Journal of Reproduction and Infertility 4 (3): 19-28, 2013
ISSN 2079-2166

DOI: 10.5829/idosi.jri.2013.4.3.8361

Evaluation of *in vitro* Capacitation of Buffalo Frozen/Thawed Sperm by Different Techniques

[1]A.R. Elkhawagah, [2]V. Longobardi, [2]B. Gasparrini, [1]G.A. Sosa, [2]G. Bifulco,
[1]M.E.A. Abouelroos, [1]A.E. Abd el-ghafar and [2]G. Campanile

[1] Department of Theriogenology, Faculty of Veterinary Medicine,
Benha University, Moshtohor-Toukh, Egypt, PO Box 13736
[2]Department of Veterinary Medicine and Animal Production,
Federico II University, Via F. Delpino 1, 80137 Naples, Italy

Abstract: This study aimed to determine the most reliable method to evaluate capacitation of buffalo frozen/thawed sperm. Frozen/thawed sperm were incubated in TALP medium in absence of capacitating agents (control) and in presence of 10 µg/ml heparin for 2 and 4 hrs. Capacitation was assessed by Trypan blue/Giemsa after lysophosphatidylecholine (LPC) exposure, chlortetracycline (CTC) fluorescence assay and immune-localization of tyrosine phosphorylated protein. Furthermore, we evaluated the effect of heparin on penetration, cleavage rates and kinetics of embryo development after heterologous IVF. The percentage of LPC-induced acrosome reacted (AR)-sperm increased (P<0.05) with heparin compared to the control after 2 hrs (28.2 vs 24.4%, respectively) and 4 hrs (35.1 vs 32.0 %, respectively). No differences in CTC pattern B (capacitated sperm) were found between groups and incubation times (on average 63%). On the contrary, heparin decreased (P<0.01) the percentage of tyrosine phosphorylation pattern A after 2 and 4 hrs (34.3 and 35.3%, respectively) compared to the control (54.5 and 51.8%, respectively) and increased (P<0.01) that of pattern EA after 2 and 4 hrs (59.2 and 54.2 %, respectively) compared to the control group (44.7 and 45.2 %, respectively). Both cleavage and penetration rates, as well as the percentage of fast developing embryos, were higher (P<0.01) in the heparin-treated group (77.2, 80.4 and 74.0 %, respectively) compared to the control (56.6, 58.0 and 55.2 %, respectively). In conclusion, Trypan blue/Giemsa staining to evaluate LPC-induced AR and tyrosine protein phosphorylation assay can be successfully used to evaluate capacitation of buffalo frozen/thawed semen.

Key words: Buffalo · Frozen Semen · Capacitation · Trypan Blue/Giemsa · Chlortetracycline

INTRODUCTION

The water buffalo (*Bubalus bubalis*) is an irreplaceable producer of both energy and protein in developing countries. Due to the limitations of MOET programs [1] in this species, ovum pick-up (OPU) and *in vitro* embryo production (IVEP) are currently the best tools to enhance the maternal contribution to genetic improvement. Although the IVEP efficiency has greatly increased 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 [3]. 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. Capacitation allows spermatozoa to undergo the zona pellucida-induced AR and hyperactivation to fertilize oocytes. This process, which occurs *in vivo* within the female genital tract, must be induced *in vitro*. Parrish *et al*. [4] demonstrated that the glycosaminoglycan heparin capacitates bovine sperm *in vitro*. Since then heparin has been the most widely capacitating agent used in most domestic species [5-9]. The putative mechanism is that heparin functions as a

Corresponding Author: A.R. Elkhawagah, Theriogenology Department,
Faculty of Veterinary Medicine, Benha University, Moshtohor-Toukh, Egypt.
P.O. Box 13736, Tel: +201141864144, Fax: +20132460640.

ligand for a receptor localized in the sperm plasma membrane, but such a receptor is yet uncharacterized. However, heparin binding to spermatozoa appears to stimulate 1) the intracellular elevation of calcium, pH and cAMP, which seem to be necessary to initiate the signaling pathway concomitant with capacitation [10, 11] and 2) the removal of seminal plasma proteins adsorbed to the plasma membrane, which are capacitation inhibitory factors [12, 13].

Although the molecular mechanisms of capacitation are not completely elucidated, many studies have demonstrated an involvement of numerous structural and biochemical modifications in spermatozoa, such as 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 [14-16]. In addition, changes in protein phosphorylation and protein kinase activity necessary to initiate sperm-egg binding and AR occur [17,18]. Protein tyrosine phosphorylation is one of the major events associated with capacitation [19], as observed in various mammalian species such as mouse [20,21], human [22,23], bovine [24,25] and buffalo [19,25,26]; it is known to regulate many sperm function including motility [27], zona pellucida recognition and acquisition of the fertilizing ability [21, 22].

Semen cryopreservation is an important tool for assisted reproduction, although the fertility of frozen/thawed spermatozoa is reduced [28, 29]. It has been recently recognized that cryopreservation procedures (dilution, cooling, freezing/thawing) induce capacitation-like changes in spermatozoa [29, 30]. Indeed, recent studies have reported similarities between the changes associated with capacitation and cryoinjury, such as plasma membrane reorganization and fluidization and calcium influx to the spermatozoa [31]. Therefore, partially or fully cryopreserved spermatozoa demonstrate capacitation-like behavior, revealed by a greater proportion of CTC fluorescent pattern B (capacitated cells) [32] and their ability to undergo the AR or fertilize oocytes *in vitro* [31,32]. Cryocapacitation may be partly responsible for the reduced longevity and, hence fertility after artificial insemination of frozen/thawed bull semen.

Various methods have been used to differentiate capacitated and non-capacitated spermatozoa. For a long time, capacitation of both fresh and frozen/thawed sperm has been evaluated indirectly by estimating the capability of spermatozoa to acrosome react following incubation with LPC, a fusogenic lipid known to induce AR in capacitated sperm without affecting motility [4]. Later, the CTC fluorescence assay was developed. The fluorescent antibiotic CTC was used to assess the destabilization of sperm membrane [33] based on its ability to cross over the cell membrane, enter intracellular compartments and bind to free calcium ions. This method has been used to assess sperm capacitation in most domestic species [34-36], including buffalo [26, 37]. Moreover CTC technique allows to distinguish three stages of sperm activation: non capacitated, capacitated acrosome intact and capacitated acrosome reacted. Finally, as it is well established that tyrosine phosphorylation of sperm proteins is a key event of sperm capacitation, the localization of phosphotyrosine containing protein detected by an indirect immunoflourescence assay has been recently successfully used [26, 38].

Therefore, the aim of this work was to assess the most reliable method to evaluate sperm capacitation of buffalo frozen/thawed sperm because of the increasing evidence that cryopreservation results in premature capacitation of sperm [6, 39, 40] In order to do so, sperm capacitated by heparin treatment were assessed by Trypan blue/Giemsa staining after LPC induction, CTC and protein tyrosine phosphorylation assays. Furthermore, as fertilizing ability is acquired after capacitation, it was also evaluated the effect of heparin treatment on penetration and cleavage rates, as well as on the kinetics of embryo development after heterologous IVF.

MATERIALS AND METHODS

Reagents and Media: Unless otherwise stated, all reagents were purchased from Sigma (Milan, Italy).

The aspiration medium was TCM 199 supplemented with 25 mM Hepes, 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 1 mM L-glutamine, 10 µL/ml amphotericin B (H199) and 2% bovine serum (BS). The *in vitro* maturation (IVM) medium was TCM 199 supplemented with 15% BS, 0.5 µg/ml FSH, 5 µg/ml LH, 0.8 mM L-glutamine and 50 µg /ml gentamycin. The *in vitro* fertilization (IVF medium) was Tyrode albumin lactate pyruvate (TALP) medium containing 0.2 mM penicillamine and 0.1 mM hypotaurine. The *in vitro* culture (IVC) medium consisted of Synthetic Oviduct Fluid (SOF) medium with 30 µl/ml essential amino acids, 10 µl/ml non essential amino acids and 5 % BS.

Experimental Design: Frozen buffalo sperm from three different bulls, over 3 replicates, were used for the trial. Sperm viability and capacitation were first assessed at

thawing with the three different assays described below. Then sperm were separated by Percoll gradients and incubated in absence of capacitating agent (control group) and in presence of 10 µg/ml heparin (heparin treated group) for 2 and 4 hrs. After each incubation time, the semen assessments were repeated.

Finally, buffalo frozen/thawed sperm from the same bulls were used to fertilize bovine oocytes in presence and absence of heparin (n=219;n=212 respectively) to evaluate penetration and cleavage rate, as well as the percentage of fast developing embryos at 48 hrs post-IVF. This experiment was repeated 6 times.

Semen Preparation: Spermatozoa were prepared from frozen/thawed semen, obtained from three Mediterranean Italian buffalo bulls previously tested for IVF in our laboratory. Frozen sperm were thawed at 37°C for 40 sec separated by Percoll procedure and centrifuge (25 min at 1500 rpm).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 the IVF medium in absence (control group) or presence of 10 µg/ml heparin (heparin treated group) in a controlled gas atmosphere of 5% CO_2 at 38.5°C in humidified air for 2 and 4 hrs.

Assessment of Sperm Capacitation

Dual Staining (Trypan Blue/giemsa) Technique after LPC Treatment: Capacitation was assessed indirectly by estimating the percentage of AR-sperm after 15 min incubation with 60 µg/ml LPC, a fusogenic agent known to induce AR only in capacitated sperm [4], in a controlled gas atmosphere of 5% CO_2 at 38.5°C in humidified air. To evaluate sperm viability and AR, sperm were fixed and stained with dual staining technique [41, 42]. 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 (Advanced Automated Research Microscope System, Nikon Eclipse 90i, phase contrast at 40 and 100 magnification). 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-reacted live (ARL) 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.

CTC Staining Technique: The capacitation status of frozen/thawed buffalo sperm under different capacitating conditions was assessed by CTC fluorescent staining as reported earlier [43]. Briefly, CTC solution (750 mM CTC, 5 mM cysteine in 130 mM NaCl and 20 mM Tris HCl) was prepared freshly, pH adjusted to 7.8 and stored at 4°C under dark condition. Equal volumes of sperm suspension and CTC solution were mixed in a falcon tube at room temperature, to which 2 µl of glutaraldehyde (12.5% in 20 mM Tris-HCl, pH 7.4) were added. Eight µl of sperm suspension were placed on a clean slide, to which 2 µl of 0.22 M 1, 4-diaza-bicyclo (2,2,2) octane dissolved in glycerol: phosphate-buffered saline (9:1) were added to retard the fading of CTC fluorescence. The sperm suspension was covered with coverslip and stored at 4°C overnight in the dark. Chlortetracycline fluorescence was observed under microscope equipped with phase contrast and epifluorescent optics (Advanced Automated Research Microscope System, Nikon Eclipse 90i). Each sample was assessed twice and at least 200 spermatozoa per slide were evaluated and classified into one of three CTC staining patterns described by Fraser *et al.* [33]: 1) uniform bright fluorescence over the whole head (uncapacitated spermatozoa, pattern F); 2) fluorescence-free band in the post-acrosomal region (capacitated spermatozoa, pattern B); and 3) dull fluorescence over the whole head except for a thin punctuate band of fluorescence along the equatorial segment (acrosome reacted spermatozoa, pattern AR).

Immuno-localization of Protein Tyrosine Phosphorylation: Localization of phosphotyrosine containing protein was detected using an indirect immunoflourescence assay as described by Tardif *et al*. [38]. After each incubation period, semen was centrifuged (300 g, 10 min), fixed in 2% (v/v) formaldehyde for 1 h at 4 °C and centrifuged again at 300 g for 10 min. The sperm pellets were incubated overnight at 4 °C in modified phosphate buffer saline (mPBS: 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, 137 mM NaCl, 5.5 mM glucose and 1.0 mM pyruvate, pH 7.4) containing 2% (w/v) BSA. After centrifugation (250 g, 10 min, 25 °C), the sperm pellets were resuspended and diluted 1:10 in mPBS. Twenty µl of sperm suspension were smeared onto a slide, air dried and permeabilized with absolute ethanol for 5 min. The permeabilized spermatozoa were incubated with primary antibody (Sigma, Cat no: T1325; diluted 1:10 in tris buffered saline-TBS: 20 mM Tris-HCl, 0.8% NaCl, pH 7.6) for 1 h at room temperature in a humid chamber.

Excess antibodies were removed by plunging the slides 4-5 times in TBS. The slides were incubated with secondary antibody, FITC conjugated goat anti-rabbit IgG (Sigma, Cat no: F0382; diluted 1:10 in TBS) for 1 h in a dark humid chamber at room temperature Excess antibodies were removed by plunging the slides 4-5 times in TBS and the slides were mounted with 90% (v/v) glycerol. Green fluorescence was observed by epifluorescent microscope (Advanced Automated Research Microscope System, Nikon Eclipse 90i) using FITC filter (Green filter). A total of 200 spermatozoa were screened per slide and classified according to Cormier and Bailey [6]: Pattern E: a short line or triangle of fluorescence in the equatorial segment; Pattern A: uniform fluorescence over the entire acrosome and Pattern EA: fluorescence at both equatorial and anterior acrosomal regions.

Assessment of Fertilizing in Vitro Ability: Bovine ovaries were recovered from a local abattoir and transported to the laboratory in physiological saline at 30-35°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2-8 mm in diameter and only those with uniform cytoplasm and multilayered cumulus cells were selected, washed twice in the aspiration medium and once in the IVM medium. Groups of 25 COCs were matured in 400 µL of IVM medium, covered with mineral oil, in four well plates (NuncTM, Roskilde, Denmark), for 22-24 hrs at 39 °C and 5% CO_2 in air. *In vitro* matured COCs were washed and transferred, 25 per well, into 300 µl of IVF medium covered with mineral oil. Buffalo frozen sperm from the three bulls were processed as described above and after centrifugation the pellet was resuspended to a final concentration of 2 x 10^6 sperm/mL in the IVF medium and insemination was performed. Gametes were co-incubated for 20 hrs at 39 °C, in 5% CO_2 in air, then presumptive zygotes were vortexed for 2 min to remove cumulus cells in Hepes-TCM with 5% BS, washed twice in the same medium and cultured in SOF medium in a humidified mixture of 5% CO_2, 7% O_2 and 88% N_2 in air at 39 °C for further 1 day.

After approximately 48 hrs cleavage rate was assessed and the percentage of fast developing embryos, i.e. those showing more than 4 blastomeres was recorded. Then both cleaved and uncleaved oocytes were dezonated by enzymatic digestion with 2 mg/mL of pronase for 3 to 5 min, fixed with absolute ethanol overnight and stained with 4,6-diamidino-2-phenylindole (DAPI Vectashield® by Vector Laboratories, Inc., Burlingame, CA, USA) for subsequent evaluation of nuclear status using a microscope equipped with phase contrast and epifluorescent optics (Advanced Automated Research Microscope System, Nikon Eclipse 90i). The incidence of monospermic and polyspermic fertilization was assessed and oocytes with two synchronous pronuclei (2 PN) were considered as normally fertilized.

Statistical Analysis: Differences in the percentages of different categories of sperm between groups were analyzed by Chi square test. The same test was used to evaluate the differences in penetration rate, cleavage rate and kinetics of embryo development.

Person's correlation coefficient was calculated to estimate the relationship among the different sperm categories identified by the three capacitation assays and fertilizing ability.

RESULTS

Trypan Blue/giemsa Staining Following LPC Induction: The percentage of sperm with intact acrosome at thawing was 79.4% and interestingly the only difference among bulls was recorded in intact-acrosome live sperm (90.7, 71.8 and 75.7 %, respectively for bulls 1, 2 and 3; $P<0.01$). The percentage of total viable sperm at thawing was significantly lower than in treated groups whereas no differences in sperm viability were recorded either between treatment or incubation times (Table 1). Treatment with heparin significantly ($P<0.05$) increased the percentage of sperms undergoing AR after LPC induction after both 2 and 4 hrs ($P<0.05$). However, within groups this parameter showed a greater variation ($P<0.01$) in relation to the incubation time, with a progressive increase of AR-sperm at increasing incubation times.

Chlortetracycline Fluorescence Assay: As shown in Table 2, no differences were recorded in patterns F, B and AR between treatments at both incubation times (2 and 4 hrs). However, compared to time 0, in all groups pattern F significantly ($P<0.01$) decreased and pattern AR significantly ($P<0.01$) increased, while the pattern B remained unchanged. No differences were observed in pattern B between 2 and 4 hrs incubation and only in heparin treated sperm the pattern F decreased and the pattern AR increased after 4 hrs.

Table 1: Effect of heparin on the percentage of live sperm and acrosome-reacted live (ARL) sperm following LPC induction.

Incubation time	Treatment	N.	Viable n. (%)	ARL n. (%)
0	Post-thawing	1800	1674 (93.0)^{A}	245 (13.6)^{A}
2 hrs	Control	1800	1530 (98.6)^{B}	447 (24.4)^{Bx}
	Heparin	1800	1510 (98.3)^{B}	508 (28.2)^{By}
4 hrs	Control	1800	1535 (98.8)^{B}	576 (32.0)^{Cz}
	Heparin	1800	1516 (98.6)^{B}	632 (35.1)^{Cw}

^{A,B,C} values with different letters within the same column differ significantly; $P < 0.01$

^{x,y,z,w} values with different letters within the same column differ significantly; $P < 0.05$

Table 2: Chlortetracycline (CTC) fluorescence patterns in frozen/thawed buffalo sperm in relation to capacitating agent (heparin) and incubation times.

		CTC pattern (%)		
Incubation time	Treatment	Pattern F n. (%)	Pattern B n. (%)	Pattern AR n. (%)
0 h	Post-thawing	612 (34.0)^{A}	1134 (63.0)	54 (3.0)^{A}
2 hrs	Control	141 (7.8)^{B}	1117 (62.1)	524 (29.1)^{B}
	Heparin	135 (7.5)^{BCx}	1158 (64.3)	495 (27.5)^{Bx}
4 hrs	Control	121 (6.7)^{BC}	1129 (62.7)	524 (29.1)^{B}
	Heparin	100 (5.6)^{Cy}	1143 (63.5)	552 (30.7)^{By}

^{A,B,C} values with different letters within the same column differ significantly; $P < 0.01$

^{x,y} values with different letters within the same column differ significantly; $P < 0.05$

Table 3: Tyrosine phosphorylated protein pattern in frozen/thawed buffalo sperm at thawing and after incubation with or without heparin.

			Tyrosine phosphorylated protein pattern			
Incubation time	Treatment	n.	Pattern E n.(%)	Pattern A n (%)	Pattern EA n (%)	Pattern E+EA n (%)
0 h	Post-thawing	1800	93(5.2)^{A}	1021 (59.5)^{A}	630 (35.0)^{A}	723 (40.2)^{A}
2 hrs	Control	1800	12 (0.7)^{B}	981 (54.5)^{B}	792 (44.0)^{B}	804 (44.7)^{B}
	Heparin	1800	87 (4.8)^{A}	618 (34.3)^{C}	1065 (59.2)^{C}	1152 (64.0)^{C}
4 hrs	Control	1800	36 (2.0)^{C}	933 (51.8)^{B}	777 (43.2)^{B}	813 (45.2)^{B}
	Heparin	1800	141 (7.8)^{D}	636 (35.3)^{C}	975 (54.2)^{D}	1116 (62.0)^{C}

^{A,B,C,D} values with different letters within the same column differ significantly; $P < 0.01$

Protein Tyrosine Phosphorylation: The protein tyrosine phosphorylation was observed in the equatorial, acrosomal and tail regions of spermatozoa. With regard to the sperm head we found the same patterns (E, A and EA) previously described [26]. Interestingly, the localization of tyrosine phosphorylated protein in sperm tail always coincided with that in the equatorial area. Therefore, the percentage of sperm exhibiting fluorescence on tail corresponds to patterns E + EA.

Regardless of the treatment and the time, the percentage of sperm cells that did not exhibit fluorescence was very low (ranging from 0.3 to 3.3%). At thawing the pattern E was significantly higher (P<0.01) than in control group both after 2 and 4 h; however, it was similar to that recorded in heparin treated group after 2h and lower than that at 4 hrs (Table 3). In addition, at thawing pattern A was higher (P<0.01) while patterns EA and E+EA were significantly lower (P<0.01). than in all other groups.

As shown in Table 3, differences in patterns E, A, EA and E+EA were recorded among control and heparin treated groups both after 2 and 4 hrs incubation. In particular, the pattern E increased in the heparin-treated group and extending the incubation time. More interestingly, heparin treatment resulted in a decreased percentage of sperm showing pattern A and increased percentage of sperm displaying pattern EA and pattern E+EA.

Fertilizing Capability: Both cleavage and penetration rates significantly (P <0.01) increased in heparin treated group (77.2 and 80.4 %, respectively) compared to control group (56.6 and 58.0 %, respectively). Interestingly, in heparin treated group the percentage of fast developing embryos (> 4 cells at 48 hrs) was significantly higher than in the control (74.0 vs 55.2 %; P <0.01).

Correlation: The percentage of AR-sperm after LPC induction was positively correlated with CTC pattern B (r=0.6; P <0.05), with tyrosine phosphorylated protein pattern E+A (r=0.5; P <0.05) and E+EA (r = 0.5; P <0.05) and negatively correlated to pattern A (r = -0.5; P <0.05). Furthermore, tyrosine phosphorylated protein pattern A was negatively correlated with pattern E+A (r= - 0.9; P <0.01) and pattern E+EA (r = - 0.99; P <0.01), while pattern EA was positively correlated to pattern E + EA. CTC pattern B was negatively correlated with CTC pattern AR (R = 0.97; P <0.01). Finally, the pattern E+A was positively correlated to cleavage rate (r = 0.5; P =0.06).

DISCUSSION

The evidence that cryopreservation induces capacitation-like changes in mammalian sperm [6, 39, 40] led to the aim of this work. A first objective of this work was to identify the most reliable method for evaluating capacitation of buffalo frozen/thawed sperm among different techniques such as Trypan blue/Giemsa staining after LPC induction, CTC and protein tyrosine phosphorylation. In addition, in order to better comprehend the phenomenon, the fertilizing ability, known to be acquired during capacitation, was assessed using heterologous IVF. Furthermore the observation that cryopreserved sperm are prematurely capacitated, suggested to investigate whether a capacitating agent is indeed required for buffalo frozen/thawed sperm during IVF.

The results of the present study confirm that Trypan blue/Giemsa staining for evaluation of AR sperm following exposure to LPC can be used to assess capacitation of frozen/thawed buffalo sperm [44], as indicated by the increase in AR-sperm after heparin treatment for both 2 and 4 hrs. However, the effect of incubation time prevailed, as shown by the progressive increase of AR at longer incubation times in both capacitating and non capacitating conditions. This latter finding is in agreement with Kadirvel *et al.* [26] that found a spontaneous increase in capacitation and acrosome reaction in buffalo sperm incubated in non capacitating medium after 3 hrs of incubation. Time-dependent increase in spontaneous capacitation and acrosome reaction in non capacitating medium has been also reported in boar [43] and bull [45] spermatozoa.

A high percentage of spermatozoa exhibited CTC Pattern B (capacitated spermatozoa) immediately after thawing (time 0), similar to that previously reported by other authors in buffalo [26], cattle [45], boar [46] and stallion [47]. This clearly indicates that cryocapacitation occurred to a significant extent. The initial membrane damage associated with freezing and thawing seems to promote the ability of frozen/thawed spermatozoa to undergo capacitation-like changes. The freezing and thawing process may involve rearrangement of lipid membrane domains, resulting in an increase in membrane fluidity, leading to increased intracellular calcium, thus initiating the capacitation like changes in cryopreserved spermatozoa [48].

Interestingly, CTC pattern B remained unchanged by increasing incubation times, regardless of the treatment, whereas patterns F and AR showed an opposite trend. In fact, after incubation pattern F decreased while pattern AR significantly increased (P<0.01). In a previous study pattern AR at thawing, however, was higher than that here recorded (25 % vs 3%), whereas the distribution of sperm categories following incubation was similar [26]. Therefore, in the present study, although a high proportion of cryopreserved sperm were capacitated at thawing, spontaneous acrosome reaction occurred only after incubation. When medium contained heparin, a decrease (P <0.05) of pattern F together with an increase (P<0.05) of pattern AR were observed at 4 h. However, no differences were recorded in pattern B between heparin treated and control groups at both incubation times, similar to previous reports in frozen/thawed buffalo [26] and bovine [6] sperm. Consequently, CTC assay seems to be not sensible to detect any effect of the capacitating agent on frozen/thawed buffalo sperm.

Sperm capacitation has been correlated with an increase in protein tyrosine phosphorylation in various mammalian species including human [22], bull [24] and buffalo [26]. In this study, the protein tyrosine phosphorylation assay also showed that cryocapacitation occurs immediately after thawing, as indicated by the majority of the spermatozoa exhibiting the fluorescent pattern and, particularly by the high percentage of spermatozoa displaying pattern A and EA at time 0. This may be attributed to the cryopreservation-induced modifications of sperm plasma membrane as previously mentioned [48]. In particular, the high level of intracellular calcium in frozen/thawed spermatozoa may trigger the signal transduction pathways that lead to protein tyrosine phosphorylation in a non-regulated manner [48]. It was previously reported in buffalo that the distribution of tyrosine phosphorylated protein in frozen/thawed spermatozoa is similar to that of *in vitro* capacitated fresh sperm [26]. The involvement of tyrosine phosphorylated protein of acrosomal region in the process of capacitation

has been demonstrated in bull [6], boar [38], equine [49] and human [22] spermatozoa. Interestingly, the results of this study demonstrated that heparin significantly (P <0.01) decreased the percentage of spermatozoa exhibiting pattern A while increasing (P <0.01) that of sperm showing pattern EA, indicating an advancement of the capacitation process in presence of heparin [50]. In contrast, in a previous work no differences were found in protein tyrosine phosphorylation in frozen/thawed buffalo sperm exposed to capacitating and non capacitating conditions [26]. However, these authors analyzed the percentages of sperm with pattern A + EA. On the contrary, in the present work the effect of heparin was evident on the individual parameters A and EA that showed an opposite trend, highlighted by a high negative correlation.

Another important finding is the higher (P <0.01) percentage of sperm displaying tyrosine protein phosphorylation labeling on the tail region when sperm were incubated in presence of heparin for both 2 and 4 hrs. It was previously reported that buffalo spermatozoa exhibit localization of protein tyrosine phosphorylation on the tail region although predominant labeling is observed on sperm head [26]. The percentage of sperm displaying tail labeling in the present study was higher than that (10-25%) recorded by Kadirvel *et al*. [26]. Furthermore, several immune-localization experiments revealed predominant protein tyrosine phosphorylation on sperm head in bull and boar with negligible labeling on tail [6, 38]. The flagellum appears to be the major sperm compartment presenting tyrosine phosphorylated protein in mouse and human [21, 22, 51], while both head and tail associated protein tyrosine phosphorylation was observed in equine [49] and human [22] spermatozoa. Physiologically, tail associated tyrosine phosphorylation is presumably involved in the hyperactivated motility during sperm capacitation [21, 25].

Roy and Atreja [25] found a time-dependent increase in tyrosine phosphorylation of proteins in heparin-treated fresh buffalo spermatozoa. A time-dependent increase has been also reported in boar [43] and bull [6] fresh spermatozoa. In our study, after incubation, a decrease of pattern A together with an increase of pattern EA compared to time 0 were recorded; however, no differences were observed between 2 and 4 hrs.

These results indicate that the tyrosine phosphorylation patterns and the response to an AR inducer like LPC vary in frozen/thawed buffalo sperm incubated in presence of, heparin. In contrast, the CTC patterns remain unchanged. Nevertheless, a positive correlation was found between the AR-sperm after induction, CTC pattern B and tyrosine phosphorylated protein pattern E+A. However, the only parameter that was correlated with cleavage rate was the tyrosine phosphorylated protein pattern E+A. The differences recorded in protein tyrosine phosphorylation both at the head and tail compartments in heparin-treated frozen/thawed sperm indicate that heparin plays a critical role during the process of capacitation. This latter assay seems, therefore, more reliable to assess capacitation of frozen buffalo semen.

The results obtained after heterologous IVF confirm that heparin is indeed required to capacitate in vitro buffalo frozen/thawed sperm, despite the high level of cryopreservation-induced capacitation. The significantly higher rates of both penetration and cleavage confirm the importance of inducing capacitation also in frozen/thawed sperm. Another interesting results is the higher percentage of fast developing embryos recorded in presence of heparin. This is important because early cleaving embryos are known to be developmentally superior [52]. Interestingly, the chronology of embryo development is related to the time of the first cleavage division that has been recently correlated to the kinetics of sperm penetration [53]. Therefore, on the basis of the results, we may speculate that when IVF is carried out in presence of heparin sperm acquire fertilizing ability earlier and hence anticipate oocyte penetration. This is particularly important in this species, because early oocyte ageing occurs impairing IVF efficiency [3].

CONCLUSIONS

Our results confirm that cryopreservation induces capacitation of buffalo sperm. However, we demonstrated that a capacitating agent such as heparin is still required to improve in vitro fertilizing ability of frozen/thawed sperm, as shown by higher penetration and cleavage rates after heterologous IVF. The comparison among different capacitation assays showed that Trypan blue/Giemsa staining to evaluate LPC-induced AR can be successfully used for frozen/thawed semen, as indicated by higher response in the presence of heparin. Nevertheless, the most reliable assay seems to be tyrosine protein phosphorylation assay that highlights interesting differences at both the head and tail compartments in presence of the capacitating agent. On the contrary, CTC assay failed to show any changes related to the presence of heparin.

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 thank Giuseppe Albero for technical assistance.

REFERENCES

1. Drost, M., 2007. Advanced reproductive technology in the water buffalo. Theriogenol.; 68: 450-3.
2. Gasparrini, B., L. Boccia, J. Marchandise, R. Di Palo, F. George, I. Donnay and L. Zicarelli, 2006. Enrichment of *in vitro* maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development. Theriogenol; 65(2): 275-87.
3. Gasparrini, B., 2007. *In vitro* embryo production in buffalo: current situation and future perspectives. Ital J Anim Sci; 6(2): 92-101.
4. Parrish, J.J., J. Susko-Parrish, M.A. Winer and N.L. First, 1988. Capacitation of bovine sperm by heparin. Biol Reprod; 38: 1171-80.
5. Farlin, M.E., D.J. Jasko, J.K. Graham and E.L. Squires, 1993. Heparin-induced capacitation: a comparison between the bull and stallion. Equ Vet J, 25(15): 49-52.
6. Cormier, N. and J.L. Bailey, 2003. A Differential Mechanism Is Involved During Heparin-and Cryopreservation-Induced Capacitation of Bovine Spermatozoa. Biol Reprod 69: 177-85.
7. Dapino, D.G., P.E. Marini and M.O. Cabada, 2006. Effect of heparin on *in vitro* capacitation of boar sperm. Biol Res; 39: 631-9.
8. Bergqvist, A.S., J. Ballester, A. Johannisson, N. Lundeheim and H. Rodríguez- Martínez, 2007. Heparin and dermatan sulphate induced capacitation of frozen-thawed bull spermatozoa measured by merocyanine-540. Zygote; 15(3): 225-32.
9. Roy, S.C. and S.K. Atreja, 2008. Tyrosine phosphorylation of a 38-kDa capacitation-associated buffalo (Bubalus bubalis) sperm protein is induced by L-arginine and regulated through a cAMP/PKA-independent pathway. Int J Androl; 31(1): 12-24.
10. Galantino-Homer, H.L., P. Visconti and G.S. Kopf, 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3959-monophosphate-dependent pathway. Biol Reprod; 56: 707-19.
11. Visconti, P.E., H. Galantino-Homer, G.D. Moore, J.L. Bailey, X. Ning, M. Fornes and G.S. Kopf, 1998. The molecular basis of sperm capacitation. J Androl; 19: 242-8.
12. Miller, D.J., M.A. Winer and R.L. Ax, 1990. Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biol Reprod; 42: 899-915.
13. Therien, I., G.Bleau and P. Manjunath, 1995. Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. Biol Reprod; 52: 1372-9.
14. Langlais, J. and K.D. Roberts, 1985. A molecular membrane model of sperm capacitation and acrosome reaction of mammalian spermatozoa. Gam Res; 12: 183-224.
15. Parrish, J.J., J.L. Susko-Parrish and N.L. First, 1989. Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. Biol Reprod; 41: 683-99.
16. Vredenburgh-Wilberg, W.L. and J.J. Parrish, 1995. Intracellular pH of bovine sperm increases during capacitation. Mol Reprod Dev; 40: 490-502.
17. Baldi, E., M. Luconi, L. Bonaccorsi, M. Muratori and G. Forti, 2000. Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. Front Biosci; 5: 110-23.
18. Iborra, A., M. Companyó, P.Martínez and A. Morros, 2000. Cholesterol Efflux Promotes Acrosome Reaction in Goat Spermatozoa. Biol Reprod; 62: 378-83.
19. Jagan Mohanarao, G. and S.K. Atreja, 2012. Identification of NO induced and capacitation associated tyrosine phosphoproteins in buffalo (Bubalus bubalis) spermatozoa. Res Vet Sci; 93: 618-23.
20. Visconti, P.E., J.L. Bailey, G.D.D. Moore, Pan, P. Olds-Clarke and G.S. Kopf, 1995. Capacitation of mouse spermatozoa: I. Correlation between the capacitation state and protein tyrosine phosphorylation. Dev; 121: 1129-37.

21. Urner, F., G. Leppens-Luisier and D.Sakkas, 2001. Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. Biol Reprod; 64: 1350-57.
22. Sakkas, D., G.L. Luisier, H. Lucas, D. Chardonnes, A. Campana, D.R. Franken and F. Urner, 2003. Localization of tyrosine phosphorylated proteins in Human sperm and Relation to capacitation and Zona pellucida Binding. Biol Reprod; 68: 1463-69.
23. Leclerc, P., E. de Lamirande and C. Gagnon, 1996. Cyclic adenosine 3', 5' monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. Biol Reprod; 55: 684-92.
24. Galantino-Hormer, H.L., P.E. Visconti and G.S. Kopf, 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. Biol Reprod; 56: 707-19.
25. Roy, S.C. and S.K. Atreja, 2009. Capacitation-associated protein tyrosine phosphorylation starts early in buffalo (Bubalus bubalis) spermatozoa as compared to cattle. Anim Reprod Sci; 110(3): 319-25.
26. Kadirvel, G., P. Kathiravan and S. Kumar, 2011. Protein tyrosine phosphorylation and zona binding ability of in-vitro capacitated and cryopreserved buffalo spermatozoa. Theriogenol; 75(9): 1630-39.
27. Vijayaraghavan, S., K.D. Trautman, S.A. Goueli and D.W. Carr, 1997. A tyrosine-phosphorylated 55-kilodaltion motility associated bovine sperm protein is regulated by cyclic adenosine 3', 5'-monophosphates and calcium. Biol Reprod; 56: 1450-57.
28. Vishwanath, R. and P. Shannon, 2000. Storage of bovine semen in liquid and frozen state. Anim Reprod Sci; 62: 23-53.
29. Bailey, J.L., J.F. Bilodeau and N. Cormier, 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl 21: 1-7.
30. Watson, P.F., J.M. Plummer, P.S.Jones and J.C. Bredl, 1995. Localization of intracellular calcium during the acrosome reaction in ram spermatozoa. Mol Reprod Dev; 41: 513-20.
31. Maxwell, W.M. and L.A. Johnson, 1997. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Mol Reprod Dev., 46: 408-18.
32. Fuller, S.J and D.G. Whittingham, 1996. Effect of cooling mouse spermatozoa to 4 degrees C on fertilization and embryonic development. J Reprod Fertil; 108: 139-45.
33. Fraser, L.R., L.R. Abeydeera and K. Niwa, 1995. Ca2+-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol Reprod Dev; 40: 233-41.
34. Vadnais, M.L., N. Roy, R.N. Kirkwood, D.J. Specher and K. Chou, 2005. Effects of extender, incubation temperature and added seminal plasma on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. Anim Reprod Sci., 90: 347-54.
35. Albrizio, M., A.C. Guaricci, F. Maritato, R.L. Sciorsci, G. Mari, G. Calamita, G.M. Lacalandra, G.G. Aiudi, R. Minoia, M.E. Dell'Aquila and P. Minoia, 2005. Expression and Subcellular localization of the μ-opioid receptor in equine spermatozoa: evidence for its functional role. Reprod; 129: 39-49.
36. Miah, A.G., U. Salma, P.B. Sinha, M. Hölker, D. Tesfaye, M.U. Cinar, H. Tsujii and K. Schellander, 2011. Intracellular signaling cascades induced by relaxin in the stimulation of capacitation and acrosome reaction in fresh and frozen-thawed bovine spermatozoa. Anim Reprod Sci; 125: 31-40.
37. Kaul, G., G.S. Sharma, B. Singh and K.K. Gandhi, 2001. Capacitation and acrosome reaction in buffalo bull spermatozoa assessed by chlortetracycline and Pisum sativum agglutinin fluorescence assay. Theriogenol; 55: 1457-68.
38. Tardif, S., C. Dube, S. Chevalier and J.L. Bailey, 2001. Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. Biol. Reprod; 6: 784-92.
39. Bailey, J.L., J.F. Bilodeau and N. Cormier, 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl; 21: 1-7.
40. Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci; 60/61: 481-92.
41. Kovacs, A. and R.H. Foote, 1992. Viability and acrosome staining of bull, boar and rabbit spermatozoa. Biotech Histochem; 67: 119-24.

42. Boccia, L., R. Di Palo, A. De Rosa, L. Attanasio, E. Mariotti and B. Gasparrini, 2007. Evaluation of buffalo semen by Trypan blue/Giemsa staining and related fertility *in vitro*. Ital. J.Anim. Sci, 6(2): 739-42.
43. Choi, Y.J., S.J. Uhm, S.J. Song, H. Song, J.K. Park, T. Kim, C. Park and J.H. Kim, 2008.Cytochrome-c upregulation during capacitation and spontaneous acrosome reaction determines the fate of pig sperm cells: linking proteome analysis. J Reprod Dev., 54: 68-83.
44. Boccia, L., S. Di Francesco, G. Neglia, M. De Blasi, V. Longobardi and G. Campanile and B. Gasparrini, 2013. Osteopontin improves sperm capacitation and *in vitro* fertilization efficiency in buffalo (Bubalus bubalis). Theriogenol, 80: 212-17.
45. Cormier, N. and J.L. Bailey, 2003. A differential mechanism is involved during heparin and cryopreservation induced capacitation of bovine spermatozoa. Biol Reprod, 6: 177- 85.
46. Green, C.E. and P.F. Watson, 2001.Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reprod, 122: 889 -98.
47. Thomas, A.D., S.A. Meyers and B.A. Ball, 2006. Capacitation-like changes in equine spermatozoa following cryopreservation. Theriogenol, 65: 1531-50.
48. Bailey, J.L., J.F. Bilodeau and N. Cormier, 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl, 21: 1-7.
49. Pommer, A.C., J. Rutllant and S.A. Meyers, 2003. Phosphorylation of protein tyrosine residues in fresh and cryo-preserved stallion spermatozoa under capacitating condition. Biol Reprod, 68: 1208-14.
50. Gualtieri, R., R. Boni, E. Tosti, M. Zagami and R. Talevi, 2005. Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium in vitro. Reprod, 129: 51-60.
51. Lewis, B. and R.J. Aitken, 2001. Impact of epididymal maturation on the tyrosine phosphorylation pattern exhibited by rat spermatozoa. Biol Reprod, 64: 1545-56.
52. Lonergan, P., H. Khatir, F. Piumi, D. Rieger, P. Humblot and M.P. Boland, 1999. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. J Reprod Fertil; 117: 159-67.
53. Alomar, M., H. Tasiaux, S. Remacle, F. George, D. Paul and I. Donnay, 2008. Kinetics of fertilisationand development and sex ratio of bovine embryos produced using the semen of different bulls. Theriogenol; 107: 48-61.