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Chromosome Complement and Developmental Competence of Swamp Buffalo Oocytes Matured and Fertilized in Vitro

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Abstract The chromosome complement of swamp buffalo (SB) oocytes matured in vitro and competence of resulting zygotes to the blastocyst stage based on the timing of its appearance and its implications has been discussed. In Expt 1, chromosomal aneuploidy was found more prevalent in oocytes cultured in maturation conditions without pregnant mare serum gonadotropin (PMSG) addition. The completion of 1st meiosis/nuclear maturation does not always guarantee the presence of complete chromosome complement of oocytes maturing in vitro. In Expt. 2, diploidy was the result of normal fertilization while polyploidy arised from polyspermic fertilization. In Expt. 3, the blastocyst formation rate of early- cleaving embryos was significantly higher than late- cleaving embryos supporting the idea that early- cleaving embryos are more likely to have normal chromosome complement resulting from normal fertilization than late- cleaving embryos. The results provide some insight into several important aspect of SB fertilization in vitro and could be used as a criterion in the production and eventual selection of embryos for embryo transfer.

Keywords: Chromosome, oocytes, embryos, in vitro

Introduction

In bubaline, attempts for the in vitro production of large numbers of embryos from abattoir-derived oocytes both for commercial and research purposes is now on going (Palta and Chauhan, 1998; Ocampo *et al*., 2001; Gasparrini, 2002; Zicarelli, 2003). Along with the development of in vitro maturation, fertilization and culture (IVM/IVF/IVC) methods is the understanding of various factors that play an important role in the acquisition of competence of oocytes matured in vitro for successful fertilization and subsequent development of resulting embryos to transferable stage. For instance, the quality of oocytes could significantly affect the rate of fertilization (Jainudeen *et al*., 1993; Ocampo *et al*., 1993; Chauhan *et al*., 1997) together with the source and methods of inducing sperm capacitation (Totey *et al.*,

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1993b; Mamuad and Venturina, 2002). Also, the time of completion of 1st cleavage of zygotes after IVF can vary, of which those cleaving within 40 hr post insemination (hpi). are more likely to develop to the blastocyst stage than those that start to cleave later (Nandi *et al*., 1998; Ocampo *et al*., 2001). The conditions of IVC affect the proportion of presumptive zygotes that develop to the blastocyst stage (Ocampo *et al.*, 1995; Gasparrini, 2003a) and the number of cells found within similar developmental stage (Marquant-LeGuienne et al., 1989). Variations in IVF and IVC conditions can be reflected in differences in chromosomal complement within the cells of embryos with overall frequencies of abnormalities falling in 12 - 44 % (King *et al.*, 1988; Iwasaki *et al.*, 1992; Yadav *et al*., 1997).

Adoptions and applications of informations gathered from the above research outputs in the IVP of swamp buffalo embryos had resulted to a low pregnancy rates (Ocampo *et al.*, 1996a) following a series of embryo transfers. Similar observations have been reported in other bubaline species (Madan *et al*., 1994b; Neglia, 2003a; Hufana-Duran *et al.*, 2004). In other mammalian species, chromosomal anomalies in embryos produced in vitro, low cell number and timing of completion of 1st cleavage were reported to affect the subsequent competence of embryos to develop to term (Hare *et al*., 1980; Iwasaki *et al.*, 1989; Yadav *et al*., 1993). In this study, we investigated the chromosome complement of SB oocytes matured in vitro, zygotes produced after IVF and correlate the developmental competence to the blastocyst stage of zygotes produced on the timing of its completion and discuss its implications.

Materials and methods

Media

The basic media used for maturation of oocytes were tissue culture medium (TCM-199; Gibco Co., Grand Island, N.Y., USA, Cat. No. 31100-035, Lot No. 1137722) with Earle's salts, L-glutamine and modified Parker medium (mPM); a TCM-199 medium modified by adding Hepes-acid, 1.5 mg/ml; calcium lactate, 0.7 mg/ml and sodium pyruvate, 0.1 mg/ml. Both media were supplemented with 10 % and 20 % (v/v) heat-treated buffalo calf serum (BCS), respectively, 100 IU/ml penicillin G and 100 ug/ml streptomycin SO_4 with or without pregnant mare serum gonadotropin (PMSG; 10 IU/ml, Serotropin, Teikokuzoki, Tokyo, Japan). For in vitro fertilization, mPM was used and supplemented with 10 % (v/v) BCS and 5 mM caffeine (Sigma Chem Co., USA, Lot No. 127F-0395, No. C-0750). For embryo culture, mPM was used with 20 % BCS and cumulus cell monolayer prepared according to Ocampo et al. (2001).

Oocyte collection and maturation

SB ovaries were collected immediately postmortem at a local abattoir and transported to the laboratory in 0.9 % NaCl solution at 30-35 °C within 2 - 4 hr. The ovaries were pooled irrespective of the donors estrus cycle. Cumulus-oocyte complexes (COCs) were aspirated from pre-ovulatory (more than 6 mm in diameter) and small antral follicles (3-5 mm in diameter) by using an 18-gauge needle attached to a 10-ml sterile plastic syringe, washed three times in the maturation medium before selecting using a stereomicroscope (200-400 x) based on the criteria described by Leibfried-Ruthledge (1979). A group of 10-15 COCs were transferred into 50 µl droplets of maturation medium under mineral oil (Sigma Chem Co., St. Louis, USA; Lot No. 29HO129) in a 35 x 10 mm Falcon polysterene culture dish (Becton and Dickinson Labware, N.J., USA) which had been previously pre-incubated to equilibrate for at least 2 hr in a CO_2 incubator. COCs were cultured at 39 ℃ under an atmosphere of 5 % CO_2 and 95 % air with high humidity. After culture for 24 hr, the cumulus and corona cells were removed by pipetting.

Sperm preparation and fertilization in vitro

Locally processed frozen semen of SB was used for fertilization. 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 mPM + 10 % BCS 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 10 µl aliquot of the sperm suspension was introduced in droplets of 90 µl fertilization medium containing the pre-washed oocytes (10-15 oocytes/drop) to co-incubate. The final sperm concentration during fertilization consisted of 1 x 10^6sperm/ml, 5 mM caffeine and 10 % BCS.

In vitro culture

Evaluation of the viability of cleaved early- stage (2- to 4- cell) embryos was determined by co-culturing in vitro with cumulus cell monolayer. The cumulus cell monolayer was prepared from COCs that were denuded free of cumulus cells by pipetting at the end of maturation culture period to form a monolayer. Cleaved embryos were cultured in 50 µl droplets of mPM + 20%

BCS with cumulus cell monolayer for 7 days and assessed morphologically according to the developmental stage attained.

Experimental

Experiment 1. Chromosome complement of oocytes cultured in different maturation conditions. The COCs derived from pre-ovulatory follicles (< 6 mm in diameter) were cultured for another 6 hr in 50 µl droplets of TCM-199 + 10% BCS (10-15 COCs/drop) to complete the required maturation period and served as the control, whereas, the COCs cultured in TCM-199 and mPM medium with or without PMSG addition were considered as treatments.

Experiment 2. Chromosome complement of embryos after in vitro fertilization. COCs were matured in mPM with PMSG for 24 hr before denuding free of cumulus cells and subjected to fertilization in vitro. The chromosome complement of cleaved embryos (2- to 4- cell stage), 20 – 45 hpi and those that failed to cleaved were determined.

Experiment 3. Developmental competence of embryos classified according to the time of cleaving. The relationship between the time of completion of 1st cleavage and their developmental competence was determined. Embryos from early- and late- cleaving groups were those obtained between 20-30 hr and 35-45 hpi, respectively. These embryos were cultured in vitro for another 7 days in mPM + 20 % BCS with cumulus cell monolayer and assessed their developmental stage at the end of culture.

Analysis of chromosome complement and nuclear status

At the end of maturation period, representative oocytes were mounted on glass slides with cover slip supported by droplets of a paraffin-vaseline (1:12, v/v) mixture, fixed and cleared with acetic acid:ethanol solution (1:3, v/v) for 24 hr or more. Cleared specimens were stained with 1 % orcein dissolved in 40 % acetic acid in water and observed under phase contrast optics at 200-400 x magnification. Oocytes with a polar body and a 2nd metaphase plate were regarded as matured (Fig. 1). Similarly, representative oocytes at the end of maturation period, unfertilized oocytes and cleaved embryos at the end of sperm-oocyte co-culture were processed individually according to the air-drying method of Tarkowski (1966) for the chromosome investigation. Samples were transferred to 1 % sodium citrate hypotonic solution (previously warmed to 37℃) for 5-10 min and then placed into a grease-free slide. After the addition of several drops of fresh methanol-acetic acid (3:1, v/v) mixture, the samples were allowed to air dry and then stained with Giemsa. Chromosome spreads were evaluated using phase contrast optics at 200-400 x magnification.

Statistical analysis

Differences in the ploidy of SB oocytes matured in different maturation conditions, those found uncleaved and at 2- to 4- cell stages following in vitro fertilization and the differences in initial cleavage rates and percentages of cleaved embryos developing to the morula up to the blastocyst stage were analyzed by the Chi-square test. Significance was defined as $P \leq 0.05$.

Results

In Expt.1, a total of 64 and 350 COCs from pre-ovulatory and small antral follicles isolated from ovaries of slaughtered SB females were analyzed, respectively. The nuclear maturation rate of COCs taken from pre-ovulatory follicles was 45/64 (70.3 %). Of which, 31/45 (68.8 %) had a normal ploidy, 7/45 (15.5 %) were hypoploid and another 7/45 (15.5 %) presented the 1st polar body but with distorted spindle formation (DSF) and/or condensed chromosome (CCh). COCs taken from small antral follicles were distributed to different culture conditions with or without PMSG addition. The nuclear maturation rate were noticeably higher in culture conditions without PMSG addition in both media used but the percentage of oocytes analyzed with normal ploidy were higher in culture conditions with PMSG. In both cases, chromosomal aneuploidy were observed more prevalent in culture conditions without PMSG addition, indicating that completion of 1st meiosis/nuclear maturation in vitro does not always guarantee the normality of oocyte maturation in vitro (Table 1).

Table 1. Chromosome complement of oocytes cultured with or without PMSG addition.

Culture Condition	**Nuclear Maturation**	**Ploidy (%)**			**Others (%)**	
		Haploid	**Hypoploid**	**Hyperploid**	**DSF/CCh**	**GV/M-1**	**P**
Pre-ovulatory Small antral follicles TCM-199	**45/64(70.3)b**	**31(68.8)a**	**7(15.5)**	**0**	**7(15.5)**	**9(14.1)**	**0**
-	**128/140(91.4)a**	**70(54.6)a**	**20(15.6)**	**2(1.5)**	**40(31.0)a**	**12(8.6)**	**0**
+ mPM	**60/78(76.9)b**	**46(76.6)b**	**10(16.6)**	**0**	**6(10)bc**	**16(20.5)**	**2(2.5)**
-	**62/72(86.1)ab**	**50(80.6)bc**	**4(12.8)a**	**0**	**8(12.9)b**	**8(13.9)**	**0**
+	**46/60(76.6)b**	**40(86.9)c**	**2(4.3)b**	**2(4.3)**	**2(4.3)c**	**14(23.3)**	**0**

Values with different superscript in the same column differ ($P<0.05$); (-) No PMSG; (+) With PMSG

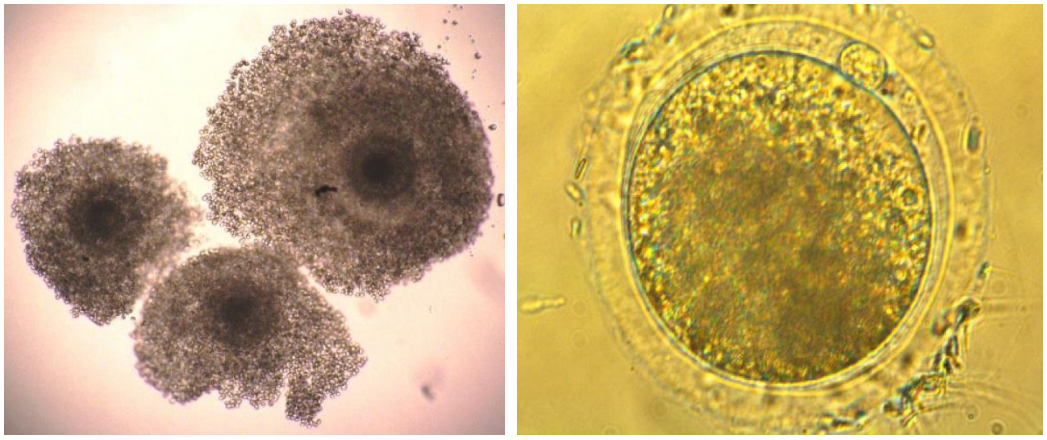

Figure 1. SB oocytes after 22 hr of culture in vitro (with moderate cumulus cell expansion), and oocyte prior to fertilization with distinct 1st polar body (Metaphase 2 stage).

In Expt 2, A total of 154 COCs were subjected to IVF after 24 hr of culture in mPM + 20 % BCS. The cleavage rate obtained 30-45 hpi was 50/154 (32.4 %), of which the rate of chromosomally normal embryos (diploid 2n = 48) was 52.0 % (26/50) and those with chromosomal abnormalities was 48.0 % (24/50). Similarly, chromosomal analysis of uncleaved oocytes (n=104) showed a 30.7 %, 32.7 % and 36.5 % haploid, polyploid and COCs at GV/M-1 stage or those with DSF/CCh, respectively (Table 2).

Table 2. Chromosome complement of oocytes and cleaved embryos after fertilization.

Status of oocytes	No. of samples examined	Ploidy (%)			Others (%)
		Haploid	Diploid	Polyploid	G/M-1 or DSF/CCh
Cleaved	50	0	26 (52.0)a	24 (8)	0
Uncleaved	104	32 (30.7)b	0	34 (2.7)	38 (36.5)

Values with different superscript in the same column differ (P<0.05).

In Expt 3, a total of 178 cleaved embryos were cultured in mPM + 20 % with cumulus cell monolayer for 7 days while observing their development to the blastocyst stage (Fig. 2). Of these, 114 (64.0 %) and 64 (36.0 %) were from early- and late- cleaving groups of embryos, respectively. The developmental rate to the morula (40.3 % vs 3.1 %) up to the blastocyst stage (14.9 % vs 3.1 %) of early- cleaving embryos was higher than late- cleaving embryos, respectively (Table 3).

Table 3. Developmental competence of embryos in relation to the completion of 1st cleavage.

		Stage of Development			
Status of Embryos	Number of embryos	>4	8- to 32-	Morula	Blastucyst
Early-	114	7 (6.1)	44 (38.6)	46 (40.3)a	17 (14.9)a
Late-	64	46 (71.8)	14 (21.8)	2 (3.1)b	2 (3.1)b

Values with different superscript in the same column differ (P<0.05)

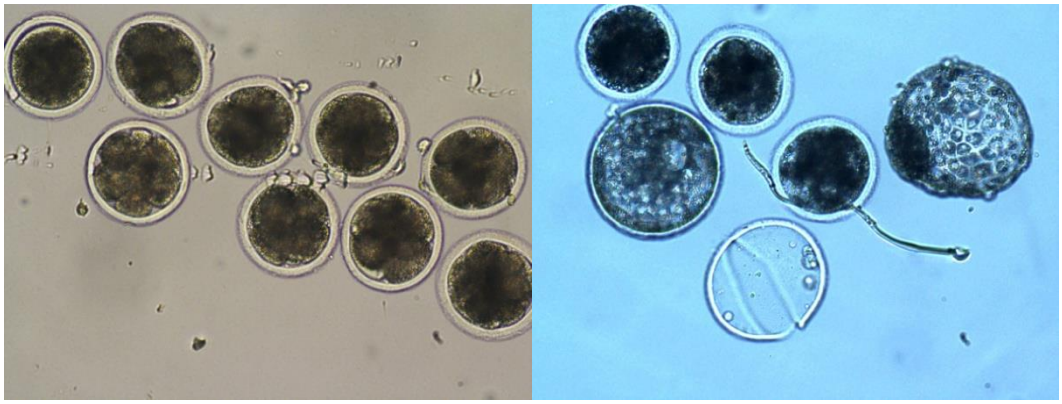

Figure 2. Cleaved SB embryos (2- to 8- cell stage), expanded blastocyst and hatched blastocyst stage embryos

Discussion

The nuclear maturation rate of SB oocytes were observed higher in culture conditions without PMSG addition, but improved normal chromosomal rate were noticed in culture conditions with PMSG addition. These findings conformed with some studies in bubaline (Totey *et al*., 1992; Jainudeen *et al.*, 1993; Purohit and Sharma, 2002a,b) though in contrast with some obtaining an improved maturation, fertilization and embryonic development (Suzuki *et al.*, 1992; Chauhan *et al.*, 1996; Gupta *et al*., 2001) following gonadotropin supplementation. Also, the addition of energy substrates in the form of calcium lactate and sodium pyruvate showed positive influence on the chromosomal normality of SB oocytes matured in vitro. In some related studies, their addition sustained oocyte viability and increased oocyte maturation in vitro (Roberts *et al.*, 2004). Others suggest that pyruvate production by cumulus cells may be important in supporting the nutrition of unfertilized and fertilized ova (Leese and Barton, 2005) or having a possible byfunctional role as an antioxidant protecting the oocytes against the stress of the in vitro environment (Gonzales-Figueroa and Gonzales-Malfino, 2005). Nonetheless, these variabilities could

also be attributed to a number of factors influencing oocyte viability in vitro including the type of serum, batch of media and hormones, variability on the selection of oocytes for maturation, expertise of the person performing the evaluation, preparation and analysis of samples and the component of the media used.

The high rate of cleaved embryos with diploid number of chromosomes following in vitro fertilization resulted from monospermic fertilization whereas those with polyploidy where the results of polyspermic fertilization suggesting that the culture conditions utilized both for maturation and fertilization could be used satisfactorily for the in vitro production of bubaline embryos for basic research. The uncleaved oocytes were the results of either failure on fertilization, polyspermic fertilization or abnormalities on maturation.

The time chosen for the selection of cleaved embryos for developmental studies was based on previous observations that 1st cleavage of buffalo zygotes in vitro occurs as early as 20 hpi but was most prevalent from 22 – 26 hpi and could still be observed at later hours between 35 – 45 hpi (Ocampo *et al*., 1996b; 2001). Similarly, 2- cell stage buffalo embryos were recovered in the oviduct 48 hr after insemination or mating in vivo (Shariffudin *et al*., 1987). The wide interval on the appearance of 1st cleavage corroborates other reports (Plante and King, 1992; Miller *et al.*, 1992) and allowed informative comparisons to be drawn between the embryos arising from early- and late-cleaving embryos. First, the early- cleaving embryos were more likely to proceed development to at least 8- to 32- cell stages within 5 days of culture that late- cleaving embryos. Second, the average number of cells produced by early- cleaving embryos as culture in vitro progresses was higher than late-cleaving embryos until their development to the blastocyst stage on the 7th day of culture. These observations clearly support the idea that early- cleaving embryos are more likely to have a normal chromosome complement resulting from normal fertilization that the late- cleaving embryos. In bovine, the incidence of chromosome abnormalities in developed embryos was found higher in the late- than early- cleaving embryos (Yadav *et al.*, 1993). The lower number of 4- celled embryos observed in the early- cleavage stage could be attributed to the existence of a chromosomal component that was readily reactive to cause penetration of available embryos and bring about earlier union of pronuclei that contribute to the formation of 4- celled embryos. These mechanism can apparently sustain relatively normal development of embryos to the 8- to 32- cell stages, the morula and finally to the blastocyst stage. This mechanism however, may not have been possible in the late- cleaving embryos due to absence or earlier deterioration of the chromosome component that should mediate penetration of the embryos and union of pronuclei associated

with the normal formation of 4- cell embryos to the blastocyst stage. These differences could also be attributed to factors such asynchronous oocyte population at a given time, the variation or penetrating ability of individual spermatozoon or defects on pronuclear formation caused by inadequacies of in vitro culture system used. Thus, the types of differences observed between early- and late- cleaving embryos (eg., rate of development, cell number) is a clear manifestations of the above factors contributory to occurrence of abnormalities in SB oocytes cultured and eventually matured in vitro.

In conclusion, oocytes cleaving early- after IVF are more likely to reach the blastocyst stage than their late- cleaving counterparts. It is an indicative of increased developmental potential and could be used as an additional criterion in the selection of embryos for transfer. This phenomenon has been observed in some species (Bavister *et al.*, 1983; Sakkas *et al*., 1998; Warner *et al.*, 1998). Apparently, the factors that control the time of 1st cleavage remained unclear although culture conditions can influence the kinetic of early development (Langendonckt *et al.*, 1997; Pinyopummintr and Bavister, 1994). Overall, the results of this study provides some insight into several important aspects of embryonic development in SB in vitro, especially on the low blastocyst formation rate and the eventual low offspring production after transfer.

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