SUPPORTING PAPERS
ISBN 979-97243-9-2

IN VITRO CAPACITATION STATUS OF MERINO RAM SPERMATOZOA

Ismaya[1] *and Phillip Summers*[2]

[1]Faculty of Animal Science, Gadjah Mada University, Yogyakarta 55281, Indonesia.
Email: ismayaismaya@yahoo.com.au
[2]Australian Institute of Tropical Veterinary and Animal Science, James Cook University, Townsville, Queensland 4811, Australia.
Email: phillip.summers@jcu.edu.au

ABSTRACT

The objective of the present study was to determine the effects of individual Merino rams on the *in vitro* capacitation status of ram spermatozoa. Four rams (eartag numbers R9, R12, R13, R16) of proven fertility were used in this study. Semen was collected by electroejaculation. The fresh semen was diluted at four dilutions (1:25, 1:20, 1:15, 1:10) in Hepes buffered synthetic oviduct fluid (HSOF). A sample of semen was collected at 0, 4, 8 and 12 hours and the capacitation status of spermatozoa determined. Capacitation status was analysed by univariate, analysis of variance to determine the effects of rams, incubation time and dilution rate on the capacitation status. During the 12 hours of incubation, progressively more spermatozoa became capacitated such that at the end of the incubation, 30.8 ± 2.5% were uncapacitated, 40.9 ± 0.9% were capacitated acrosome-intact and 29 ± 2.5% were capacitated acrosome-reacted. There were differences between rams in the capacitation profile. There was no significant effect of dilution on the capacitation rate. In conclusion, this study have established baseline of procedures for the detection of the capacitated spermatozoa using the chlortetracycline assay.

Key words: Merino Rams, Spermatozoa, In Vitro Capacitation

INTRODUCTION

Capacitation is a prerequisite that renders spermatozoa capable of achieving fertilization (Austin, 1951; Chang, 1951). Normally capacitation takes place in the female reproductive tract of animals and humans during the peri-ovulatory period but will also occur in a variety of artificial media without any contribution from the female (Yanagimachi, 1989).

Capacitation of spermatozoa *in vitro* has been widely investigated in a number of species under different circumstances including rabbits (Hafez, 1980), goats (Kaul *et al.*, 1997), hamsters (Smith *et al.*, 1998; Arnoult *et al.*, 1999; Si and Olds-Clark, 2000; Kulanand and Shivaji, 2001), pigs (Wang *et al.*, 1995; Green and Watson, 2001; Kaneto *et al.*, 2002; Suzuki *et al.*, 2002), buffaloes (Kaul *et al.*, 2001; Kitiyanant *et al.*, 2002), horses (Rathi *et al.*, 2001; Colenbrander *et al.*, 2002; Pommer and Meyers, 2002), and cattle (Iqbal and Hunter, 1995; Topper *et al.*, 1999; Coscioni *et al.*, 2001; O'Flaherty *et al.*, 2002).

Substances that have been used to facilitate the *in vitro* capacitation process of mammalian spermatozoa include Hepes-synthetic oviduct fluid (Gomez *et al.*, 1997),

ISBN 979-97243-9-2

Tyroide's albumin-lactate-pyruvate (TALP) medium (Parrish *et al.*, 1988; Green and Watson, 2001), calcium ionophore A23187 (Byrd, 1981; Kitayanant *et al.*, 2002), a high ionic strength medium (Brackett *et al.*,1982), serum albumin (Harrison *et al.*, 1982; Go and Wolf, 1985), heparin (Parrish *et al.*, 1988; Kitayanant *et al.*, 2002), caffeine (Niwa and Ohgoda, 1988), oviduct fluid (Parrish *et al.*, 1989), oviductal epithelial cells (Ellington *et al.*, 1991) and follicular fluid (McNutt and Killian, 1991).

Capacitated spermatozoa can be identified by several methods including the spermatozoa marker *Pisum sativum* agglutinin (Fabro *et al.*, 2002) and chlortetracycline (Fraser, 1995; Gomez *et al.*, 1997; Gillan *et al.*, 2000; Green and Watson, 2001; Huo *et al.*, 2002; Suzuki *et al.*, 2002; Aires *et al.*, 2003).

The aim of the present study was to determine the effects of individual rams, incubation time and dilution rate in Hepes synthetic oviduct fluid (HSOF) medium on capacitation status of ram spermatozoa.

MATERIAL AND METHODS

Table 1.Composition of Hepes-synthetic oviduct fluid (HSOF) medium.

Constituents	Concentration of HSOF * (mg/ 100ml)	Supplier
NaCl	1007.7 mM (629.4)	Sigma, USA
KCl	7.16 mM (53.4)	BDH Chemicals, Australia
$CaCl_2$	1.17 mM (12.99)	Ajax Chemicals, Australia
$MgCl_2$	0.49 mM (4.7)	Sigma,USA
$NaHCO_3$	25.07 mM (210.6)	Ajax Chemicals, Australia
L-Lactic acid	3.3 mM (36)	Sigma, USA
HEPES	15 mM (357.45)	Sigma, USA
Sodium pyruvate	0.33 mM (3.63)	Sigma, USA
BSA-V	3.2 mg/ml (320)	Sigma, USA
KH_2PO_4	1.19 mM (16.2)	Ajax Chemicals, Australia
D-Glucose	4 mM (81)	Ajax Chemicals, Australia
Penicillin-G	75 µg /ml (7.5)	Sigma, USA
Streptomycin	50 µg/ml (5)	Sigma, USA
Kanamycin monosulfate	120 µg/ml (12)	Sigma, USA
Pyruvic acid	60 µg/ml (6)	Sigma, USA
Distilled water	100ml	

* Modified from Tervit *et al.*, 1972

Animals

Four rams (eartag numbers R9, R12, R13, R16) of proven fertility were used in this study. Semen was collected three times at intervals of two weeks and spermatozoa analysed for capacitation status.

Collection of ram semen

Semen was collected from adult rams by electroejaculation using standard procedures (Evans and Maxwell, 1987). The ram was manually restrained on its side within a building out of direct sunlight and the penis extruded. The penis was kept extruded by placing a piece of cotton gauze posterior to the glans penis to hold the extended penis and to direct the glans into a 15 ml sterile plastic centrifuge tube (Rohre/tube; Sarstedt, Germany). The collection tubes were kept in a polystryene box at about 39 ^{o}C. Electroejaculation was achieved by stimulation of the internal male

SUPPORTING PAPERS
ISBN 979-97243-9-2

accessory glands and nerves to the penis with a rectal probe connected to the mobile electrical stimulator (Electrojec; Ratex Instruments, Mitcham, Victoria). The electrical stimuli were given in a three seconds on and three seconds off pattern, with a gradual increase in voltage from zero volts to the optimum desired peak (five volts) then returning to zero volts. An electroejaculation attempt was terminated if semen was not obtained after 16 stimulations. Semen was collected no more than twice from a particular ram within a 7 day period. At the completion of a semen collection, a small amount of antiseptic cream was applied to the glans penis before allowing the penis to retract into the prepuce. The prepuce and penis was gently massaged for about one minute to reduce any swelling that may have developed and to reduce any discomfort the ram may have experienced.

Sperm preparation and analysis

The fresh semen was diluted at four dilutions (1:25, 1:20, 1:15, 1:10) in Hepes buffered synthetic oviduct fluid (HSOF) (see Table 1) in microcentrifuge tubes (Eppendorf tubes). The diluted semen samples were held on a microscope warm stage set at 39 °C (LEC Instruments; Scoresby, Victoria). This temperature was selected in order to approximate the temperature within the reproductive tract of the ewe. A sample of semen was collected at 0, 4, 8 and 12 hours and the capacitation status of spermatozoa determined.

Chlortetracycline assay for capacitation

The chlortetracycline (CTC)-fluorescence assay as described by Gillan *et al.*(1997) was used to assess the capacitation status of spermatozoa. The CTC staining solution was prepared prior to each experiment. It contained 750 µM CTC-HCl in stock buffer (stored at 4 °C), 20 mM Tris, 130mM NaCl, and 5 mM L-cysteine (all reagents from Sigma, USA). A 50µl sample of spermatozoa suspension was placed in a light-protected eppendorf tube and an equal volume of CTC staining solution was added. After thorough mixing for 30 seconds, a 10µl sample of filtered glutaraldehyde (EM grade; 1% v/v in 1 M Tris, pH 7.8) was added to fix the spermatozoa. A 10µl sample of this uniformly mixed suspension was placed onto a clean microscope slide and 10 µl of 1.4-diazabicyclo 2.2.2] octane (DABCO, 0.22 M, Sigma, USA) dissolved in glycerol: PBS (9:1) was added to retard photobleaching. A coverslip was placed on the sample, and excess fluid was removed by compression and the edges of the coverslip were sealed with colourless nail varnish. The slides were examined at 40 x magnification with a fluorescence microscope (Leitz Wetzlar, Germany) and 100 spermatozoa evaluated, unless otherwise specified.

In this study, three categories of capacitation status were identified:

- Uncapacitated spermatozoa (Figure 1.A). A bright band of yellow fluorescence present on the head and on the mid-piece of the spermatozoon.
- Capacitated acrosome-intact spermatozoa (Figure 1.B). A bright band of fluorescence was present on the anterior portion of the head and on the mid-piece whereas the post acrosomal region was non-fluorescent.
- Capacitated acrosome-reacted spermatozoa (Figure 1.C). A bright band of fluorescence was present only on the mid-piece and the head of the spermatozoon was non-fluorescent.

ISBN 979-97243-9-2

(A) (B) (C)

spermatozoa: Uncapacitated *spermatozoa (A), Capacitated acrosome-intact spermatozoa (B) and Capacitated acrosome-reacted spermatozoa (C)*

Statistical analysis

All data were analysed using the SPSS software program (SPSS 11.0 Brief Guide, New Jersey, USA). Capacitation status was analysed by univariate, analysis of variance to determine the effects of rams, incubation time and dilution rate on the capacitation status. The level of significance was considered to be $P \leq 0.05$. The differences between means were tested by the Least Significant Difference test.

RESULTS AND DISCUSSION

Immediately after dilution of semen in HSOF medium, most spermatozoa (93.5 ± 2.2 %) were not capacitated with a small percentage (6.4 ± 3.1%) being capacitated and acrosome-intact. During the 12 hours of incubation, progressively more spermatozoa became capacitated such that at the end of the incubation, 30.8 ± 2.5% were uncapacitated, 40.9 ± 0.9% were capacitated acrosome-intact and 29 ± 2.5% were capacitated acrosome-reacted (Figure 2).

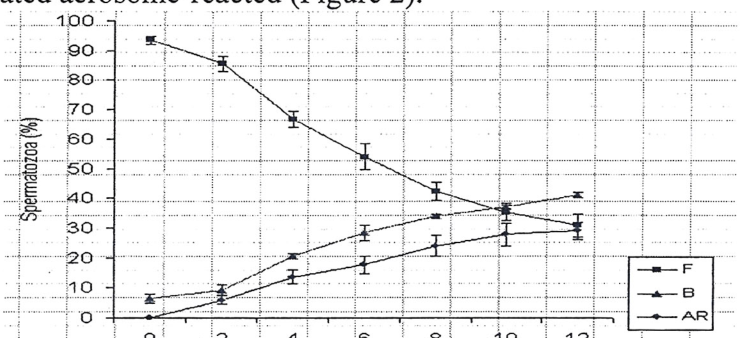
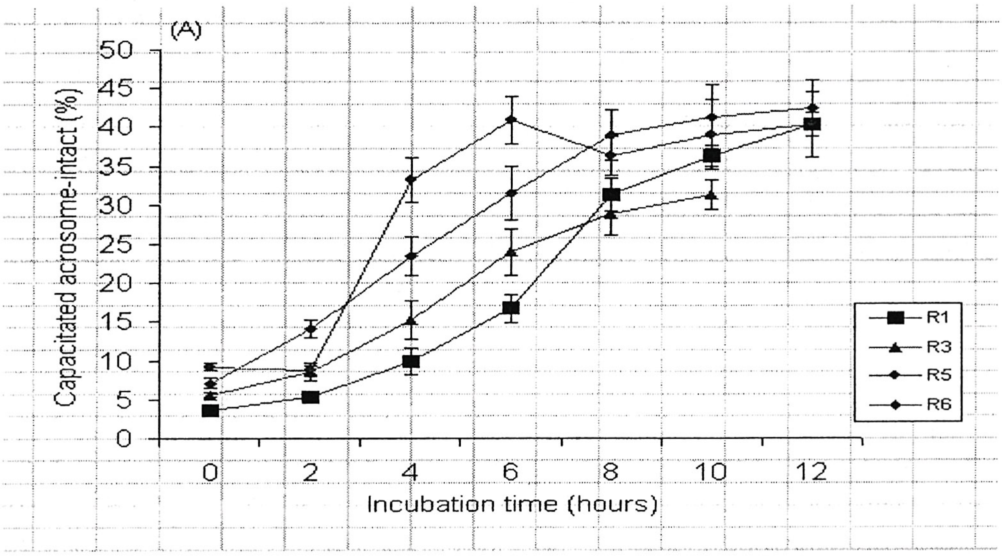


Figure 2. Relationship between uncapacitated (UC), capacitated acrosome-intact (CAI) and capacitated acrosome-reacted (CAR) ram spermatozoa during in vitro culture in HSOF medium. The results are the mean (SEM) for four rams (R9, R12, R13, R16) with three replicates for each ram.

There were differences between rams in the capacitation profile. This difference was present between four and six hours of incubation where R5 had significantly more capacitated acrosome-intact (Figure 3) and between four and 10 hours of incubation

SUPPORTING PAPERS
ISBN 979-97243-9-2

where R5 had significantly more capacitated acrosome-reacted spermatozoa than the other rams (Figure 3). There was no significant effect of dilution on the capacitation rate (Figure 4).



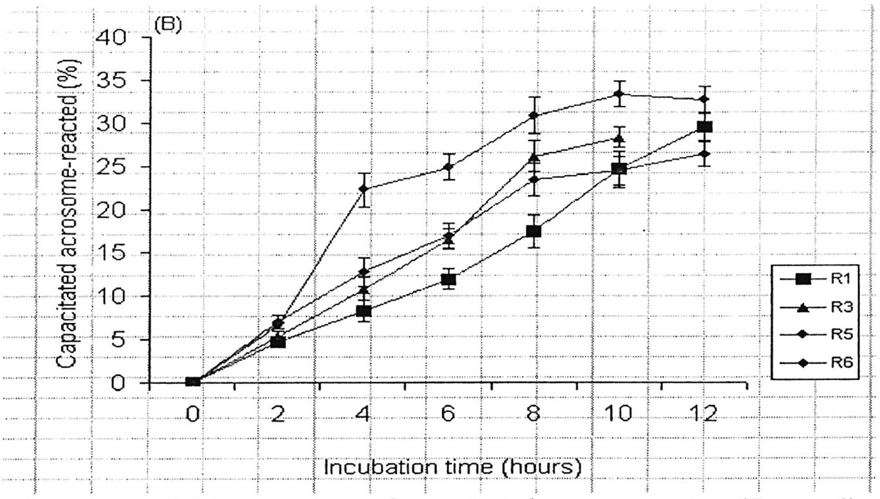
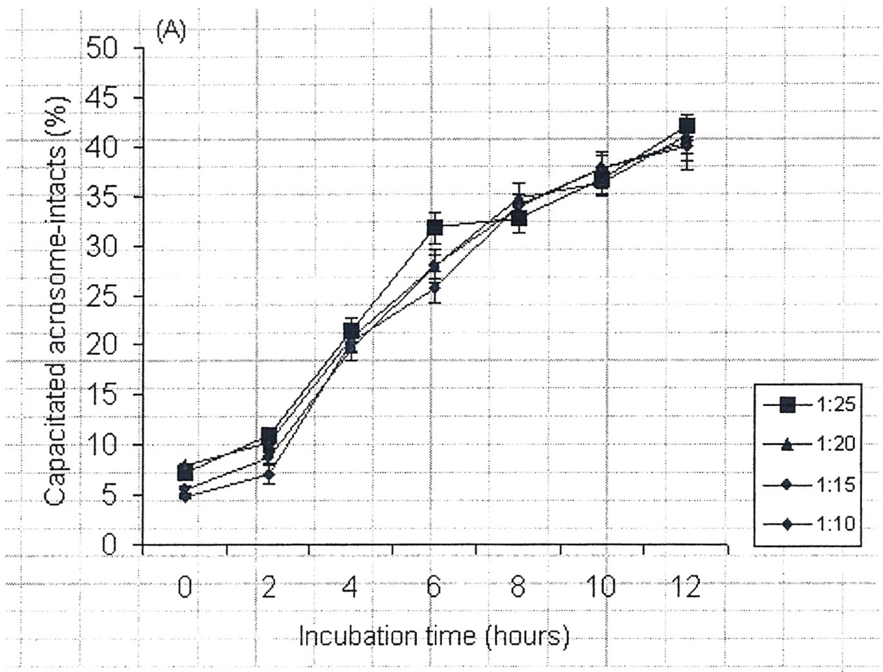


Figure 3. The mean (SEM) percentage of capacitated acrosome-intact (Figure A) and capacitated acrosome-reacted (Figure B) spermatozoa from four rams (R1, R3, R5, R6) during in vitro culture in HSOF medium. There were three replcates for each ram.
** Indicates a significant difference (P< 0.05) between R5 and the other rams*

ISBN 979-97243-9-2



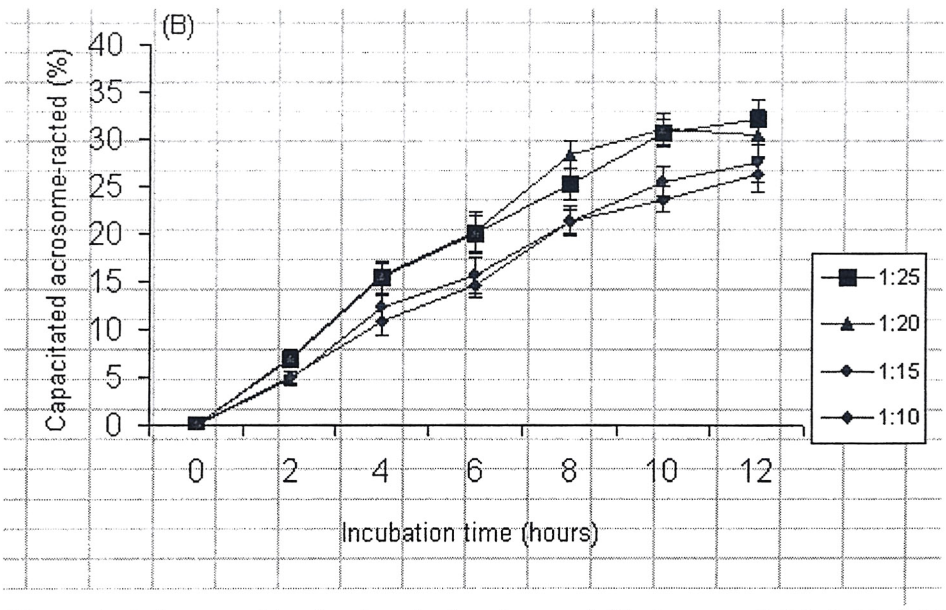


Figure 4. Influence of in vitro incubation time and dilution rate (1:25, 1:20, 1:15, 1:10) of semen on the percentage of spermatozoa that had undergone capacitation but were acrosome-intact (Figure A) and that had undergone the acrosome reaction (Figure B). The results are the mean (SEM) of three replicates for each ram (R1, R3, R5, R6).

As expected, the majority of spermatozoa in a fresh semen sample were uncapacitated and following incubation in a physiological medium, the percentage of capacitated spermatozoa increased with incubation time. What determines why some spermatozoa are capacitated at say four hours after insemination and others are uncapacitated after 12 hours of incubation is not known but presumably relates to the fact that there is a heterogenous population of spermatozoa in a semen sample. In addition, there were differences between rams in the capacitation rate particularly between four to 10 hours of incubation.

In this study was an attempt to find out more about capacitation *in vitro.* Much of the research reported in the literature has been on capacitation in the oviducts and the acrosome reaction induced by close association with the oocyte (McNutt and Killian, 1991; Florman *et al.*, 1998; Arnoult *et al.,* 1999; Fazeli *et al.*, 1999) but the results in this study show that capacitation and the acrosome reaction will occur in *in vitro* by using HSOF medium.

Acrosome-reacted spermatozoa have a very short life span of several minutes (Yanagimachi, 1994) and therefore it is highly unlikely that acrosome-reacted spermatozoa in the posterior half of the reproductive tract could fertilise an oocyte. It also calls into question the role of the so-called sperm reservoir in the cervix (Mattner, 1966) particularly when only about 20% of spermatozoa in the cervix are motile 6 hours after mating and considerably less are motile 24 hours after mating.

CONCLUSION

In conclusion, this study have established baseline information on the procedures for the detection of the capacitated spermatozoa *in vitro* using the chlortetracycline assay, and there were differences between rams in the capacitation profile.

REFERENCES

Aires VA, Hinsch E and Hinsch KD (2003) Effect of genistein upon capacitation of cryopreserved bovine spermatozoa. *Andrologia* 35: 2-12

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M and Florman HM (1999) Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proceedings of theNational Academy of Science USA* 96: 6757-6762

Austin CR (1951) The capacitation of mammalian sperm. *Nature (Lond.)* 170: 326 Byrd W (1981) *In vitro* capacitation and the chemically induced acrosome reaction in bovine spermatozoa. *Journal of Experimental Zoology* 215: 35-46

Chang MC (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes.*Nature (Lond)* 168: 697-698

Colenbrander B, Brouwers J.F.H.M, Neild D.M, Stout T.A.E, da Silva P and Gadella B.M (2002) Capacitation dependent lipid rearrangements in the plasma membrane of equine sperm. *Theriogenology* 58: 341-345

Coscioni AC, Reichenbach HD, Schwartz J, LaFalci, VSN, Rodrigues JL and Brandelli A (2001) Sperm function and production of bovine embryos *in vitro* after swim-up with different calcium and caffeine concentration. *Animal* Reproduction Science 67: 59-67

Ellington JE, Padila AW, Vredenburgh WH, Dougherty EP and Foote RH (1991) Behavior of bull spermatozoa in bovine uterine tube epithelial cell coculture: an *in vitro* model for studying the cell interactions of reproduction. *Theriogenology* 35; 977-987

Fabro G, Rovasio RA, Civalero S, Frenkel A, Caplan SR, Eisenbach M and Giojalas L C (2002) Chemotaxis of capacitated rabbit spermatozoa to follicular fluid

SUPPORTING PAPERS
ISBN 979-97243-9-2

revealed by a novel directionality-based assay. *Biology of Reproduction* 67: 1565-1571

Fazeli A, Duncan AE, Watson PF and Holt WV (1999) Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biology of Reproduction* 60: 879-886

Florman HM, Arnoult C, Kazam IG, Li C and O'Toole CM (1998) A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. *Biology of Reproduction* 59: 12-26

Fraser LR (1995) Cellular biology of capacitation and the acrosome reaction. *Humam Reproduction* (Suppl) 1: 22-30

Funahashi H (2002) Induction of capacitation and acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system. *Reproduction* 124: 857-864

Gillan L, Evans G and Maxwell WMC (1997) Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. *Reproduction, Fertility andDevelopment* 9: 481-487

Gillan L, Evans G and Maxwell WMC (2000) The interaction of fresh and frozen-thawed ram spermatozoa with oviduct epithelial cells *in vitro. Reproduction,*Fertility and Development 12: 237-244

Gomez MC, Catt JW, Gillan L, Evans G and Maxwell WMC (1997) Effect of culture , incubation and acrosome reaction of fresh and frozen-thawed ram spermatozoa for *in vitro* fertilization and intracytoplasmic sperm injection.Reproduction and Fertility 9: 665-673

Green CE and Watson PF (2001) Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reproduction* 122: 889-898 Hafez ESE (1980) Functional anatomy of female reproduction. In. *Reproduction in Farm Animals*. Ed. ESE Hafez.4th ed. Lea and Febiger. Philadelphia. pp 30-82

Huo LJ, Yue KZ and Yang ZM (2002) Characterization of viability, mitochondrial activity, acrosomal integrity and capacitation status in boar sperm during *in vitro* storage at different ambient temperatures. *Reproduction, Fertility and Development* 14: 509-514

Iqbal N and Hunter AG (1995) Effect of various capacitation systems on bovine sperm motion characteristics, acrosome integrity and induction of hyperactivation. *Journal of Dairy Science* 78 : 91-102

Kaneto M, Harayama H, Miyake M and Kato S (2002) Capacitation-like alterations in cooled boar spermatozoa: assessment by the chlortetracycline staining assay and immunodetection of tyrosine-phosphorylated sperm proteins. *AnimalReproduction Science* 73: 197-209

Kaul G, Sharma GS, Singh B and Gandhi KK (2001) Capacitation and acrosome reaction in buffalo bull spermatozoa assessed by chlortetracycline and *Pisum sativum* agglutinin fluorescence assay. *Theriogenology* 55: 1457-1468 Kaul G, Singh S, Gandhi KK and Anand SR (1997) Calcium requirement and time course of capacitation of goat spermatozoa by chlortetracycline assay. *Andrologia* 29:243-251.

Kitiyanant Y, Chaisalee B and Pavasuthipaisit K (2002) Evaluation of the acrosome reaction and viability in buffalo spermatozoa using two staining methods: the effects of heparin and calcium ionophore A23187. *International Journal of Andrology* 25: 215-222

Kulanand J and Shivaji (2001) Capacitation-associated changes in protein tyrosine phosphorylation, hyperactivation and acrosome reaction in hamster spermatozoa.*Andrologia* 33: 95-104

Mattner PE (1966) Formation and retention of the spermatozoa reservoir in the cervix of ruminants. *Nature* 212: 1479-1480

McNutt TL,and Killian GJ (1991) Influence of bovine follicular and oviduct fluids on sperm capacitation *in vivtro. Journal of Andrology* 12: 244-252

Niwa K and Ohgoda O (1988) Synergestic affect of caffeine and heparin on *in vitro* fertilization of cattle oocytes mature in culture. *Theriogenology* 30: 733-741 O'Flaherty CM, Beorlegui NB and Beconi MT (2002) Lactate dehydrogenase-C4 is involved in heparin- and NADH-dependent bovine sperm capacitation. *Andrologia* 34: 91-97

Parrish JJ, Susko-Parrish JL, Handrow RR, Sims MM and First NL (1989) Capacitation of bovine spermatozoa by oviduct fluid. *Biology of Reproduction* 40: 1020-1025

Parrish, JJ, Susko-Parrish JL,Winer MA and First NL (1988) Capacitation of bovine sperm by heparin. *Biology of Reproduction* 38: 1171-1180

Pommer AC, Linfor JJ and Meyers SA (2002) Capacitation and acrosomal exocytosis are enhanced by incubation of stallion spermatozoa in a commercial semen extender. *Theriogenology* 57: 1493-1501

Rathi R, Colenbrander B, Bevers MM and Gadella BM (2001) Evaluation of *in vitro* capacitation of stallion spermatozoa. *Biology of Reproduction* 65: 462-470

Si Y and Olds-Clarke P (2000) Evidence for the involvement of calmodulin in mouse sperm capacitation. *Biology of Reproduction* 62: 1231-1239

Smith TT, Christine A, McKinnon-Thompson and Wolf DE (1998) Changes in lipid disffusibility in the hamster sperm head plasma membrane during capacitation *in vivo* and *in vitro. Molecular Reproduction and Development* 50: 86-92

Suzuki K, Asano A, Erikson B, Niwa K, Nagai T and Rodriguez-Martinez H (2002) Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma cumulus-oocyte-complexes-conditioned medium and hyaluronan.*International Journal of Andrology* 25: 84-93

Tervit HR, Whitingham DG and Rowson LEA (1972) Successful culture *in vitro* of sheep and cattle ova. *Journal of Reproduction and Fertility* 30: 493-497

Topper EK, Killian GJ, Way A, Engel B and Woelders H (1999) Influence of capacitation and fluid from the male and female genital tract on the zona binding ability of bull spermatozoa. *Journal of Reproduction and Fertility* 115 : 175-183

Wang WH, Abeydeera LR, Fraser LR and Niwa K (1995) Functional analysis using chlortetracycline fluorescence and *in vitro* vertilization of frozen-thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. *Journal of Reproduction and Fertility* 104: 305-313

Yanagimachi R (1989) Sperm capacitation and gamete interaction. *Journal of Reproduction and Fertility (Suppl.)* 38: 27-33

Yanagimachi R (1994) Mammalian Fertilization. In *The Physiology of Reproduction* Vol. Second edn., edited by E Knobil and JD Neill, Raven Press, Ltd., New York.pp. 189-318