39. Harrison RA, Ashworth PJ, Miller NG. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Mol. Reprod. Dev. 1996; 45:378-391.
40. Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D *et al*. Capacitation of mouse spermatozoa: protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Mol. Reprod. Dev. 1995; 121:1139-1150.
41. Parrish JJ, Susko-Parrish JL, First NL. Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. Biol Reprod. 1989; 41:683-699.
42. Vredenburgh WWL, Parrish JJ. Intracellular pH of bovine sperm increases during capacitation. Mol Reprod Dev. 1995; 40:490-502.
43. Arnoult C, Zeng Y, Florman HM. ZP3-dependent activation of sperm cation channels regulates acrosomal secretion during mammalian fertilization. J Cell Biol. 1996; 134:637-645.
44. Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM. A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: A tale of two channels. Biol Reprod .1998; 59:12-26.
45. Leclerc P, de Lamirande E, Gagnon C. Gagnon. Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic Biol Med. 1997; 22:643-656.
46. de Lamirande E, Tsai C, Harakat A, Gagnon C. Involvement of re-active oxygen species in human sperm arcosome reaction induced by A23187, lysophosphatidylcholine, and biological fluid ultrafiltrates. J Androl. 1998; 19:585-594.