

In Vitro Motility, Velocity and Capacitation Status of Merino Ram Spermatozoa

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Abstract. The objective of the present study was to determine the effects of Merino rams on the in vitro motility, velocity and capacitation status of ram spermatozoa. Four rams of proven fertility were used in this study. Semen was collected by electro ejaculation. The fresh semen was diluted at four dilutions in Hepes buffered synthetic oviduct fluid. A sample of semen was collected and the motility, velocity and capacitation status of spermatozoa determined and analyzed. The results study showed that motility and velocity of Merino rams spermatozoa were not significantly different. The mean average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) were 91.2 $\mu\text{m/s}$, 72.2 $\mu\text{m/s}$ and 134 $\mu\text{m/s}$, respectively. Amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and linearity (LIN) was 5.5 μm , 19.5 Hz, and 54.7%, respectively. During the incubation time, progressively more spermatozoa became capacitated in such that at the end of the incubation, 40.9 \pm 0.9% was capacitated acrosome-intact and 29 \pm 2.5% was capacitated acrosome-reacted. In conclusion, the motility and velocity of Merino spermatozoa were similar among rams. There were differences among rams in the capacitation profile; however there was no significant effect of incubation time and dilution rate on the capacitation status.

Keywords: Merino rams, spermatozoa, motility, velocity, capacitation in vitro

Abstrak. Penelitian ini bertujuan menentukan efek biri-biri Merino pada motilitas in vitro, kecepatan dan kapasitas spermatozoa biri-biri. Empat biri-biri dengan kesuburan baik digunakan dalam penelitian ini. Semen dikumpulkan dengan elektroejakulasi. Semen segar diencerkan dengan empat kali dilusi dalam cairan Hepes buffered synthetic oviduct. Sample semen dikumpulkan, lalu status motilitas, kecepatan dan kapasitas spermatozoa ditentukan dan dianalisa. Hasil penelitian menunjukkan bahwa motilitas dan kecepatan spermatozoa biri-biri Merino tidak banyak berbeda. Rata-rata average path velocity (VAP) 91.2 $\mu\text{m/s}$, straight-line velocity (VSL) 72.2 $\mu\text{m/s}$ dan curvilinear velocity (VCL) 134 $\mu\text{m/s}$. Amplitude of lateral head displacement (ALH) 5.5 μm , beat cross frequency (BCF) 19.5 Hz and linearity (LIN) 54.7%. selama masa inkubasi, lebih banyak spermatozoa dikapabilitas sedemikian rupa sehingga sehingga di akhir inkubasi 40.9 \pm 0.9% reaksi kapasitas lengkap and 29 \pm 2.5% reaksi kapasitas akrosom. Disimpulkan bahwa motilitas dan kecepatan spermatozoa sama antar biri-biri. Ada perbedaan antar biri-biri dalam profil kapasitas; namun masa inkubasi dan tingkat dilusi tidak berpengaruh besar terhadap status kapasitas

Kata kunci: biri-biri Merino, spermatozoa, motilitas, kecepatan, kapasitas in vitro

Introduction

The motility and longevity of spermatozoa within the female reproductive tract are important factors if spermatozoa are to reach the oviducts and fertilize oocytes. Strong progressive motility is particularly important if spermatozoa are to pass through the mucosal folds and mucus of the cervix and the folds and mucus of the utero-tubal junction. The

development of computer-aided semen analysis (CASA) has taken away much of the subjective assessment of spermatozoa and enables the objective assessment of the detailed movement characteristics of spermatozoa under a variety of physiological and other experimental conditions. Examples of studies that have used computer-aided semen analysis to define the motility and velocity characteristics of spermatozoa include rams

(Bag et al., 2002), boars (Vasquez et al., 2002), mice (Si and Olds-Clarke, 2000) and camels (Al-Qarawi et al., 2002).

Capacitation is a prerequisite that renders spermatozoa capable of achieving fertilization (Philip et al., 2005; Platt et al., 2009). 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 (Conejo-Nava et al., 2003; Kardivel et al., 2009; Sebkova et al., 2012).

Substances that have been used to facilitate the in vitro capacitation process of mammalian spermatozoa include oviductal epithelial cells (Ellington et al., 1991) and follicular fluid (McNutt and Killian, 1991). Tyroide's albumin-lactate-pyruvate (TALP) medium (Green and Watson, 2001), calcium ionophore A23187 (Kitayanant et al., 2002), heparin (Kitayanant et al., 2002; Zicarelli et al., 2009), Chlortetracycline (Hereros et al., 2005; Grasa et al., 2006; Kadervel et al., 2009; Ded et al., 2010; Oh et al., 2010; Kato et al., 2011)

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 motility, velocity and capacitation status of Merino ram spermatozoa.

Materials and Methods

Animals

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

Collection of ram semen

Semen was collected from adult rams by electroejaculation using standard procedures. 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 polystyrene box at about 39°C. Electroejaculation was achieved by stimulation of the internal male 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) 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.

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 50 μl 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 10 μl sample of filtered glutaraldehyde (EM grade; 1% v/v in 1 M Tris, pH 7.8) was added to fix the spermatozoa. A 10 μl 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 cover slip was placed on the sample, and excess fluid was removed by compression and the edges of the cover slip were sealed with colourless nail varnish. The slides were examined at 40 x magnification with a fluorescence microscope (Leitz Wetzlar, Germany) and 100 spermatozoa were evaluated, otherwise specified.

In this study, three categories of capacitation status were used to identify (Ismaya and Summers, 2006), (1) Uncapacitated spermatozoa (Figure 1.A). A bright band of yellow fluorescence present on the head and on the mid-piece of the spermatozoon, (2) 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, (3) 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.

Statistical analysis

All data were analysed using the SPSS software program (SPSS 11.0 Brief Guide, New Jersey, USA). Motility and velocity of sperm

were analyzed by mean and standard deviation. 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 among means were tested by the Least Significant Difference test.

Results and Discussion

The mean semen volume and motility of spermatozoa was 0.89 ml and 81.7%, and the semen varied in color from milky to thick creamy. The percentage of motile spermatozoa in incubated undiluted semen varied between rams. Two rams were similar with spermatozoa having a short period of motility whereas the other two rams had spermatozoa that were motile for a considerably longer period of time. However the motility of spermatozoa in semen diluted in HSOF and incubated at 39°C was similar in all rams, and there was no significant difference between the rams. In addition there was no significant effect of the dilution rate on the motility and longevity of spermatozoa.

The mean percentage of motile spermatozoa and the percentage showing progressive and rapid motility were 78.4, 54.5 and 64.1%, respectively. The mean percentage of static spermatozoa was 21.6%.

The mean average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) of sperm were 91.2 $\mu\text{m/s}$, 72.2 $\mu\text{m/s}$ and 134 $\mu\text{m/s}$. Amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and linearity (LIN) of sperm were 5.5 μm , 19.5 Hz, and 54.7%, respectively.

Immediately after dilution of semen in HSOF medium, most spermatozoa (93.5 \pm 2.2%) were not capacitated with a small percentage (6.4 \pm 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, $30.8 \pm 2.5\%$ were uncapacitated, $40.9 \pm 0.9\%$ were capacitated acrosome-intact and $29 \pm 2.5\%$ were capacitated acrosome-reacted (Figure 2).

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 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).

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 determined why some spermatozoa were capacitated at four hours after insemination and others were uncapacitated after 12 hours of incubation was not known but presumably related to the fact that there is a heterogeneous population of spermatozoa in a semen sample. In addition, there were differences among rams in the capacitation rate particularly between four and 10 hours of incubation.

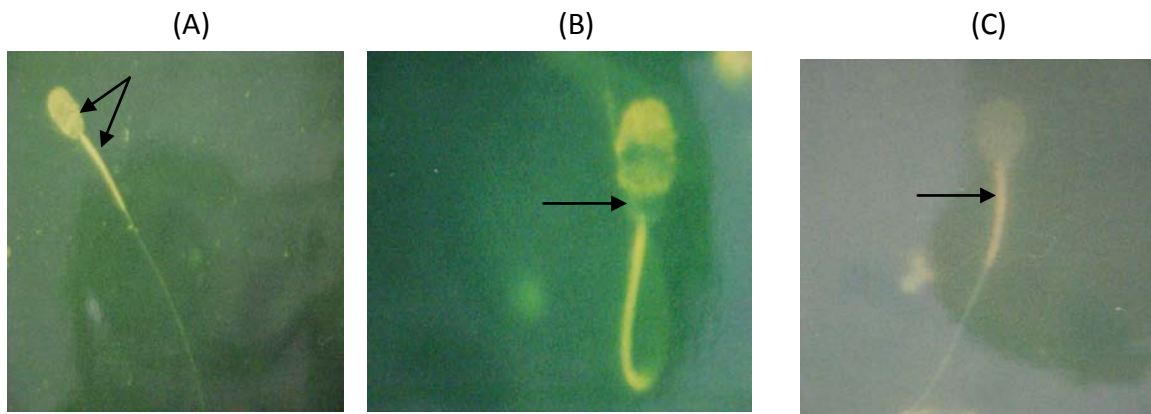


Figure 1. Capacitation status of spermatozoa: uncapacitated Spermatozoa(A), capacitated acrosome-intact spermatozoa (B) and capacitated acrosome -reacted spermatozoa (C)

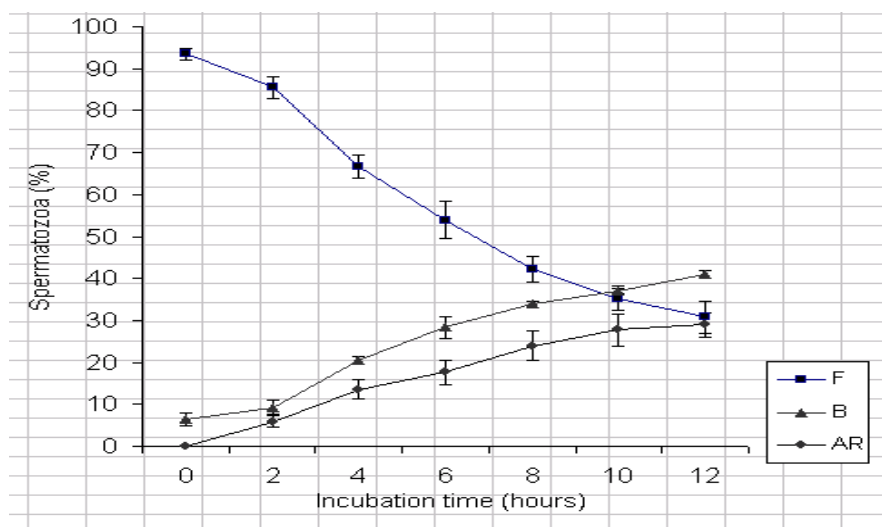


Figure 2. Relationship between uncapacitated (F), capacitated acrosome-intact (B) and capacitated acrosome-reacted (AR) ram spermatozoa during in vitro culture in HSOF medium. The results are the mean (SEM) for four rams (R9, R12, R13, R16) with three replicates for each ram.

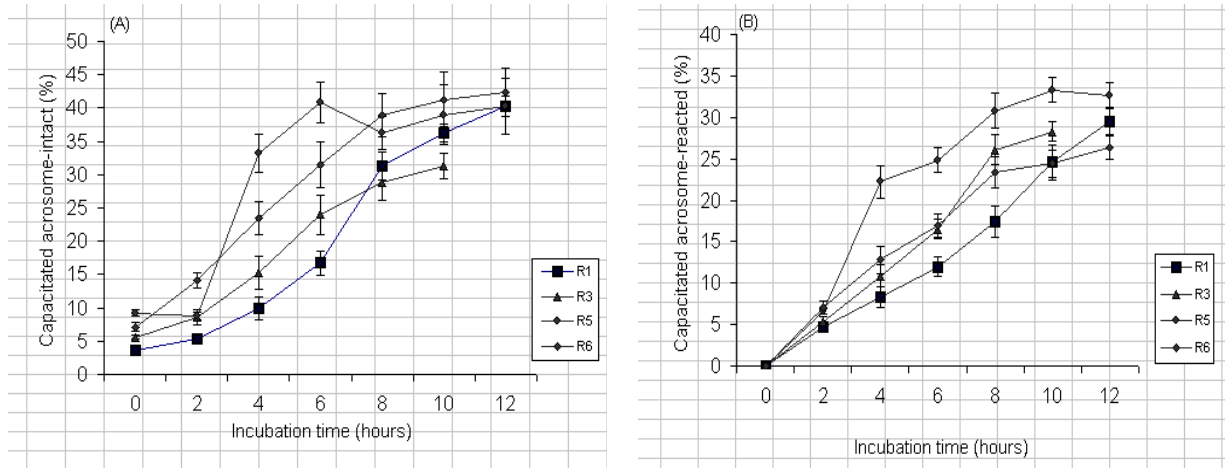


Figure 3. The mean (SEM) percentage of capacitated acrosome-intact (Figure A) and capacitated acrosome-reacted (Figure B) spermatozoa from four rams (R1, R3, R5, R6) during in vitro culture in HSOF medium. There were three replicates for each ram.

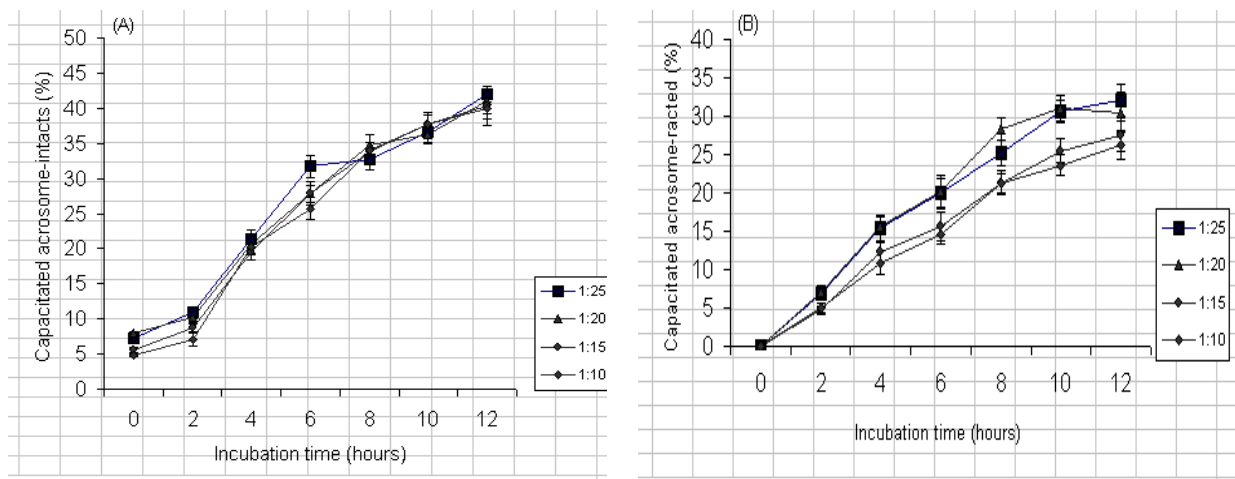


Figure 4. Influence of in vitro incubation time and dilution rate (1:25, 1:20, 1:15, 1:10) of semen on the percentage of spermatozoa that had undergone capacitation but were acrosome-intact (Figure A) and that had undergone the acrosome reaction (Figure B). The results are the mean (SEM) of three replicates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

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 (Florman et al., 1998; Arnoult et al., 1999; Fazeli et al., 1999; Erikson et al., 2007) 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; Kato and Nagao, 2009) and therefore it is highly unlikely that acrosome-reacted spermatozoa in the posterior half of the reproductive tract can fertilize 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.

Conclusions

The motility and velocity of Merino sperm were similar among rams and there were differences among rams in the capacitation profile, but not in incubation time and dilution rate.

References

- Al-Qarawi AA, HA Abdel-Rahman, SA El-Mougy and MS El-Belely. 2002. Use of new computerized system for evaluation of spermatozoal motility and velocity characteristics in relation to fertility levels in dromedary bulls. *Anim. Reprod. Sci.* 74:1-9.
- Arnoult C, IG Kazam, PE Visconti, GS Kopf, M Villaz and HM Florman. 1999. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. In: *Proceedings of the National Academy of Science. USA, 1999.* 96:6757-6762.
- Bag S, A Joshi, SMK Naqvi, PS Rawat and JP Mittal. 2002. Effect of freezing temperature, at which straws were plunged into liquid nitrogen, on the post-thaw motility and acrosomal status of ram spermatozoa. *Anim. Reprod. Sci.* 72: 175-183.
- Conejo-Nava J, R Fierro, CG Gutierrez and M Betancourt. 2003. Membrane status and in vitro capacitation of porcine sperm preserved in long-term extender at 16 °C. *Archives of Andrology.* 49:287-295.
- Ded L, P Dostalova, A Dorosh, K Dvorakova-Hortova, and J Peknicova. 2010. Effect of estrogens on boar sperm capacitation. *Reproductive Biol. and Endocrinol.* 8:87-93.
- Ellington JE, AW Padila, WH Vredenburgh, EP Dougherty and RH Foote. 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
- Erkson DW, AI Way, RP Bertolla, DA Chapman, and GJ Killian. 2007. Influence of osteopontin, casein and oviductal fluid on bovine sperm capacitation. *Anim. Reprod.* 4:103-112.
- Fazeli A, AE Duncan, PF Watson and WV Holt. 1999. Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biol. Reprod.* 60:879-886.
- Florman HM, C Arnoult, IG Kazam, C Li and CM 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: 12-26.
- Gillan L, G Evans and WMC Maxwell. 1997. Capacitation status and fertility offresh and frozen-thawed ram spermatozoa. *Reproduction, Fertility and Development.* 9: 481-487.
- Grasa P, JA Cebrian-Perez and T Muino-Blanco. 2006. Signal transduction mechanisms involved in in vitro sperm capacitation. *Reprod.* 32:721-732.
- Green CE and Watson PF. 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reprod.* 122:889-898.
- Herrerros MG, IM Aparicio, I Nunez and LJ Garcia-Marin. 2005. Boar sperm velocity and motility patterns under capacitation and non-capacitating incubation conditions. *Theriogenology.* 63:795-805.
- Ismaya and P Summers. 2006. In vitro capacitation status of Merino ram spermatozoa. In: *Proceedings of the 4th International Seminar on Tropical Animal Production.* Faculty of Animal Science Gadjah Mada University, Yogyakarta Indonesia. Pp. 46-54.
- Kato Y and Y Nagao. 2009. Effect of PVC on capacitation status and embryonic development in cattle. *Theriogenology.* 72: 624-635.
- Kato Y, S Shoei and Y Nagao. 2011. Capacitation status of activated bovine sperm cultured in media containing methyl-B-cyclodextrin affects the acrosome reaction and fertility. *Zygote.* 19:21-30.
- Kardivel G, S Kumar, A Kumaresan and P Kathiravan. 2009. Capacitation status and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Anim. Reprod. Sci.* 116:244-253.
- Kitiyant Y, B Chaisalee and K Pavasuthipaisit. 2002. Evaluation of the acrosome reaction and viability in buffalo spermatozoa using two staining methods: the effects of heparin and calcium ionophore A23187. *Int. J. Androl.* 25: 215-222.
- McNutt TL and GJ Killian. 1991. Influence of bovine follicular and oviduct fluids on sperm capacitation in vitro. *J. of Andrology* 12: 244-252.
- Oh SA, YJ Park, YA You, EA Mohamed, and MG Pang. 2010. Capacitation status of stored boar spermatozoa is related to litter size of sows. *Anim. Reprod. Sci.* 121: 131-138.
- Platt MD, AM Salicioni, DF Hunt, and PE Visconti. 2009. Use of differential isotop labeling and mass spectrometry to analyze capacitation-associated changes in the phosphorylation status of mouse sperm protein. *J. Proteome Res.* 8:1431-1440.

- Sebkova N, M Cerna, L Ded, J Peknicova, and K Dvorakova-Hortova. 2012. The slower the better: How sperm capacitation and acrosome reaction is modified in the presence of estrogens. *Reproduction*. 143: 297-307.
- Si Y and P Olds-Clarke. 2000. Evidence for the involvement of calmodulin in mouse sperm capacitation. *Biol. Reprod.* 62: 1231-1239.
- Topper EK, GJ Killian, A Way, B Engel and H Woelders. 1999. Influence of capacitation and fluid from the male and female genital tract on the zona binding ability of bull spermatozoa. *J. Reprod. and Fertility*. 115:175-183.
- Vazquez JM, EA Martinez, I Parrilla, MA Gil, X Lucas and J Roca. 2002. Motility characteristics and fertilizing capacity of boar spermatozoa stained with Hoechst 33342. *Reprod. Domestic Anim.* 37: 369-374.
- Yanagimachi R. 1994. Mammalian Fertilization. In: *The Physiology of Reproduction*. 2nd ed. Edited by E Knobil and JD Neill, Raven Press, Ltd., New York. pp. 189-318.
- Zicarelli L, E Mariotti, SD Francesco, S Velotto, M Rubessa and G Neglia. 2009. Effect of bull on in vitro sperm capacitation induced by different agents in buffalo species (*Bubalus bubalis*). *J. Anim. Sci.* 8:177-179.