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Evaluation of bioassay using in vitro matured water buffalo oocytes in predicting bull sperm fertility

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The ability to penetrate zona-intact oocyte has been a very useful test of sperm fertility. In this study, the accuracy of bioassay in predicting sperm fertility using water buffalo oocytes matured in vitro have been evaluated. In Experiment 1, length of sperm-oocyte co-incubation was evaluated based on percentage penetration. The observed mean penetration rate using 10 hr and 24 hr co-incubation time had no difference, including monospermic fertilization rate. In Experiment 2, the effect of caffeine, theophylline alone or in combination were evaluated based on percentage penetration rate. Neither conditions with capacitating agents used showed no significant differences in the mean penetration rates (but was higher significantly against the control ($P<0.05$). In Experiment 3, 5 bulls were evaluated on their fertilizing capability. The mean penetration rates observed among the bulls evaluated had no significant differences (34.44 ± 8.68 to 48.61 ± 2.32). The results of this study suggest that the in vitro matured oocyte bioassay may be a useful tool in assessing bull sperm fertility.

Key words: assay test, bulls, sperm, capacitation, oocytes, co-incubation

Introduction

Acrosome reaction of the sperm served as the definitive evidence for the completion of sperm maturation, collectively called as "capacitation" (Yanagimachi, 1981). The endpoint for sperm capacitation occurs when the spermatozoa penetrated an intact zona pellucida of unfertilized homologous ova. The use of light mictoscopic procedures for analysis of acrosome reaction of either living, motile sperm or in fixed and stained preparations is limited whether or not the acrosome reactions considered were truly "physiological". A physiological acrosome reaction (PAR) is the condition wherein the spermatozoon was able to penetrate the intact-zona pellucida of a matured egg, whether ovulated or matured in vitro. In mouse (Bleil and Wasserman, 1983) and hamster (Jacobs et al., 1984), PAR occur only on the

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surface of zona pellucida This condition makes PAR quite doubtful because of the presence of large number of spermatozoa swimming freely in the fertilization medium suspension. Thus, the penetration of zona pellucida became the most appropriate endpoint for studies on sperm penetrating capability. In most fertility studies, the response being recorded is the percentage of penetrated eggs. Analysis of percentage data are most accurate in the range of 30 – 70 % (Snedecor and Cochram, 1980). For the percent egg penetration to remain within this range, data on the appropriate sperm concentration and its interactions with other variables (eg., capacitating agent, bulls) is needed. Also, the time of sperm-egg co-incubation time must be strictly controlled, since spontaneous activation of the egg may occur during prolonged egg exposure to sperm (Binor et al., 1980).

Thus, the use of a physiologically appropriate end point in fertility studies, that is the penetration of zona pellucida, the use of large number of homologous oocytes matured in vitro is going to be beneficial. It has been demonstrated in many mammalian species that oocytes matured in vitro retained their biological and physical characteristics that includes sperm penetration and lectin binding, cortical reaction-mediated block to polyspermy (Fukui et al., 1988a,b; Blottner et al., 1990; Coy et al., 1993) that are relevant in fertility studies. In this study, we present an assay test for predicting buffalo bull fertility using oocytes matured in vitro.

Materials and Methods

Reagents

Unless specified, all chemicals, reagents and hormones were purchased from Sigma (St Louis, Mo, USA).The basic media for maturation of oocytes is a defined tissue culture medium with Earle's salts and L-glutamine (Krisher et al., 1999), for fertilization is a Brackett-Oliphant medium (BO; Brackett and Oliphant, 1975) and for culture is the modified synthetic oviductal fluid medium (mSOF, Tervit et al., 1972).

Oocyte collection and maturation in vitro

Buffalo ovaries were collected at a local abattoir and transported to the laboratory within 5 hr in sterile physiological saline solution (0.9 % NaCl, w/v) supplemented with antibiotics (0.1 mg/ml Penicillin and 500 ug/ml Streptomycin) maintained at a temperature of 35-37º C. The follicular oocytes were aspirated from 3-5 mm follicles using an 18- gauge needle attached to a 10- ml disposable plastic syringe. The follicular aspirates were pooled in a test tube maintained at 37º C in a water bath. After 5 min, part of the follicular fluid was decanted and the sediment searched for cumulus-

oocyte complexes (COCs) using a stereomicroscope at low magnification. Oocytes with intact, unexpanded cumulus cell investments and with evenly granulated ooplasm were selected for use in the experiments. Selected COCs were washed twice in Hepes buffered TCM-199 with Earles salt supplemented with 10 % fetal calf serum (FCS). The COCs were then placed in droplets of maturation medium consisting of TCM-199 + 10 % FCS + 5.5 mg/ml Na pyruvate, 0.02 IU/ml FSH, 1 µg/ml estradiol-17 β, 10 ng/ml EGF, 50 µg/ml gentamycin sulfate with cysteamine.

Sperm preparation and fertilization

Locally processed frozen semen of water buffaloes from the Sperm Processing Unit of the Philippine Carabao Center stationed at Digdig, Carranglan, Nueva Ecija were used for in vitro fertilization (IVF). For each trial, straw containing 0.5 ml semen was thawed in a water bath at 39°C for 15 sec and processed by the swim-up method. Briefly, the semen suspension was put in a test tube, added with 5 ml of fertilization medium + 10 % calf serum and centrifuged (460 g for 5 min) for washing (2x). The sperm pellet was then layered with 2 ml of fertilization medium and kept in the incubator for 1 hr. Afterwards, about 1.8 ml of the upper portion of the semen suspension was recovered and washed using the same fertilization medium. The sperm pellet was re-suspended in the same medium to give an initial sperm concentration of 10 x 10^6 sperm/ml. Then, a 20 µl aliquot of the sperm suspension was introduced in droplets of 80 µl fertilization medium containing the pre-washed oocytes (10-15 oocytes/drop) to co-incubate. The final sperm concentration during fertilization consisted of 2 x 10^6sperm/ml, 5 mM caffeine or theophylline (4.5 mg/ml) and 10 % calf serum. The motility rate of sperm during insemination was more than 50 %.

Experimental

Experiment 1. Effect of length of sperm-oocyte co-incubation on the fertilization of IVM buffalo oocytes. Oocytes were exposed to spermatozoa for 10 hr or 24 hr, before collecting and denuded free of extra sperms found still attached to the zona pellucida by pipetting. Afterwards, the oocytes were cultured in mSOF for another 6 hr before examining for the evidence of fertilization. The sperm used was from bull No.

Experiment 2. Effect of capacitating agent on fertilization of IVM buffalo oocytes. The fertilizing ability of spermatozoa exposed to theophylline or theophylline + caffeine (was evaluated by exposing the oocytes for 24 hr, before transferring in mSOF medium for another 6 hr, then fixed for fertilization analysis.

Experiment 3. Bull-specific effect on the fertilization of IVM oocytes. In the establishment of a functional IVF protocol, it is necessary to have a

common source of semen. It has been demonstrated that spermatozoa from individual bulls differ in their ability to fertilize in vitro (Chauhan et al., 1998.). In this experiment, we tried to determine which of the available semen processed in the Sperm Processing Center of PCC is more capable of fertilizing IVM oocytes for eventual use in the AI program.

Statistical Design

The oocytes were assigned randomly to treatments of each experiments upon collection and selection based on the criteria described by Ocampo et al., (2001). The percentages of penetrated oocytes and monospermic fertilization were analyzed by ANOVA ($P<0.05$).

Results and Discussion

A total of 90 oocytes were co-incubated with semen from the same bull but of different exposure time (Table 1). The percentage penetration rate was higher when sperm-oocytes were co-incubated for 24 hr (39.15±2.1)) although showed no difference when using 10 hr (27.38±10.4) co-incubation time. Similarly, the monospermic fertilization rate showed no difference but was higher when using a shorter sperm-oocyte co-incubation time. Before, using sperm concentration of 1 x 10^7/ml, a high incidence of polyspermy was reported when 12 -16 hr sperm-oocyte co-incubation (Bacci et al., 1991) was used but eventually reduced when co-incubation was lessened. In another study, a co-incubation time of 24 hr with 2 x 10^6/ml resulted to a better fertilization rates compared with 1-4 x 10^6/ml (Totey et al., 1993). Others have used high sperm concentration with a sperm-oocyte co-incubation of 6 hr and improved fertilization rates without affecting the post-fertilization development to blastocyst stage (Gasparrini, 2001). Our earlier output suggested the use of 2 x 10^6/ml when using frozen-thawed semen yielding a 50.0 % fertilization rate or better without increasing the occurrence of polyspermic fertilization (Ocampo et al., 2001; 2015). In this experiment, a sperm concentration of 2 x 10^6/ml was used resulting to less than 50.0% penetration rate. The result was in contrast to our previous experiments and could be attributed to the quality of semen used from the same bull after thawing.

Table 1. Effect of sperm-oocyte co-incubation time on the penetration of buffalo oocytes.

Time (hr)	No. of trials	No. of oocytes (%) inseminated	penetrated	monospermic	polyspermic	unfertilized	others*
10	4	44	12 (27.38±10.4)	8 (66.7)	4 (33.7)	29 (65.9)	3 (6.8)
24	3	46	18 (39.15±2.1)	11 (61.1)	7 (38.9)	23 (50.0)	5 (10.9)

*oocytes were either degenerated, M-1 or parthenote.

In the induction of sperm capacitation in buffalo, heparin (Totey et al., 1993; Boni, 1994; Chauhan et al., 19980 and caffeine (Ocampo et al., 2001a,b) have routinely been used. The sperm can either be pre-incubated (Madan et al., 1994; Chauhan et al., 1997; Boni et al., 1999) with capacitating agent or added directly to the fertilization medium (Totey et al., 1993; Gasparrini et al., 2000; Ocampo et al., 2015). In this experiment, theophylline and caffeine alone/or in combination were evaluated on their influence in the penetration of oocytes (Table 2). The penetration rate of oocytes by sperm directly added to the fertilization medium with theophylline, caffeine or in combination (48.61±2.32 to 54.68±6.48))was significantly higher than the control (10.23±3.24)). The monospermic fertilization showed no difference ranging from 58.8 % - 76.0 %. Other researchers that used theophylline as the capacitating agent had varying results in terms of penetration and monospermic fertilization (Atabay et al.,2010; Aquino et al., 2013). Nonetheless, our results showed that neither capacitating agent could be used to induce capacitation but needs further screening in relation to the optimum concentration required to necessitate its use for IVF in relation to the source of sperm.

Table 2. Effect of capacitating agent on the penetration of buffalo oocytes
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Agent used	No. of trials	No. of oocytes (%) inseminated	penetrated	monospermic	polyspermic	unfertilized	others*
Control	4	50	5 (10.23±3.24)[a]	3 (60.0)	2 (40.0)	45 (90.0)	0
Caffeine	6	104	51 (49.45±6.67)[b]	30 (58.8)	21 (41.2)	53 (51.0)	0
Theophylline	4	50	25 (48.61±2.32)[b]	19 (76.0)	6 (24.0)	5 (30.0)	20 (20.0)
Theophylline + Caffeine	3	62	33 (54.68±6.48)[b]	22 (66.7)	11 (33.3)	23 (37.1)	6 (9.7)

[a,b] Values differ significantly (P<0.05); *oocytes were either degenerated, GVBD or M-1

In Experiment 3, a total of 237 oocytes were inseminated in vitro using semen from 5 buffalo bulls of different breeds. No significant differences in the mean penetration rate (34.44±8.68 to 48.61±2.32) between bulls were observed (Table 3). Lowest monospermic fertilization rate was obtained from bull # BR090983 (65.0%) with the highest observed from bull # 3CL03003 (81.5%).

Table 3. Bull effect on the penetration of buffalo oocytes.

Bull No.	No. of trials	No. of oocytes (%) inseminated	penetrated	monospermic	polyspermic	unfertilized	others*
3CL03003	4	40	16 (41.96±3.41)	13 (81.5)	3 (18.8)	18 (45.0)	6 (15.0)
GR1323	3	34	11 (34.44±8.68)	8 (72.7)	3 (27.3)	21 (61.8)	2 (5.9)
BR090983	3	42	20 (45.43±12.68)	13 (65.0)	7 (35.0)	15 (35.7)	7 (16.7)
2CL0097	4	50	25 (48.61±2.32)	19 (76.0)	6 (24.0)	19 (38.0)	6 (12.0)
2GP10071	3	71	29 (43.27±5.34)	21 (72.4)	8 (27.6)	42 (59.1)	0

*oocytes were either degenerated, GVBD, M-1 or parthenote

A high degree of variability in bovine and ovine IVF have been observed after using semen from different source, indicating differences in sperm capacitation, acrosome reaction and fertilization (Iritani et al., 1986; Fukui et al., 1988a,b). In buffalo, similar variations on the fertilizing capability and subsequent embryonic development in vitro among individual bulls have been reported (Chauhan et al., 1998). In this study, the fertilizing capability of semen from selected bulls showed no difference providing further evidence on the usefulness of the bioassay used. The protocol adopted on handling and processing of semen samples have been standardized to minimized discrepancies that may influence the expected result. Similarly, the sperm motility prior to insemination and the final sperm concentration in the droplets of fertilization medium have been strictly monitored before introducing the oocytes. The results provided further support to the recommendations of the Sperm Processing Unit of PCC on the use of semen from the bulls tested for AI program of the agency.

In conclusion, the sperm penetration assay using IVM oocytes has an extended range considering the penetration rates observed in this study. In an assay designed to determine male-associated traits (such as intrinsic differences in fertility or responses to capacitating agent), limiting female contribution to variability is important. It is known that female differences contribute significantly to experimental variance in IVF and embryo culture research (Bavister, 1986). Nonetheless, the use of pooled oocytes from various ovaries of slaughtered females greatly facilitate and simplify the analysis of results. Also, it is important to compare bull responses to various

capacitating agents using both zona-free and zona-intact oocytes in determining the accuracy of fertility studies. Both assay could be used in determining the egg penetration percentage and the number of penetrating sperms/egg in order to increase accuracy in assessing fertility (Aitken and Elton, 1986). Therefore, the presence/availability of large number of homologous oocytes derived from slaughtered females will facilitate large scale bioassays of buffalo bull(s) fertility using ejaculated, epididymal or frozen-thawed semen.

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