Development of *In Vitro* Produced Buffalo (*Bubalus bubalis*) Embryos in Relation to Time

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ABSTRACT : The objective of the present study was to examine the developmental rates, and the stage of development in relation to time since fertilization, of *in vitro* produced buffalo embryos. Buffalo cumulus-oocyte complexes obtained from slaughterhouse ovaries were matured and fertilized *in vitro*. The fertilized oocytes (n=248) were then co-cultured with buffalo oviductal epithelial cells and evaluated for the developmental stages on Days 2, 4, 6, 7, 8, 9 and 10 post-insemination. The peak of 4-cell stage embryos was observed on Day 2 (63.7 %), whereas Day 4 was marked by peaks of 6-8-cell stage embryos (20.9%) and 16-cell stage embryos to early morulae (50%). On Days 6, 7, 8, 9, and 10 post-insemination, 49.5, 48.3, 38.3, 33.8 and 33.4% embryos were found to be at morula/compact morula stages, 8.8, 12.5, 25.4, 6.0 and 1.2% at early blastocyst/blastocyst stages, 0, 6.8, 7.2, 15.3 and 2.0% at expanded blastocyst stage and 0, 1.6, 4.8, 19.3 and 38.5% hatching/hatched blastocyst stages, respectively. The peaks of early blastocyst/blastocyst, expanded blastocyst and hatching/hatched blastocyst stages were observed on Days 8, 9 and 10, respectively. The percentages of oocytes which initially became arrested and subsequently degenerated were 3.6, 4.8, 10.4, 14.5, 21.3 and 24.5% on Days 4, 6, 7, 8, 9 and 10 post-insemination, respectively.
(Key Words : Buffalo, Embryo, IVM, IVF, Oocyte)

INTRODUCTION

In vitro maturation, fertilization and culture (IVMFC) have become valuable techniques for production of bovine embryos for use in embryo transfer, nucleus transfer studies and for production of transgenic animals. The IVMFC methodology is being routinely used for production of bovine embryos on an industrial scale (Gordon, 1991; Lu and Polge, 1992). The technique involves collection of oocytes from slaughterhouse ovaries, maturation of selected immature oocytes in media containing serum and/or gonadotropins (Fukui and Ono, 1989), fertilization by frozen-thawed *in vitro* capacitated spermatozoa (Parrish et al., 1986) and culture of cleaved embryos in media containing oviduct or cumulus cells upto morula and blastocyst stage (Goto et al., 1988; Eyestone and First, 1989). Bovine IVMFC system has been found to be an excellent tool for studying the early embryonic development in terms of the cleavage kinetics (Van Soom et al., 1992; Grisart et al., 1994) and timing of embryo development (Xu et al., 1992; Grisart et al., 1994). Although an IVMFC system similar to that in cattle has been developed for production of buffalo embryos upto blastocyst stage by us (Madan et al., 1994; Chauhan et al., 1997) and others (Totey et al., 1992), there is no information available on cleavage kinetics and early embryonic development in relation to time in buffalo. The present investigation was, therefore, undertaken to study the developmental rates, and the stage of development in relation to time since fertilization, of IVMFC-derived buffalo embryos.

MATERIALS AND METHODS

All chemicals and media were purchased from Sigma Chemical Co., St. Louis, MO, USA) unless otherwise indicated. FSH-P was from Schering-Plough (Kenilworth, NJ, USA). Mineral oil was from Squibb and Sons (Princeton, NJ, USA) and the 0.22 and 0.45 μm filters were from Millipore (Molsheim, France). Disposable Petri dishes were from Becton, Dickinson & Co. (Lincoln Park, NJ, USA).

Oocyte collection and *in vitro* maturation

Buffalo ovaries were collected in an abattoir and were transported to the laboratory in 0.9% normal saline at 32 to 37℃ within 4 h. Follicular oocytes (2 to 6 mm in diameter) were aspirated with 20-gauge needle attached to

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a 5-ml glass syringe. The *in vitro* maturation of buffalo oocytes was carried out by a method followed routinely in the laboratory (Madan et al., 1994). The aspiration medium consisted of TCM-199 supplemented with 10% fetal bovine serum (FBS) and phosphate buffer saline (PBS) supplemented with 0.3% bovine serum albumin (BSA) at a 1:1 ratio. Only compact cumulus-oocyte complexes (COCs) with an unexpanded cumulus mass having more than 5 layers of cumulus cells, and with homogenous cytoplasm were taken for the study. The culture medium consisted of TCM-199+10% FBS+5 μg/ml FSH-P. After washing with the culture medium, the COCs (10-15 oocytes) were placed in 50-μl droplets of the culture medium, covered with paraffin oil in a 35 mm Petri dish and cultured for 24 h in a CO_2 incubator (5% CO_2 in air) at 38.5℃.

Sperm preparation and *in vitro* fertilization

The spermatozoa used for IVF throughout the study were from the same batch and the same donor, and had been tested for IVF earlier. The spermatozoa were prepared for insemination as described earlier (Chauhan et al., 1997). Briefly, two straws of frozen-thawed ejaculated buffalo semen were washed with BO (Brackett and Oliphant, 1975) medium containing heparin (10 μg/ml) without BSA. The sperm were suspended for swim-up in BO medium containing 10 μg/ml heparin and 10 mM caffeine. Progressively motile spermatozoa were placed in 100 μl droplets of BO medium containing 5 mg/ml BSA, 10 μg/ml heparin and 5 mM caffeine in a Petri dish, covered with mineral oil and placed in CO_2 incubator for 1 h at 38.5℃ before insemination of *in vitro* matured oocytes. After 24 h of *in vitro* culture the oocytes were

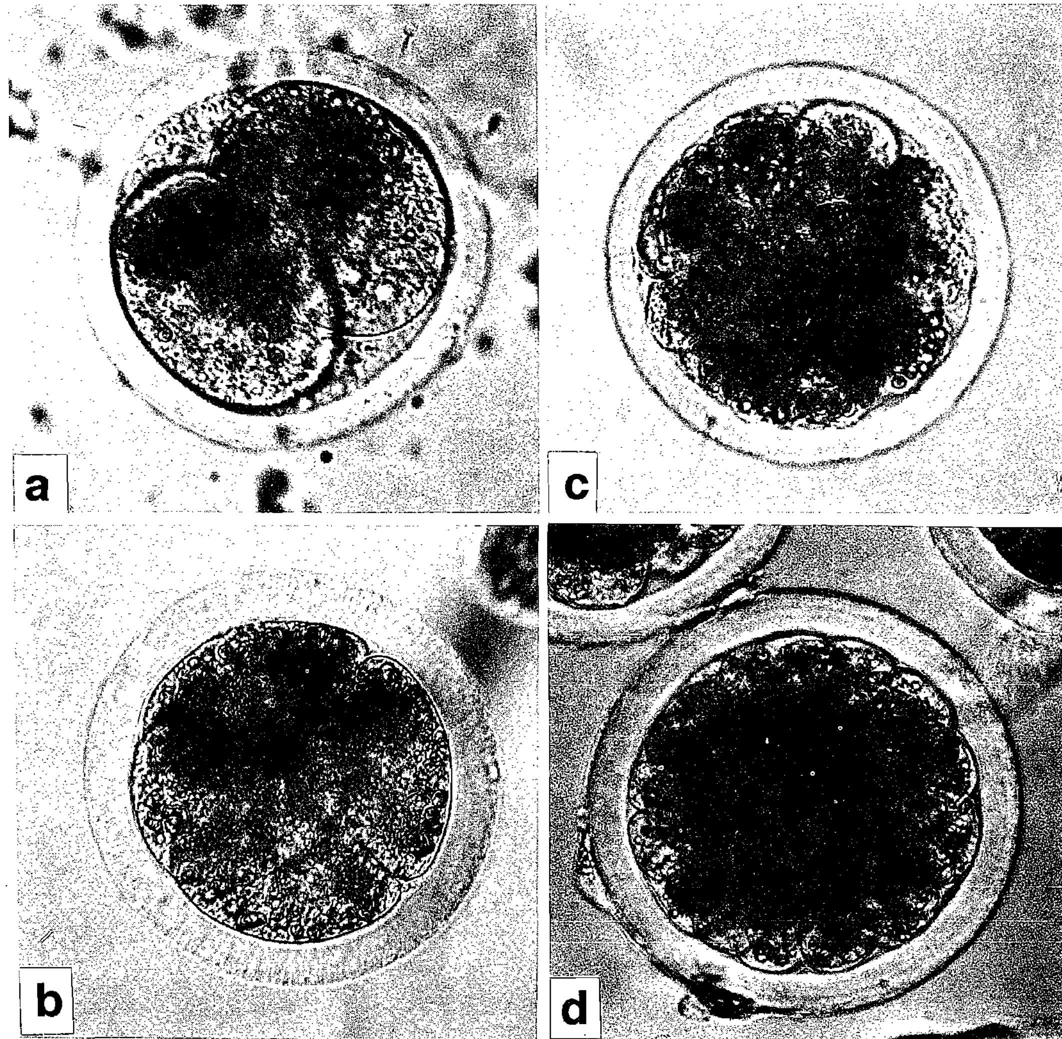

Figure 1. Buffalo embryos produced by *in vitro* maturation, fertilization and culture technique. a) two-cell stage (× 400), b) four-cell stage (× 400), c) sixteen-cell stage (× 400) and d) morula (× 400).

washed in BO medium and introduced into 100 μl droplets of processed buffalo spermatozoa (10 to 12 oocytes/droplet) and were left for 6 h in CO_2 incubator at 38.5℃. At the end of sperm-oocyte incubation, the oocytes were separated from sperm droplets and washed with TCM-199+10% FBS and then cultured for 2 days.

Preparation of buffalo oviductal cells and embryo culture

Buffalo oviducts were collected from freshly slaughtered animals having ovaries with freshly formed corpora lutea and brought to the laboratory in isotonic saline at 4℃. The oviducts were freed from ligaments and adjoining blood vessels, and 2 cm of the ampullary and isthmic regions were excised. Using a pair of forceps as a clamp, epithelial cells were expressed from the isthmus towards the ampulla into a Petri dish containing TCM-199 +10% FBS. The cells were disaggregated into small clusters by repeated flushings through 26-gauge needle, and the resultant cell suspension was allowed to settle at the bottom of Petri dish. The cells were picked up from there and washed twice with 5 ml of TCM-199 containing 10% FBS. The cell suspensions were transferred to 200-μl droplets of TCM-199+10% FBS and cultured in CO_2 incubator at 38.5℃. After 24 h, the cell clusters were washed three times in TCM-199+10% FBS and

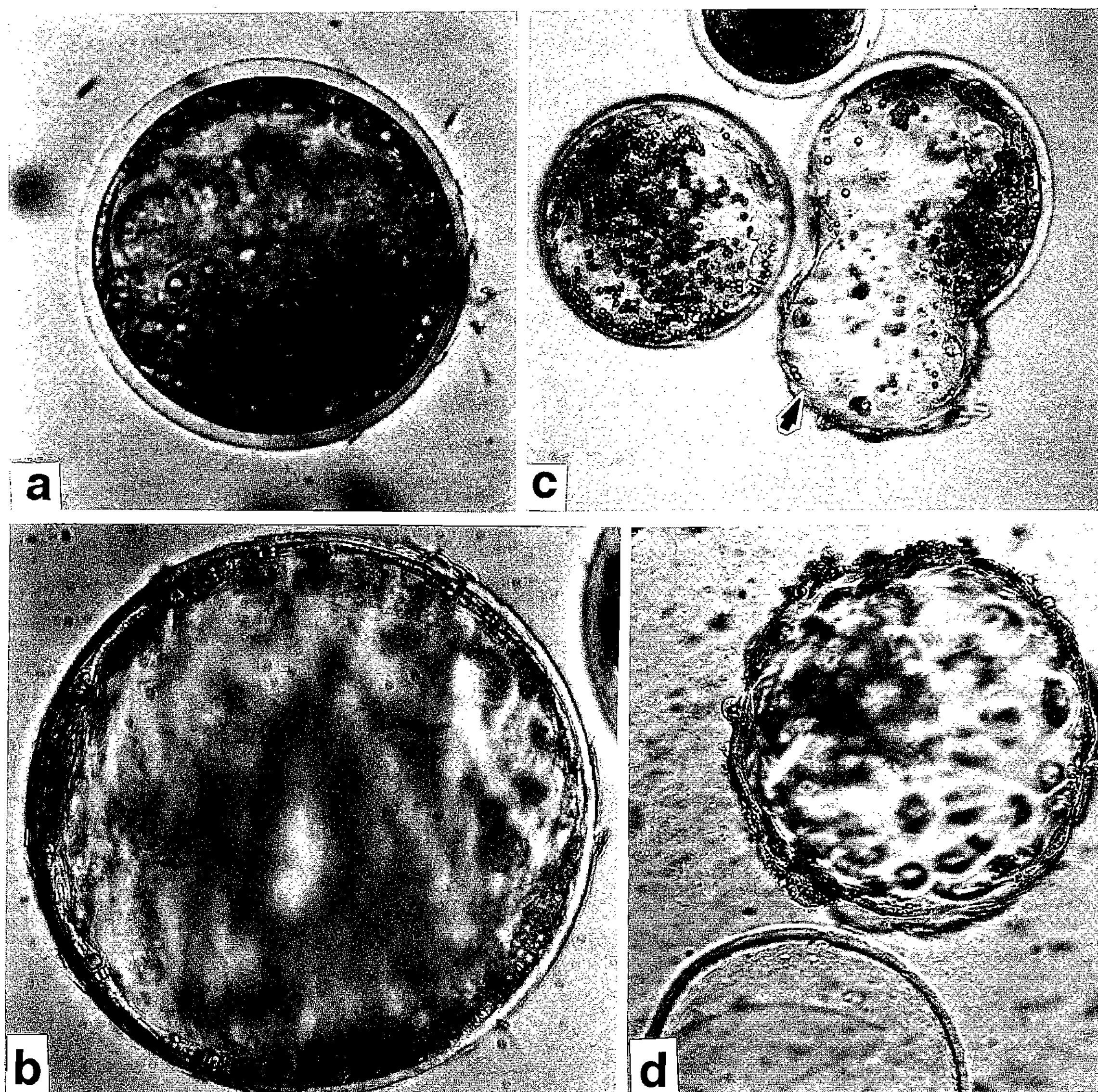

Figure 2. Buffalo embryos produced by *in vitro* maturation, fertilization and culture technique. a) blastocyst (× 400), b) expanded blastocyst (× 400), c) blastocyst under the process of hatching (× 200) and d) hatched blastocyst (× 200).

transferred to each of the culture droplets (approximately 50 cell clusters per 50-μl droplet) for co-culture with fertilized oocytes. The fertilized oocytes were then co-cultured for 10 days at 38.5℃ in CO_2 incubator in TCM-199+10% FBS and evaluated under the phase contrast microscope with DIC attachment on Days 2, 4, 6, 7, 8, 9 and 10 post insemination.

The following morphological criteria were employed in the evaluation of buffalo embryos developed *in vitro*.

2-16 cell stages: embryos with 2, 4, 8 or 16 blastomeres were termed as 2-cell (figure 1a), 4-cell (figure 1b), 8-cell and 16-cell stages (figure 1c), respectively.

Morula: an embryo with > 32 cells in which individual blastomeres were difficult to discern from one another and the cellular mass occupied most of the perivitelline space (figure 1d).

Compact morula: a morula in which compaction of blastomeres was clearly visible and individual blastomeres had coalesced.

Early blastocyst: an embryo in which formation of blastocoel had just started. Visual differentiation between trophoblast and the inner cell mass may be possible at this stage.

Blastocyst: an embryo with a well defined blastocoel and with a pronounced differentiation of the outer trophoblast layer and the darker, more compact inner cell mass. The embryo occupied most of the perivitelline space (figure 2a).

Expanded blastocyst: a blastocyst which had expanded and in which zona thinning was clearly discernable (figure 2b).

Hatching/hatched blatocyst: a blastocyst which had partly (figure 2c) or completely come out of the ruptured zona (figures 2d).

RESULTS AND DISCUSSION

At the time of the first examination on Day 2 post insemination, out of a total of 248 fertilized oocytes taken for the study, 63.7% of embryos had reached 4-cell stage, 14.1% had progressed to 6-8 cell stage whereas 22.1% were still at 2-cell stage (table 1). By Day 4 post insemination 50.0% embryos were found to be between 16 -cell and early morula stages, whereas 14.9% had become morulae/compact morulae. A substantial proportion (27.3 %) of embryos was, however, still at 4-8-cell stage. Formation of blastocoel had begun by Day 6 post

Table 1. Development of buffalo embryos produced by *in vitro* maturation, fertilization and culture technique, in relation to time

Stage of embryo (n=248)	Days post insemination						
	2	4	6	7	8	9	10
2-cell stage	55 (22.1)	11 (4.4)	4 (1.6)	–	–	–	–
4-cell stage	158 (63.7)	15 (6.0)	12 (4.8)	–	–	–	–
6-8-cell stage	35 (14.1)	52 (20.9)	21 (8.4)	5 (2.0)	–	–	–
16-cell to early morula	–	124 (50.0)	52 (20.9)	45 (18.1)	24 (9.6)	10 (4.0)	–
Morula/compact morula	–	37 (14.9)	123 (49.5)	120 (48.3)	95 (38.3)	84 (33.8)	83 (33.4)
Early blastocyst/blastocyst	–	–	22 (8.8)	31 (12.5)	63 (25.4)	15 (6.0)	3 (1.2)
Expanded blastocyst	–	–	–	17 (6.8)	18 (7.2)	38 (15.3)	5 (2.0)
Hatching/hatched blastocyst	–	–	–	4 (1.6)	12 (4.8)	48 (19.3)	95 (38.3)
Degenerated embryos	–	9 (3.6)	12 (4.8)	26 (10.4)	36 (14.5)	53 (21.3)	61 (24.5)

Data from 12 replicates were pooled.

insemination in 8.8% of embryos, whereas 70.4% embryos were still in 16-cell to early morula stages. By Day 7 post insemination the percentage of early blastocysts/blastocysts had increased to 12.5%. Some blastocysts had commenced expansion (6.8%) and hatching (1.6%). There were only 9.6% embryos between 16-cell and morula stages, and a majority of embryos had developed to morulae/compact morulae (38.3%) and early blastocysts/blastocysts (25.4%) by Day 8 post insemination. The interval between Day 8 and Day 9 post insemination was marked by a high rate of expansion and hatching resulting in a decrease in the proportion of early blastocysts/blastocysts to 6.0%, and an increase in the proportion of expanded and hatching/hatched blastocysts to 15.3 and 19.3%, respectively. By Day 10 post insemination 38.3% of embryos had either already hatched or were in the process of hatching, whereas a substantial proportion had become arrested at morula/compact morula stages (33.4%). Over the progress of culture period the percentage of oocytes which initially became arrested and subsequently began degenerating increased from 3.6% on Day 2 post insemination to 24.5% on Day 10 post insemination.

The rates of *in vitro* blastocyst production in the present study are similar to those reported by others on Day 7 post insemination (Totey et al., 1996) and by us on Day 9 post insemination (Chauhan et al., 1997). Our results are also comparable in terms of peaks of 4-cell, 16-cell to early morula, and morula/compact morula stages on Days 2, 4 and 6 post insemination, respectively, with those reported in cattle (Plante and King, 1992; Van Soom et al., 1992; Xu et al., 1992). The highest percentages of early blastocysts/blastocysts/expanded blastocysts were, however, observed on Day 8 post insemination in our study, compared to the peak of blastocysts/expanded blastocysts seen on Day 7 post insemination in cattle (Xu et al., 1992). Our results are different from those reported in this study, also in terms of a substantial proportion of buffalo embryos being arrested in morula/compact morula stages on Days 7, 8 and 9 post insemination (48.3, 38.3 and 37.8%, respectively), compared to 3.6, 3.0 and 0.8%, respectively, in cattle (Xu et al., 1992). This may partly be due to differences in the developmental rates between cattle and buffalo and partly because of differences in culture conditions.

The present study confirms the results of earlier reports in terms of wide variations in developmental rates and quality of cattle (Van Soom et al., 1992) and buffalo embryos (Totey et al., 1996). Time is an important parameter of embryo development. Earlier studies in cattle have shown that the embryos which undergo cleavage earlier have a greater chance of developing into morulae/blastocysts, and the faster the embryos cleaved, the more chance (upto 70%) they had of becoming morulae/blastocysts by Day 8 (Plante and King, 1992; Van Soom et al., 1992). More slowly dividing embryos developed at a very low rate and reached only the 9-16-cell stage or even earlier stages (Grisart et al., 1994). Similar results have been observed in IVMFC of buffalo oocytes by Totey et al. (1996) who found that buffalo embryos which completed first cleavage before 30 h post insemination were more likely to develop into blastocysts (25%) than those which completed first cleavage after 30 h post insemination (7.8%). The fast-cleaving and fast-developing blastocysts have been found to be superior to slow-cleaving and slow-developing blastocysts in terms of cell numbers in cattle (Goto et al., 1992) and buffalo (Totey et al., 1996). The pregnancy rates have also been reported to be lower for single Day 8 IVMFC-derived blastocysts than those for Day 7 blastocysts (Monson et al., 1992). These observations, in combination with results of the present study highlight the need to screen and select fast-cleaving emgryos for obtaining blastocysts of superior quality and higher viability. Although prolonging the culture period from Day 7 to Day 8 resulted in a two-fold increase in the blastocyst yield in the present study, there is a need to exercise caution as retardation of embryo development has been shown to be the principal cause of repeat breeding in cattle (Gustafsson, 1985). This becomes all the more important in case of IVMFC-derived embryos as the rate of development of these embryos is slower than that of those which develop *in vivo,* especially after the 8-cell stage (Barnes and Eyestone, 1990). Besides this, blastulation starts at a very early stage in IVMFC-derived embryos, resulting in these embryos having lower cell numbers and low viability compared to embryos produced *in vivo* (Iwasaki et al., 1990).

The developmental rate of IVMFC-derived buffalo embryos, as observed in the present study is slower than that of those obtained from superovulated buffaloes *in vivo* reported by us earlier (Singla et al., 1992). Similar results have been reported in cattle (Grisart et al., 1994). However, an accurate estimation of the differences between developmental rates *in vivo* and *in vitro* is very difficult due to 1) variations in the time of ovulation and fertilization *in vivo* and 2) possible inaccuracies in the

morphological evaluation of IVMFC-derived embryos in comparison to those developed *in vivo,* because of significantly lower number of cells in the former (Iwasaki et al., 1990).

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