Italian Journal of Animal Science

ISSN: (Print) 1828-051X (Online) Journal homepage: http://www.tandfonline.com/loi/tjas20

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To cite this article: Luigi Zicarelli, Evelina Mariotti, Serena Di Francesco, Salvatore Velotto, Marcello Rubessa & Gianluca Neglia (2009) Effect of bull on in vitro sperm capacitation induced by different agents in buffalo species (Bubalus bubalis), Italian Journal of Animal Science, 8:sup2, 177-179, DOI: 10.4081/ijas.2009.s2.177

To link to this article: https://doi.org/10.4081/ijas.2009.s2.177

Effect of bull on *in vitro* sperm capacitation induced by different agents in buffalo species (*Bubalus bubalis*)

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ABSTRACT - The aim of this work was to evaluate the effect of bull on the efficacy of different capacitating agents in buffalo. Spermatozoa derived from 4 different bulls were incubated in absence of a capacitating agent, in presence of 0.01 mM heparin and in 20% buffalo estrous serum (BES) for 2 hours. Sperm were then exposed to lysophosphatidylcholine, an agent that induces acrosome reaction only on capacitated spermatozoa. A double staining technique with Trypan-blue/Giemsa was used to evaluate viability and acrosome status of spermatozoa fixed in 37% formaldehyde. The efficiency of capacitation was evaluated by assessing the percentage of acrosome-reacted (AR) spermatozoa in the treated groups. A bull effect on sperm capacitation *in vitro* was demonstrated, as indicated by differences in the percentage of AR sperm among bulls regardless of the treatment. In particular when heparin was used as capacitating agent bull C gave higher percentages of AR sperm than bull A (22.7 *vs.* 14.0%, respectively) with intermediate values for bulls B and D (16.7 and 19.1%, respectively), whereas when BES was used bull D was the most efficient one (22.1, 21.9, 25.0, and 35.0% for bulls A, B, C and D, respectively).

Key words: Buffalo, Sperm capacitation, Heparin, Buffalo estrus serum.

Introduction - In the last few years interest in buffalo breeding has largely increased worldwide, due to the critical role that this species plays in many climatically disadvantaged agricultural systems. The success of buffalo breeding highly depends on the genetic improvement that can be achieved through the application of reproductive biotechnologies. It has been proven that, due to low and inconsistent response to multiple ovulation and embryo transfer (MOET) treatments (Misra, 1997; Zicarelli, 1997) in buffalo, the *in vitro* embryo production (IVEP) technology is the best tool to enhance genetic progress through the maternal contribution. Fertilization has been often considered the most critical step of the IVEP procedures in buffalo, as cleavage rates lower than those obtained in other domestic species have been widely reported (Galli *et al*., 2000, Neglia *et al*., 2003, Gasparrini *et al*., 2004). Many factors may affect the *in vitro* fertilization efficiency, such as the sperm viability and capability, the adequate *in vitro* environment for gametes survival, the appropriate time of insemination, the duration of gametes co-incubation, the presence of cumulus cells, and the acquisition of the oocyte developmental competence during the complex process of cytoplasmic maturation. The quality of frozen semen has been previously considered the major factor impairing *in vitro* fertilization (IVF), based on the demonstration of several damages of the male gamete occurring following cryopreservation (Meur *et al*., 1988), together with

the drastic reduction of cleavage rate reported with frozen compared to fresh semen (Totey *et al*., 1992). Currently, the quality of frozen semen has improved, as indicated by similar fertility parameters, recorded for fresh compared to frozen semen (Wilding *et al*., 2003), suggesting that other factors may negatively affect fertilization. However, the overall improvement of the cryopreserved sperm quality did not eliminate another serious impediment, the so-called "bull effect", consisting in the high degree of variation of the buffalo bulls fertilizing capability *in vitro* (Totey *et al*., 1993). Sperm need to undergo capacitation to acquire the fertilizing ability; this process that *in vivo* occurs within the female genital tract, must be induced *in vitro*. Although several agents have been proven to induce sperm capacitation *in vitro*, heparin is still the most widely used as it gives more consistent results regardless of the bull in other species (Boccia, 2006). An earlier work demonstrated that an improvement of capacitation can be achieved by incubating buffalo sperm with some biological fluids, such as buffalo estrus serum (BES) and the follicular fluid (FF) recovered from a pool of dominant follicles (Boccia *et al*., 2005). However, the study was carried out using sperm derived from a single bull previously tested for IVF, without taking into account the potential differences among bulls. Therefore, the aim of this work was to evaluate the bull effect on the efficacy of different capacitating agents, such as heparin and BES in buffalo species, which was indirectly assessed by estimating the capability of spermatozoa to acrosome react.

Material and methods - Frozen-thawed sperm from 4 Italian Mediterranean buffalo bulls were treated by swim-up in order to select only the motile and live population. Spermatozoa were assessed immediately after swim-up separation, to evaluate the incidence of acrosomal loss in non-treated cells (time 0). The remaining spermatozoa were incubated in the fertilization medium, a modified TALP (Lu *et al.*, 1987) supplemented with 0.2 mM penicillamine and 0.1 mM hypotaurine, in three different conditions for 2 hours: in absence of a capacitating agent (control), in presence of 0.01 mM heparin and in 20% BES.

After incubation with these agents, sperm were exposed for 15 min to 60 μg mL^{-1} of lysophosphatidylcholine, an agent known to induce acrosome reaction only on capacitated spermatozoa. Trypan blue was used first to differentiate live from dead spermatozoa and the dried smears were then fixed in 37% formaldheyde and stained with Giemsa for acrosome evaluation by microscopic examination. The evaluation of sperm cells was performed at 40x and 100x oil immersion magnification. The percentage of acrosome-reacted spermatozoa in each group was used to assess the efficiency of capacitation under different incubation conditions. The experiment was repeated two times. Differences between groups were analyzed by Chi Square test.

Results and conclusions - Out of the total number of sperm analyzed, acrosomal loss was observed on average only in 4% of the sperm population at time 0; this may be accounted for by either damages preceding cell death or freezing-induced capacitation. Results on sperm capacitation, indicated by the percentages of acrosome-reacted sperm in each group are shown in Table 1.

Regardless of the bull, treatment with BES resulted in a significantly ($P<0.01$) higher incidence of acrosome reaction compared with heparin, which is the capacitating agent currently used in the IVF system. However, both treatments improved capacitation compared to the control, confirming previous observations (Boccia, 2006). The presence of a certain percentage of AR sperm in the control is accounted for by the presence of bovine serum albumin in the medium, which is known to act as a cholesterol acceptor and hence to facilitate capacitation. Furthermore, a bull effect on sperm capacitation *in vitro* was demonstrated, as indicated by differences in the percentage of AR sperm among bulls regardless of the treatment. In particular when heparin was used as a capacitating agent, bull C gave higher percentages of AR sperm than bull A with intermediate values for bulls B and D, whereas when BES was used bull D was the most efficient bull. These results demonstrated that the bull affects the efficacy of the capacitation agent; in fact BES was

Table 1. Percentage of acrosome-reacted (AR) sperm in relation to the bull and capacitation agent.

Treatments	Control n (%)	Heparin n (%)	BES n (%)	Total n (%)
Bull A	220 (6.4) ^{AY}	250 (14.0) ^{aXx}	267 (22.1) ^{AXy}	737 (14.2) ^{Aa}
Bull B	215 (5.1) ^{AY}	246 (16.7) ^{abX}	265 (21.9) ^{AX}	726 (14.7) ^{Aab}
Bull C	199 (9.5) ^{ABY}	203 (22.7) ^{bX}	184 (25.0) ^{bX}	586 (19.1) ^{bc}
Bull D	234 (14.1) ^{BY}	246 (19.1) ^{abY}	206 (35.0) ^{BaX}	686 (22.7) ^{Bc}
Total	868 (8.8) ^{Y}	945 (18.1) ^{X}	922 (26.0) ^{Z}	

Values within columns with different superscripts are significantly different ($^{a,b}P<0.05$; ^{A,B} $P<0.01$).
Values within rows with different superscripts are significantly different (^{x,y} $P<0.05$; ^{X,Y,Z} $P<0.01$).

superior to heparin in 2 out of the 4 bulls tested (A and D), whereas no differences were found among treatments for the other two bulls (B and C). On the other hand, the efficacy of heparin to induce sperm capacitation *in vitro* was demonstrated for 3 out of the 4 bulls; when bull D was used, heparin failed to improve capacitation, as indicated by the similar percentages of AR sperm with respect to the control, whereas BES significantly improved this parameter.

In conclusion our results indicate that in order to improve *in vitro* fertilization efficiency in buffalo it may be worth to assess the efficacy of different capacitating agents in relation to the bull enrolled in the program.

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