Effect of Methyl-B-Cyclodextrin (MBCD) on In Vitro Capacitation of Buffalo Frozen/Thawed Sperm

Article · March 2014

DOI: 10.6000/1927-520X.2014.03.01.3

8 authors, including:

Ahmed Elkhawagah
Benha University
15 PUBLICATIONS **15** CITATIONS
SEE PROFILE

Bianca Gasparrini
University of Naples Federico II
167 PUBLICATIONS **2,422** CITATIONS
SEE PROFILE

Gamal Abd El Raheem Sosa
Benha University
87 PUBLICATIONS **148** CITATIONS
SEE PROFILE

Angela Salzano
University of Naples Federico II
43 PUBLICATIONS **64** CITATIONS
SEE PROFILE

Effect of Methyl-B-Cyclodextrin (MBCD) on *In Vitro* Capacitation of Buffalo Frozen/Thawed Sperm

A.R. Elkhawagah[1], V. Longobardi[2], B. Gasparrini[2,*], G.A. Sosa[1], A. Salzano[2], M.E.A. Aboul-roos[1], A.E. Abd El-Gaffar[1] and L. Zicarelli[2]

[1]*Theriogenology Department, Faculty of Veterinary Medicine, Benha University, Moshtohor – Toukh, PO Box 13736, Egypt*

[2]*Department of Veterinary Medicine and Animal Production, Federico II University, Via F. Delpino 1, 80137 Naples, Italy*

Abstract: The aim of this study was to determine the effect of Methyl-B-Cyclodextrin (MBCD) on capacitation of buffalo sperm. Frozen/thawed semen was incubated in the absence of capacitating agents (negative control), in the presence of 10 µg/ml heparin (positive control) and of 1, 2, 4 and 8 mg/ml MBCD for 2 and 4h. At each incubation time, sperm motility was evaluated by phase contrast microscopy. Capacitation was assessed by the sperm ability to undergo acrosome reaction after lysophosphatidylcholine treatment, evaluated with viability by Trypan blue-Giemsa. After 2 h capacitation increased (P<0.01) in MBCD groups (39.2±1.4, 44.5±3.3, 56.7±1.5 and 62.5±3.8, with 1, 2, 4 and 8 mg/ml MBCD, respectively) compared to the negative and positive controls (27.5±1.0 and 28.0±0.8, respectively). Likewise, after 4 h the percentage of live capacitated sperm was higher at increasing concentration of MBCD (31.0±0.7, 34.5±1.7, 42.0±1.9, 49.2±2.8, 62.3±1.5 and 70.8±1.7 in negative control, positive control and with 1, 2, 4 and 8 mg/ml MBCD, respectively; P<0.01). After 2h sperm motility was lower (P<0.01) in 4 and 8 mg/ml MBCD groups (43.3±2.1 and 25.0±3.2, respectively) than in negative control, positive control, 1 and 2 mg/mlMBCD groups (55.0±1.8, 48.3±2.8, 61.7±2.8, 56.7±1.1, respectively). After 4 h the lowest sperm motility was observed with higher MBCD concentrations (40.0±0.0, 46.7±4.2, 51.7±4.6, 50.0±0.0, 40.0±3.7 and 6.7±1.1, in negative control, positive control, 1, 2, 4 and 8 mg/ml MBCD, respectively; P<0.01). In conclusion, MBCD improved sperm capacitation in a dose-dependent manner while decreasing the sperm motility at higher concentrations.

Keywords: Buffalo, Frozen semen, Methyl-B-Cyclodextrin, capacitation.

INTRODUCTION

The water buffalo (*Bubalus bubalis*) is an irreplaceable producer of both energy and protein in developing countries. In the current scenario, the best tool to enhance the maternal contribution to genetic improvement is ovum pick-up (OPU) and *in vitro* embryo production (IVEP). In fact, although the efficiency of both multiple ovulation and OPU-IVEP in this species is mainly affected by the low number of oocytes recovered, the latter technology gives higher embryo yields in long term programs [1]. Despite the great improvement of IVEP efficiency recorded in recent years in buffalo [2], the cleavage rate is still lower than in most domestic species, suggesting that *in vitro* fertilization (IVF) is a critical step [1]. Many factors are known to affect IVF efficiency, such as the sperm quality, the bull, the environment, the appropriate time of insemination, as well as an appropriate capacitation of frozen-thawed sperm. Indeed, sperm need to undergo capacitation to acquire fertilizing ability. This process, which occurs *in vivo* within the female genital tract, must be induced *in vitro*. It is not possible to rule out that sperm capacitation is impaired in the buffalo IVF system currently used.

Mammalian sperm undergo a series of molecular, biochemical and physiological modifications in the female reproductive tract that are collectively known as capacitation that enables the spermatozoa to interact with egg investment at the right time at the right place [3,4]. The capacitation-associated modifications in spermatozoa include changes in plasma membrane structure, mainly cholesterol efflux, leading to an increase in membrane fluidity, bicarbonate (HCO_3^-), intracellular pH, Ca^{2+} and cAMP levels. In addition, changes in protein phosphorylation and protein kinase activity occur that are necessary to initiate sperm-egg binding and acrosome reaction (AR) [5,6]. Uterine and oviductal fluids contain sterol-binding proteins that facilitate sperm capacitation *in vivo* [7,8]. It has been reported that bovine serum albumin (BSA), high-density lipoproteins and newly discovered non-physiological cholesterol acceptors called beta-cyclodextrins facilitate *in vitro* cholesterol removal from sperm [9].

Beta-cyclodextrins (β-CDs) are non-physiological cyclic heptasaccharides consisting of 7β (1–4)-glucopyranose units**,** having a high affinity for sterols,

*Address correspondence to this author at the Department of Veterinary Medicine and Animal Production, Federico II University -Via F. Delpino 1, 80137 Naples, Italy; Tel: +390812536284; Fax:+39081292981;
E-mail: bgasparr@unina.it

ISSN: 1927-5196 / E-ISSN: 1927-520X/14

that are often used to investigate the role of cholesterol in somatic cell types [10-13]. Beta-cyclodextrins have the ability to release cholesterol from a variety of cells in culture [10,11,14] and selectively extract cholesterol from the plasma membrane in preference to other membrane lipids [15]. It has been reported that the order of potency in accepting cholesterol is methyl-β-cyclodextrin (M-β-CD) > OH-propyl-β-cyclodextrin (2-OH-p-β-CD) > β-cyclodextrin (β-CD) [10]. Although β-CD are not biological molecules of the female reproductive tract, they can be used as highly efficient cholesterol acceptors to investigate the role of cholesterol release as an early event of *in vitro* sperm capacitation and AR [6,16]. It has been demonstrated that the addition of β-CDs (M-β-CD and 2-OHp-β-CD) to the capacitating medium promotes the release of cholesterol from sperm plasma membrane and induces capacitation and AR in a protein free medium in mouse [17,18], boar [19], bull [4,18,20,21], buck [6] and human [22]. It has also been reported that β-CDs increase protein tyrosine phosphorylation in the absence of BSA with a pattern similar to that observed in media containing BSA, depending on cAMP and on the presence of $NaHCO_3$ in the capacitation medium [18]. Furthermore, the pre-incubation of bovine sperm with MBCD has been shown to decrease the frequency of live sperm, although enhancing the capacitation and AR of live sperm and promoting *in vitro* fertilization and embryo development [21].

Therefore, the aim of the present study was to evaluate the efficiency of different concentrations of MBCD (1, 2, 4 and 8 mg/ml), at two incubation times (2 and 4 h), to induce *in vitro* capacitation of frozen-thawed buffalo sperm. This was indirectly assessed by estimating the capability of spermatozoa to acrosome react following incubation with lysophosphatidylcholine (LPC), a fusogenic lipid known to induce AR in capacitated sperm without affecting motility.

MATERIAL AND METHODS

Unless otherwise stated, all reagents were purchased from Sigma (Milan, Italy).

Spermatozoa were prepared from frozen/thawed semen, obtained from a Mediterranean Italian buffalo bull previously tested for IVF in our laboratory. Frozen sperm were thawed at 37°C for 40 sec and selected by centrifugation (25 min at 1500 rpm) on a Percoll discontinuous gradient (45–80%). The pellets were reconstituted into 2 ml of sperm TALP medium and centrifuged twice, at 1100 and 900 rpm for 10 min. After centrifugation, the sperm pellets were re-suspended to a final concentration of 20×10^6/ml in Tyrode albumin lactate pyruvate (TALP) medium containing 0.2 mM penicillamine and 0.1 mM hypotaurine (IVF medium) modified according to the experimental groups. In particular, sperm were incubated in the IVF medium in the absence of capacitating agents (negative control), with the standard concentration of heparin (10 µg/ml, positive control), and with different concentrations of MBCD (1, 2, 4 and 8 mg/ml) in a controlled gas atmosphere of 5% CO_2 at 38.5°C in humidified air for 2 and 4 h.

Samples of semen were assessed immediately after thawing to evaluate motility, viability and the incidence of acrosomal loss in non-treated cells. Following incubation, sperm motility was examined by phase-contrast microscopy at 40 and 100 magnification on a clean and dry glass slide maintained on thermo-regulated stage at 37°C. On the basis of the mass activity semen motility was evaluated as previously described [23] with slight modifications, i.e. graded into scores from1 to 10.

Capacitation was assessed indirectly by estimating the percentage of acrosome-reacted sperm after 15 min incubation with 60 µg/ml LPC, a fusogenic agent known to induce AR only in capacitated sperm [24] in a controlled gas atmosphere of 5% CO_2 at 38.5°C in humidified air. To evaluate the sperm viability and the AR, sperm were fixed and stained with dual staining technique [25,26]. In particular, Trypan blue was used first to differentiate live from dead spermatozoa, then the dried smears were fixed in 37% formaldehyde and stained with Giemsa for acrosome evaluation by microscopic examination. At least 200 sperm cells were counted for each group. Based on staining characteristics of sperm cells we differentiated four categories: acrosome-intact live (AIL), acrosome-intact dead (AID), acrosome-lost live (ALL) and acrosome-lost dead (ALD). We recorded as live only the sperm displaying both head and tail viable and as dead those with either the head or the tail unviable. The experiment was repeated three times.

Statistical Analysis

Data on sperm capacitation, motility and viability were analyzed using General liner Model (GLM) of SPSS version (16.0). The model included the effects of treatments (negative control, positive control and 1, 2, 4 and 8 mg/ml MBCD), incubation time (2 h vs 4 h) and their interaction. When analysis of variance showed a

significant effect, the post-hoc test of Duncan was employed to detect the differences among treatments.

RESULTS

Effect of MBCD on Sperm Viability

The percentage of intact-acrosome live sperm at thawing was high (536/600 = 89.3%), indicating the good quality of semen used for this trial, and the percentage of acrosome-reacted live sperm was very low (4.5%). Sperm viability remained high both after 2 h (98.7 ± 0.5, 99.5 ±0.3, 99.8 ± 0.2, 100.0 ± 0.0, 100.0 ± 0.0 and 99.8 ± 0.2, in the negative and positive controls, and with 1, 2, 4 and 8 mg/ml MBCD, respectively) and 4 h incubation (99.5 ± 0.5, 100.0 ± 0.0, 99.8 ± 0.2, 99.3 ± 0.2, 100.0 ± 0.0 and 100.0 ± 0.0, in the negative and positive controls, and with 1, 2, 4 and 8 mg/ml MBCD, respectively).

Effect of MBCD on the Percentage of Live Capacitated Sperm

The capacitation rate was influenced by both the treatment (P<0.01) and the incubation time (P<0.01). As shown in Table **1**, after 2 h incubation no differences in capacitation, indicated by the percentages of AR sperm after induction with LPC, were observed between the negative and the positive control. On the contrary, it was demonstrated that MBCD promotes capacitation of frozen/thawed buffalo sperm in a dose-dependent manner, as indicated by higher percentages (P<0.01) of AR at increasing concentrations of the compound. After 4 h incubation an increase (P<0.01) in the percentages of capacitation was also recorded in the positive control compared to the negative control (Table **1**). Furthermore, a linear increase (P<0.01) in the capacitation rate was recorded in the MBCD treated groups.

Effect of MBCD on Sperm Motility

Sperm motility was also affected by both the treatment (P<0.01) and the incubation time (P<0.01). A negative effect on sperm motility was observed with the two highest concentrations of MBCD, with the lowest (P<0.01) value recorded with 8 mg/ml MBCD (Table **2**). This effect was overwhelming after 4 h incubation: 4 mg/ml of MBCD significantly decreased (P<0.01) sperm motility compared to the other groups; however, the poorest motility was recorded with 8 mg/ml of MBCD.

DISCUSSION

The results of the present study demonstrated that MBCD triggers the capacitation process of frozen/thawed buffalo sperm in a dose and time-dependent manner. In fact, also at the lower concentration tested (1 mg/ml) MBCD increased capacitation rate compared to both the control groups, to reach double values with the highest concentration tested (8 mg/ml) at both incubation times. The results showed a progressive improvement of capacitation at increasing concentrations of MBCD. The capacitation process was also positively influenced by the incubation time, with higher values after 4 h. Interestingly, following 2 h incubation heparin failed to increase capacitation compared to the negative control, i.e. the group devoid of capacitating agents, whereas an increase was registered after 4 h. This finding is in agreement with previous reports [24,27], indicating that heparin requires longer time to induce capacitation of buffalo frozen/thawed sperm. In addition, it is worth

Table 1: Effect of MBCD on the Percentage of Live Capacitated Sperm After 2 and 4 h Incubation

Groups	2hr	4hr	Total
	Mean±SE	Mean±SE	Mean±SE
Negative control	27.50 ± 0.96^{AX}	31.00 ± 0.68^{AY}	29.25 ± 0.79^{A}
Positive control	28.00 ± 0.77^{AX}	34.50 ± 1.74^{BY}	31.25 ± 1.34^{A}
1 mg/ml MBCD	39.17 ± 1.35^{B}	42.00 ± 1.86^{C}	40.58 ± 1.18^{B}
2 mg/ml MBCD	44.50 ± 3.34^{B}	49.17 ± 2.82^{D}	46.83 ± 2.19^{C}
4 mg/ml MBCD	56.67 ± 1.54^{Cx}	62.33 ± 1.50^{Ey}	59.50 ± 1.33^{D}
8 mg/ml MBCD	62.50 ± 3.77^{C}	70.83 ± 1.70^{F}	66.67 ± 2.34^{E}
Total	43.06 ± 2.39^{X}	48.31 ± 2.52^{Y}	45.68 ± 1.75

^{A,B,C,D,E,F}Values with different superscripts within the same column differed significantly; P < 0.01.
^{X,Y}Values with different superscripts within the same raw differed significantly; P < 0.01.
^{x,y}Values with different superscripts within the same raw differed significantly; P < 0.05.

Table 2: Effect of MBCD on Sperm Motility at 2 and 4 h Incubation

Groups	2hr	4hr	Total
	Mean±SE	Mean±SE	Mean±SE
Negative control	55.00 ± 1.83^{ACX}	40.00 ± 0.00^{AY}	47.50 ± 2.42^{A}
Positive control	48.33 ± 2.79^{BC}	46.67 ± 4.21^{AB}	47.50 ± 2.42^{A}
1 mg/ml MBCD	61.67 ± 2.79^{A}	51.67 ± 4.59^{B}	56.67 ± 2.97^{B}
2 mg/ml MBCD	56.67 ± 1.05^{AX}	50.00 ± 0.00^{BY}	53.33 ± 1.12^{B}
4 mg/ml MBCD	43.33 ± 2.11^{B}	40.00 ± 3.65^{A}	41.67 ± 2.07^{C}
8 mg/ml MBCD	25.00 ± 3.16^{DX}	6.67 ± 1.05^{CY}	15.83 ± 3.19^{D}
Total	48.33 ± 2.21^{X}	39.17 ± 2.81^{Y}	43.75 ± 1.86

^{A,B,C,D}Values with different superscripts within the same column differed significantly; $P < 0.01$.
^{X,Y}Values with different superscripts within the same raw differed significantly; $P < 0.01$.

pointing out that the negative control contained BSA to preserve viability and it is known that BSA itself may trigger the capacitation process. Cholesterol efflux from the sperm plasma membrane is a key molecular event of capacitation [28]. When cholesterol is removed from the sperm plasma membrane the cholesterol/ phospholipid ratio of the membrane decreases, the lipid concentration changes, and hence the membrane architecture and fluidity modifies and the cAMP-signalling cascade is triggered.

It is likely that the promoting effect of MBCD on buffalo sperm capacitation is due to the cholesterol efflux induced on sperm plasma membrane. It was demonstrated that MBCD mediates cholesterol release from mammalian sperm in a concentration-dependent manner [18] and this may account for the linear increase of capacitation recorded in the present study. Our results are in agreement with previous studies that demonstrated the promoting effect of β-cyclodextrins on the release of cholesterol from sperm plasma membrane and hence on the induction of capacitation and AR in a protein free medium in the mouse [17,18], boar [19] and bull [4,18,21]. However, in this work we evaluated the effect of MBCD on the capacitation of frozen/thawed buffalo sperm. It is known that cryopreservation promotes the premature capacitation of spermatozoa [29,30] that may lead to the reduced longevity of cryopreserved spermatozoa in the female reproductive tract, without affecting the *in vitro* fertilizing ability. It was reported that MBCD improves fertilizing capacity of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells [31,32]. Furthermore, the pre-incubation of bovine frozen/thawed sperm with MBCD affects viability and capacitation status of the sperm and promotes fertilization *in vitro* [21].

Another finding of the present study is the significant decrease of motility recorded at greater concentrations of MBCD (4 and 8 mg/ml). This deleterious effect on sperm motility is particularly evident after 4 h incubation with the highest concentration of MBCD, not exceeding 10%. Therefore, MBCD at the two concentrations that determined the highest capacitation rates (4 and 8 mg/ml) negatively affected sperm motility. It was previously reported that when porcine sperm are treated with MBCD an increase in deteriorated cells is detected which is mirrored with reduction of sperm motility [33]. In the same study MBCD at concentrations >1 mM rendered sperm cells immotile. A decreased sperm motility was also observed by other authors [18] with concentrations higher than 1 mM on mouse sperm. In our study the deleterious effect on motility was recorded at higher concentrations (> 4 mg/ml, corresponding to 3 mM), despite the fact that sperm were frozen/thawed. Furthermore, sperm viability remained high in all groups, both after 2 h (99.6% on average) and 4 h (99.8% on average) incubation. In this trial we evaluated capacitation indirectly, by assessing the percentage of acrosome reacted sperm after exposure to LPC, a fusogenic agent known to induce AR only on capacitated sperm cells [25]. However, we cannot rule out that spontaneous acrosome reaction occurred after MBCD treatments. This is worth investigating as acrosome-reacted sperm are not able to bind to the zona pellucida of the oocyte and are, therefore, unable to fertilize the oocytes.

In conclusion, it was demonstrated that MBCD promotes capacitation/AR of buffalo frozen-thawed sperm in a concentration-dependent manner. It was also observed that at concentrations higher than 4 mg/ml MBCD significantly decreases sperm motility,

without though affecting sperm viability. Further studies are needed to distinguish whether MBCD induces capacitation or spontaneous acrosome reaction and, hence, to identify the best concentration and incubation time for pretreatment of sperm prior to *in vitro* fertilization, to assess the effect of MBCD on fertilizing ability of buffalo frozen-thawed sperm.

ACKNOWLEDGEMENTS

This work was supported by POR CAMPANIA FSE 2007/2013. “Sviluppo di Reti di Eccellenza tra Università, Centri di Ricerca, Imprese”. PROGETTO CARINA “Sicurezza, sostenibilità e competitività nelle produzioni agroalimentari della Campania”CUP B25B09000080007. The authors gratefully acknowledge Giuseppe Albero for technical assistance.

REFERENCES

[1] Gasparrini B. *In vitro* embryo production in buffalo: current situation and future perspectives. Ital J Anim Sci 2007; 6 (2): 92-101.

[2] Gasparrini B, Boccia L, Marchandise J, Di Palo R, George F, Donnay I, Zicarelli l. Enrichment of *in vitro* maturation medium for buffalo (Bubalusbubalis) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development. Theriogenology 2006; 65(2): 275-87. http://dx.doi.org/10.1016/j.theriogenology.2005.05.036

[3] Yanagimachi R. Gamete manipulation for development: new methods for conception. Reprod Fertil Dev 2001; 13: 3-14. http://dx.doi.org/10.1071/RD00047

[4] Kato Y, Shoei S, Nagao Y. Capacitation status of activated bovine sperm cultured in media containing methyl-β-cyclodextrin affects the acrosome reaction and fertility. Zygote 2010; 19: 21-30. http://dx.doi.org/10.1017/S0967199410000304

[5] Baldi E, Luconi M, Bonaccorsi L, Muratori M, Forti G. Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. Front Biosci 2000; 5: 110-23. http://dx.doi.org/10.2741/baldi

[6] Iborra A, Companyó M, Martínez P, Morros A. Cholesterol Efflux Promotes Acrosome Reaction in Goat Spermatozoa. Biol Reprod 2000; 62:378-83. http://dx.doi.org/10.1095/biolreprod62.2.378

[7] Ehrenwald E, Foote RH, Parks JE. Bovine oviductal fluid components and their potential role in sperm cholesterol. Mol Reprod Dev 1990; 25:195-204. http://dx.doi.org/10.1002/mrd.1080250213

[8] Shadan S, James PS, Howes EA, Jones R. Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar sperm. Biol Reprod 2004; 71: 253-65. http://dx.doi.org/10.1095/biolreprod.103.026435

[9] Gadella BM, Tsai PS, Boerke A, Brewis IA. Sperm head membrane reorganisation during capacitation. The Internation. J Dev Biol 2008; 52: 473-80. http://dx.doi.org/10.1387/ijdb.082583bg

[10] Kilsdonk EPC, Yancey P, Stoudt G, Bangerter FW, Johnson WJ, Phillips MC, *et al.* Cellular cholesterol efflux mediated by cyclodextrins. J Biol Chem 1995; 270: 17250-56. http://dx.doi.org/10.1074/jbc.270.29.17250

[11] Yancey PG, Rodrigueza WV, Kilsdonk EPC, Stoud GW, Johnson WJ, Phillips MC, *et al.* Cellular cholesterol efflux mediated by cyclodextrins: demonstration of kinetic pools and mechanism of efflux. J Biol Chem 1996; 271: 16026-34. http://dx.doi.org/10.1074/jbc.271.27.16026

[12] Barenholz Y. Sphingomyelin and cholesterol: From membrane biophysics and rafts to potential medical applications. Subcell Biochem 2004; 37: 167-215. http://dx.doi.org/10.1007/978-1-4757-5806-1_5

[13] Chiu PC, Chung MK, Tsang HY, Koistinen R, Koistinen H, Seppala M, Lee KF, and Yeung WS. Glycodelin-S in human seminal plasma reduces cholesterol efflux and inhibits capacitation of spermatozoa. J Biol Chem 2005; 280: 25580-89. http://dx.doi.org/10.1074/jbc.M504103200

[14] Zeng WX, Terada T. Effects of Methyl-Beta-Cyclodextrin on Cryosurvival of Boar Spermatozoa. J Androl 2001; 22 (1): 111-18.

[15] Ohtani Y, Irie T, Uekama K, Fukunaga K, Pitha J. Differential effects of α˜, β˜, and γ-cyclodextrins on human erythrocytes. Eur J Biochem 1989; 186: 17-22. http://dx.doi.org/10.1111/j.1432-1033.1989.tb15171.x

[16] Companyó M, Iborra A, Villaverde J, Martínez P, Morros A. Membrane fluidity changes in goat sperm induced by cholesterol depletion using beta-cyclodextrin. Biochim Biophys Acta 2007; 1768: 2246-55. http://dx.doi.org/10.1016/j.bbamem.2007.06.011

[17] Choi YH, Toyoda Y. cyclodextrin remove cholesterol from mouse sperm and induces capacitation in a protein-free medium. Biol Reprod 1998; 59: 1328-33. http://dx.doi.org/10.1095/biolreprod59.6.1328

[18] Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, *et al*. Cholesterol Efflux-mediated Signal Transduction in Mammalian Sperm. J Biol Chem 1999; 274 (5): 3235-42. http://dx.doi.org/10.1074/jbc.274.5.3235

[19] Funahashi H. Effect of methyl-B-cyclodextrin and fertilization promoting peptide on capacitation of boar spermatozoa in a protein-free medium. J Reprod Dev 2002; 48(1): 57-63. http://dx.doi.org/10.1262/jrd.48.57

[20] Dinkins MB, Brackett BG. Chlortetracycline staining patterns of frozen-thawed bull spermatozoa treated with β-cyclodextrins, dibutyrylcAMP and progesterone. Zygote 2000; 8: 245-56. http://dx.doi.org/10.1017/S0967199400001040

[21] Nagao Y, Ohta Y, Murakami H, Kato Y. The effects of methyl-β-cyclodextrin on in-vitro fertilization and the subsequent development of bovine oocyte. Zygote 2010; 18(4): 323-30. http://dx.doi.org/10.1017/S0967199409990396

[22] Cross NL. Effect of methyl-Beta-cyclodextrin on the acrosomal responsiveness of human sperm. Mol Reprod Dev1999; 53: 92-98. http://dx.doi.org/10.1002/(SICI)1098-2795(199905)53:1<92::AID-MRD11>3.0.CO;2-Q

[23] Vale WG. Sperm Cryopreservation. Third Course on Biotechnology of Reproduction In Buffaloes, Caserta, Italy. In: Bubalus bubalis. J. Buffalo Sci. and Tech 1997; suppl. 4: 129-140.

[24] Parrish JJ, Susko-Parrish JL, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38: 1171-80. http://dx.doi.org/10.1095/biolreprod38.5.1171

[25] Kovacs A, Foote R H. Viability and acrosome staining of bull, boar and rabbit spermatozoa. Biotech Histochem 1992; 67: 119-24. http://dx.doi.org/10.3109/10520299209110020

[26] Boccia L, Di Palo R, De Rosa A, Attanasio L, Mariotti E, Gasparrini B. Evaluation of buffalo semen by Trypan blue/Giemsa staining and related fertility *in vitro*. Ital J Anim Sci 2007; 6 (suppl 2): 739-42.

[27] Boccia L, Di Francesco S, Neglia G, De Blasi M, Longobardi V, Campanile G, Gasparrini B. Osteopontin improves sperm capacitation and *in vitro* fertilization efficiency in buffalo (Bubalus bubalis). Theriogenology 2013; 80 (3): 212-7. http://dx.doi.org/10.1016/j.theriogenology.2013.04.017

[28] Moseley FLC, Jha KN, Björndahl L, Brewis IA, Publicover SJ, Barratt CLR, *et al*. Induction of human sperm capacitation varies between incubation media; an effect that is not associated with proteinkinase A activation. Mol Hum Reprod 2005; 11: 523-29. http://dx.doi.org/10.1093/molehr/gah188

[29] Bailey J, Bilodeau J, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl 2000; 21: 1-7.

[30] Watson PF. The causes of reduce fertility with cryopreserved semen. Anim Reprod Sci 2000; 60: 481-92. http://dx.doi.org/10.1016/S0378-4320(00)00099-3

[31] Takeo T, Hoshii T, Kondo T, Toyodome H, Arima H, Yamamura K, Irie T, Nakagata N. Methyl-beta-cyclodextrin improves fertilizing ability of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells. Biol Reprod 2008; 78(3): 546-51. http://dx.doi.org/10.1095/biolreprod.107.065359

[32] Takeo T, Nakagata N. Reduced Glutathione Enhances Fertility of Frozen/Thawed C57BL/6 Mouse Sperm after Exposure to Methyl-Beta-Cyclodextrin. Biol Reprod 2011; 85: 1066-72. http://dx.doi.org/10.1095/biolreprod.111.092536

[33] Van Gestel RA, Helms JB, Brouwers JF, Gadella BM. Effects of methyl-beta-cyclodextrin-mediated cholesterol depletion in porcine sperm compared to somatic cells. Mol Reprod Dev 2005; 72: 386-95. http://dx.doi.org/10.1002/mrd.20351