***In vitro* maturation, fertilization and development of follicular oocytes from buffalo (*Bubalus bubalis*)**

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Summary. Cumulus–oocyte complexes, 5596, were cultured for 24 h in either TCM-199 or Ham's F-10 with or without gonadotrophins and supplemented with either 20% buffalo oestrous serum (BES) or fetal calf serum (FCS). The maturation rates of oocytes cultured in TCM-199 or Ham's F-10 medium supplemented with 20% BES were 47.4 ± 17.8 and 44.8 ± 25.6 , respectively. Addition of luteinizing hormone (LH) ($5 \mu\text{g ml}^{-1}$) significantly improved the maturation rate in the Ham's F-10 medium supplemented with 20% BES (76.8 ± 18.3), but follicle-stimulating hormone (FSH) ($0.5 \mu\text{g ml}^{-1}$) and oestradiol ($1 \mu\text{g ml}^{-1}$) failed to synergize with LH (71.7 ± 19.5). In the TCM-199 system, LH failed to enhance the maturation rate but the addition of FSH and oestradiol significantly enhanced the proportion of mature oocytes (42.7 ± 1.4 and 81.7 ± 14.5 , respectively; $P < 0.05$). Frozen–thawed spermatozoa prepared in Bracket and Oliphant (BO) medium and treated with $5 \text{ mmol caffeine l}^{-1} + 10 \mu\text{g heparin}$ showed a higher fertilization rate (29.8%) than those treated in Hepes–Talp and treated with $10 \mu\text{g heparin ml}^{-1}$ (19.6%). Fertilization rate was significantly improved when fresh ejaculated spermatozoa treated with $5 \text{ mmol caffeine l}^{-1}$ and $10 \mu\text{g heparin}$ in BO medium (50%) was used. Rate of cleavage and development were also higher when *in vitro* fertilization was carried out with fresh ejaculated spermatozoa treated with caffeine and heparin (34.1 and 36.8%, respectively) than with frozen–thawed spermatozoa (27.0 and 22.0%, respectively).

Development rate was enhanced when fertilized ova were cultured in ligated rabbit oviduct (28.0%) than when co-cultured on oviductal cell monolayers (8.2%). The results indicate that oocytes cultured in medium supplemented with BES and gonadotrophins reveal high rates of maturation and development to the blastocyst stage after fertilization with fresh ejaculated spermatozoa.

Keywords: oocytes; maturation; fertilization; culture; buffalo

Introduction

The lower reproductive capacity of domestic buffalo than of cattle is a major impediment to using buffalo for milk and meat production worldwide. This is particularly relevant in Asia, where 95% of the world population of domestic buffaloes is found. Buffalo is the mainstay of the Indian dairy industry; 55% of the milk production is contributed by 19 million buffaloes. Several international organizations have emphasized the potential of buffaloes in the agricultural economy of many developing nations in Asia, but domestic buffalo continues to be neglected despite its superiority over cattle under the harsh environments of the tropics.

Recent advances in embryo transfer technology have allowed progress towards increasing the number of offspring produced from genetically superior buffaloes. The application of bovine embryo transfer technology to buffalo has had only a limited success (Vlahov *et al.*, 1985; Kurup, 1988; Karaivanov *et al.*, 1990; Madan, 1990). However, there is slow, yet steady, progress in terms of superovulatory response and embryo recovery (Mishra *et al.*, 1991).

In vitro fertilization may prove useful for obtaining large numbers of embryos for transfer. The first ruminant offspring from *in vitro* fertilization was a bull calf born in 1981 (Bracket *et al.*, 1982). Since then, fertilization has been accomplished *in vitro* in many species. Little information is available on *in vitro* maturation and fertilization of buffalo oocytes (Majumdar *et al.*, 1988; Singh *et al.*, 1989; Totey *et al.*, 1991). The low reproductive capacity of buffalo, for example in terms of total number of follicles in the ovary, high percentage of atretic follicles, changes in acrosomal proteins and membrane damage during freezing, is a major problem. Surti buffaloes have 12 000 primordial follicles and Nilli Ravi have 19 000 primordial follicles in contrast to hundred thousands in cattle (Samad & Nasser, 1979). This study was conducted as part of a long-term investigation of *in vitro* fertilization mechanisms in buffalo. We investigated a suitable protocol for *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of buffalo oocytes and determined the developmental competence of these embryos *in vitro* and *in vivo*.

Materials and Methods

Collection of ovaries

Ovaries from sexually mature buffalo, which were probably well past their reproductive prime, were obtained at a local slaughterhouse within 2–3 h of slaughter and transported to the laboratory at 25–30°C in normal saline supplemented with 50 µg gentamycin ml⁻¹. Ovaries were washed four times in fresh phosphate-buffered saline supplemented with 50 µg gentamycin ml⁻¹. Precautions were taken to minimize bacterial contamination by conducting procedures in highly sterile conditions.

Oocyte recovery

Small antral follicles (2–6 mm diameter) were aspirated into 10 ml disposable syringes fitted with 18-gauge needles. The follicular fluid aspirates were pooled in a sterile 50 ml centrifuge tube and held for an additional 30 min. Cumulus–oocyte complexes, 5596, were isolated under a low-power magnification zoom stereo microscope (SMZ-2, Nikon, Japan) and washed five times in Tyrode's lactate medium buffered with 25 mmol Hepes l⁻¹ and supplemented with 3 mg BSA, fraction V ml⁻¹ and 0.25 mmol sodium pyruvate l⁻¹. Only oocytes enclosed with compact cumulus cells were used for maturation. Aseptic precautions were taken during these procedures. Number of usable oocytes varied from 20 to 60 per 100 ovaries. Owing to a low recovery rate, each treatment was studied individually. Experiments were performed between November and January (1987–1990).

Oocyte maturation

Cumulus–oocyte complexes were assigned to one of the following eight treatment groups and cultured for 24 h (ten oocytes in 50 µl droplets) under sterile paraffin oil at 39°C and a moist atmosphere of 5% CO₂ in air.

- (1) Ham's F-10 medium plus 10% fetal calf serum (FCS), 0.5 µg oFSH ml⁻¹ (NIDDK, NHPP) and 5 µg oLH ml⁻¹ (NIDDK, NHPP) + 1 µg oestradiol ml⁻¹ (Sigma Chemical Co.);
- (2) Ham's F-10 medium plus 20% BES (prepared from blood taken during oestrus and heat-inactivated at 56°C for 30 min);
- (3) Ham's F-10 medium plus 20% BES, 5 µg oLH ml⁻¹ and 1 µg oestradiol ml⁻¹;
- (4) Ham's F-10 medium plus 20% BES, 0.5 µg oFSH ml⁻¹, 5 µg oLH ml⁻¹ and 1 µg oestradiol ml⁻¹;
- (5) TCM-199 plus 10% FCS, 0.5 µg oFSH ml⁻¹, 5 µg oLH ml⁻¹ and 1 µg oestradiol ml⁻¹;
- (6) TCM-199 plus 20% BES;
- (7) TCM-199 plus 20% BES, 5 µg oLH ml⁻¹ and 1 µg oestradiol ml⁻¹;
- (8) TCM-199 plus 20% BES, 0.5 µg oFSH ml⁻¹, 5 µg oLH ml⁻¹ and 1 µg oestradiol ml⁻¹.

Maturation percentage was assessed after 24 h in culture by fixing 2697 oocytes in acetic acid:ethanol (1:3) and staining with 1% orcein in 45% acetic acid. Stage of meiosis was determined under a phase contrast microscope (Microphot: FXA, Nikon, Japan). The remaining oocytes were scored by cumulus mass expansion and secretion of first polar body and subjected to fertilization.

Sperm preparation and IVF

Fresh ejaculate or frozen–thawed spermatozoa were used for *in vitro* insemination by two methods. In the first method (Parrish *et al.*, 1986) fresh ejaculate or frozen–thawed semen (three to four 0.5-ml straws of buffalo frozen

semen thawed in water at 35–37°C for 1 min) was washed twice in Hepes–Talp medium supplemented with 6 mg BSA ml⁻¹ essentially free of fatty acids (Sigma Chemical Co.) and 1 mmol sodium pyruvate l⁻¹ (pH 7.4) by centrifugation at 833 *g* for 10 min each. A sperm pellet was then carefully layered under 1 ml Hepes–Talp for swim up; 1 h later the top 0.8 ml of medium was removed and centrifuged again for 10 min. The sperm pellet was resuspended to 200 µl of Hepes–Talp. Sperm motility and concentration were assessed with a haemocytometer. The medium for fertilization was a modified Tyrode's medium (pH 7.4). Droplets (48 µl) were prepared under sterile paraffin oil and equilibrated with 5% CO₂ in air for at least 2 h before use. A 2-µl aliquot of sperm suspension was added (to a final concentration of 1 × 10⁶ spermatozoa ml⁻¹) to droplets of fertilization medium containing 5–10 oocytes, 10 µg intestinal mucosal heparin ml⁻¹ (176 USP units ml⁻¹, Sigma Chemical Co.) or 10 µmol penicillamine l⁻¹ plus 2 µmol sodium metabisulfite l⁻¹, 1 µmol hypotaurine l⁻¹ and 20 µmol adrenaline l⁻¹ or heparin plus 10 µmol penicillamine l⁻¹, 2 µmol sodium metabisulfite l⁻¹, 1 µmol hypotaurine l⁻¹ and 20 µmol adrenaline l⁻¹.

In the second method (Niwa & Ohgoda, 1988) fresh ejaculated or frozen–thawed semen were washed twice in BO medium (Bracket & Oliphant, 1975) containing 10 mmol caffeine l⁻¹ without BSA. The sperm pellet was resuspended in BO medium as used for washing to give a 2 × 10⁶ spermatozoa ml⁻¹. Matured oocytes were washed twice with BO medium and placed into 50-µl droplets of BO medium supplemented with 20 mg BSA (essentially free of fatty acids), 20 µg heparin and covered with sterile paraffin oil and equilibrated with 5% CO₂ in air for at least 2 h before use. The sperm suspension (50 µl aliquot) was introduced into 50 µl of the medium which gave a final concentration of 1 × 10⁶ spermatozoa ml⁻¹, 10 mg BSA ml⁻¹, 10 µg heparin ml⁻¹ and 5 mmol caffeine l⁻¹.

Spermatozoa and oocytes were incubated for 22–24 h in a CO₂ incubator at 39°C and oocytes were then washed, mounted, fixed and stained with orcein for examination of sperm penetration and two pronuclei.

Embryo culture

After 22 h co-incubation of sperms and eggs, ova were removed from fertilization droplets, stripped of cumulus cells by repeated pipetting through a small-bore pipette and assigned randomly either to culture in ligated rabbit oviduct or to co-culture with 7 day-old oviductal cell monolayer in TCM-199 supplemented with 10% FCS and 50 µl gentamycin ml⁻¹. The embryos were examined under the microscope every 24 h.

Data analysis

Oocyte maturation was indicated in the fresh state by cumulus expansion, the presence of a polar body and metaphase II plate. The maturation rate was recorded as the total number of oocytes that matured over the total number of oocytes cultured. Fertilization rate was assessed by observing male and female pronuclei and cleavage rate by two-cell and four-cell stage. Development of cleaved eggs to blastocysts and morula was observed on day 5–6.

Statistical analyses were made by χ^2 test.

Results

Overall yield of the oocytes recovered by aspiration of buffalo ovaries was quite low compared with that in cattle. The maximum number of usable oocytes recovered per ovary using follicles of 2–6 mm diameter was 0.4. Average number of total oocytes per ovary was 0.7. 40.8% of the oocytes (0.3 per ovary) were atretic (Table 1).

Table 1. Oocytes recovered by aspiration for buffalo *in vitro* fertilization

	Total	Per ovary
Number of ovaries	12 263	—
Oocytes	8995	0.73
Nonatretic (usable)	5323 (59.0%)	0.43
Atretic	3672 (41.0%)	0.31

The medium supplemented with 20% BES and gonadotrophins resulted in marked expansion of cumulus cells characteristic of matured ova (Figs 1 and 2). Maturation rate was significantly higher ($P < 0.05$) in both media when 20% BES was added with LH, FSH and oestradiol than in media supplemented with 20% BES alone (Table 2). Addition of 5 µg ml⁻¹ LH in Ham's

Fig. 1. Immature buffalo oocytes ($\times 85$).

Fig. 2. Buffalo oocyte after maturation: expansion of cumulus cells is visible ($\times 85$).

Table 2. Influence of serum and gonadotrophins on *in vitro* maturation of buffalo oocytes

Medium	Serum	Hormones	Total oocytes	Maturation % mean $\pm$ SD
Hams F-10	FCS	oFSH + oLH + oestradiol	967	69.2 $\pm$ 18.4**
	BES	—	401	44.8 $\pm$ 25.6*
	BES	oLH	954	76.8 $\pm$ 18.3**
	BES	oFSH + oLH + oestradiol	1736	71.7 $\pm$ 19.5**
TC-199	FCS	oFSH + oLH + oestradiol	1043	63.6 $\pm$ 16.19
	BES	—	188	47.4 $\pm$ 17.8*
	BES	oLH	140	42.7 $\pm$ 1.4
	BES	oLH + oFSH + oestradiol	167	81.7 $\pm$ 14.5**

* < ** P < 0.05.

FCS: fetal calf serum; BES: buffalo oestrous serum; oFSH: ovine follicle stimulating hormone; oLH: ovine luteinizing hormone.

Table 3. Fertilization and cleavage rate using frozen–thawed and fresh ejaculated semen for buffalo *in vitro* fertilization

Medium	Total oocytes	Number fertilized (%)	Number cleaved (%)	% Morula: blastocyst
Hepes-Talp				
Frozen semen				
Heparin	682	134 (19.6)	12 (8.9)	—
Penicillamine + hypotaurine + adrenaline	225	25 (11.1)	—	—
Heparin + penicillamine + hypotaurine + adrenaline	67	6 (8.9)	—	—
Fresh ejaculated semen				
Heparin + penicillamine + hypotaurine + adrenaline	58	5 (8.6)	—	—
Penicillamine + hypotaurine + adrenaline	88	13 (14.7)	1 (7.6)	—
BO medium				
Frozen semen				
Caffeine + heparin	885	264 (29.8)	73 (27.6)	22.0
Penicillamine + hypotaurine + adrenaline	45	8 (17.7)	1 (12.5)	—
Fresh ejaculated semen				
Caffeine + heparin	692	346 (50.0)	118 (34.1)	36.8
Penicillamine + hypotaurine + adrenaline	157	49 (31.2)	13 (26.5)	30.0

F-10 medium supplemented with 20% BES enhanced the maturation rate but addition of FSH or oestradiol failed to synergize with LH in Ham's F-10 medium. However, addition of 5 μ g LH ml⁻¹ in TCM-199 failed to enhance the rate of maturation, but FSH and oestradiol did so significantly (P < 0.05).

A low fertilization rate (19.6%) was achieved with frozen semen capacitated with 10 μ g heparin ml⁻¹ in Hepes–Talp medium. This rate was not significantly increased by stimulating sperm motility with penicillamine + hypotaurine + adrenaline either alone or in combination with heparin (11.1 and 8.95%, respectively). In addition fertilization rate did not improve when fresh ejaculated semen was used in Hepes–Talp; it was significantly enhanced when frozen semen was capacitated with 5 mmol caffeine l⁻¹ plus 10 μ g heparin ml⁻¹ in BO medium (29.8%; P < 0.05), and further increased when fresh ejaculated spermatozoa were capacitated in the same manner (50.0%; P < 0.05) (Table 3 and Fig. 3).

Fig. 3. Fertilized buffalo ovum, showing male and female pronucleus ($\times 250$).

Table 4. Fertilization rate using freshly ejaculated, frozen and diluted semen stored at 4°C for 24 h of buffalo bull no. PR 54

Semen	Number of oocytes inseminated	Oocytes fertilized	Fertilization %
Fresh	35	27	77.1*
Diluted and stored at 4°C for 24 h	20	7	35.0***
Frozen	30	7	23.3**b

* < ** $P < 0.05$; ^a < ^b $P < 0.05$.

The rate of cleavage was low (8.9%) after insemination with frozen–thawed semen treated with 10 µg heparin ml⁻¹ in Hepes–Talp. However, cleavage rate was significantly ($P < 0.05$) increased to 27.6% when frozen–thawed spermatozoa were capacitated with caffeine plus heparin in BO medium and further increased to 34.1% with fresh ejaculated semen treated with caffeine plus heparin in BO medium. Similarly, development of embryos to morula and blastocyst was also increased from 0% to 22.0% and 36.8% when frozen and fresh ejaculated semen was capacitated in caffeine and heparin, respectively (Table 3). Fertilization rate was 17.7% when frozen–thawed spermatozoa treated with penicillamine and hypotaurine and adrenaline were used. Fertilization rate was increased significantly ($P < 0.05$) to 31.2% when fresh ejaculated semen was used. Cleavage rate was also significantly increased from 12.5% (frozen–thawed spermatozoa) to 26.5% (fresh ejaculated spermatozoa).

Fresh ejaculated semen, diluted semen stored at 4°C and frozen semen of one particular bull (PR 54) from the same collection for buffalo IVF were used to confirm the low fertilization rate with frozen semen. A high fertilization rate (77.1%) was observed when fresh ejaculated semen was used, whereas fertilization rate was reduced significantly to 35.0% and 23.3% ($P < 0.05$) when

diluted and frozen–thawed semen was used, respectively. These results confirmed that preservation and freezing of buffalo semen reduces the fertilization capacity of spermatozoa significantly (Table 4).

Buffalo oviductal cells in monolayer culture did not improve the proportion of embryos cleaving past the four-cell stage. Since most of the embryos did not go beyond the four-cell stage, it is yet to be confirmed whether a classical block at the four-cell stage occurs in *in vitro* fertilized buffalo embryos. However, there was a significant improvement when embryos were co-cultured in ligated rabbit oviduct (11% versus 38.6%). Twenty-eight percent of embryos developed into morulae and blastocyst compared with 8.2% in the oviductal monolayer system (Table 5). The peak of embryo development into blastocysts occurred 7 days after insemination when embryos were co-cultured with oviductal monolayer system and 6 days after culture in ligated rabbit oviduct respectively (Fig. 4).

Table 5. Comparison of early development in buffalo ova after incubation in ligated rabbit oviduct and buffalo oviduct cell monolayer

	Total oocytes	Cleavage (%)	Morula: blastocyst (%)
Rabbit oviduct	313	121 (38.6)	34 (28.0)
Oviduct cell monolayer	384	45 (11.0)	4 (8.2)

Fig. 4. Buffalo ovum cultured in ligated rabbit oviduct for 5 days after *in vitro* fertilization: expanded blastocysts and morulae are visible ($\times 85$).

Discussion

The present study was undertaken to develop an efficient method for IVF in buffaloes. Overall yield of total and usable oocytes per ovary was low in buffalo compared with cattle, in which the number of oocytes per ovary is up to 10 (Gordon & Lu, 1990). The low recovery of oocytes per ovary in buffalo can be attributed to the low average number of primordial follicles in the ovaries (12 636

in cycling buffalo and 10 132 in non-cycling buffalo) (Danell, 1987). Samad and Nasser (1979) reported that the Surti breed of buffalo has 12 000 primordial follicles and the Nilli Ravi breed has 19 000 primordial follicles in contrast to one hundred thousand in cattle ovary.

The average frequency of atresia for all the buffaloes was reported to be as high as 70.6%. However, more information is required on the incidence and extent of follicular atresia in relation to follicular size and the reproductive status of the buffalo that are being used as the source of ovaries.

Eight protocols for oocyte maturation were investigated. When selected oocytes were cultured in the presence of 20% BES and gonadotrophins, a significant increase in maturation rate was observed compared with that obtained with 20% BES alone. The effect of adding FSH, LH, oestradiol and progesterone during maturation of bovine oocytes have been studied by many workers (Hensleigh & Hunter, 1985; Stubbing *et al.*, 1988). Our study on buffalo IVM demonstrates that addition of hormones resulted in a significant increase in maturation rate. Present data agree with the report of Fukushima and Fukui (1985) and Younis *et al.* (1989), who found that the maturation rate of bovine oocytes was significantly increased by the addition of LH or a combination of LH, FSH and oestradiol compared with controls with no added hormone. However, it was observed that 5 μg LH ml^{-1} in Ham's F-10 medium supplemented with 20% BES significantly enhanced the rate of maturation compared with medium lacking LH, and the addition of FSH and oestradiol failed to synergize with LH. In contrast, addition of LH in TCM-199 supplemented with 20% BES failed to enhance the rate of maturation but addition of FSH and oestradiol significantly enhanced the rate of maturation.

Although various procedures for capacitating bull spermatozoa have been used for IVF, the most effective to date is the method of Parrish *et al.* (1984), who demonstrated that heparin could increase the frequency of IVF. However, this was not confirmed in buffaloes. Fertilization rate was very low (19.6%) when frozen-thawed spermatozoa treated with 10 μg heparin ml^{-1} in HEPES-Talp medium were used. Similar results were observed in IVF in buffalo by Singh *et al.* (1989), although maturation rate was more than 75%. There was no improvement in fertilization rate when frozen-thawed spermatozoa treated with penicillamine, hypotaurine and adrenaline or in combination with heparin in HEPES-Talp medium were used.

Fertilization rate was improved when preparation of frozen-thawed spermatozoa was carried out in BO medium and treated with 5 mol caffeine l^{-1} and 10 μg heparin. Fertilization rate was further improved when fresh ejaculated semen was treated with caffeine and heparin in BO medium (50%). It has been observed that the IVF rate of bovine oocytes increased significantly when caffeine was added to heparin in the fertilization medium, suggesting a synergistic effect between these chemicals in stimulating IVF of bovine oocytes (Ohgoda *et al.*, 1987; Niwa & Ohgoda, 1988; Park *et al.*, 1989).

However overall fertilization rate with frozen-thawed semen in buffalo IVF is quite low compared with that reported for bovine IVF. It may be due to leakage of various enzymes like acrosin and hyaluronidase on preservation. Enzyme release has generally been recognized as cellular injury, whereby membranes become inactivated or destroyed resulting in loss of cellular material (Ganguli, 1979). There is evidence from electron microscopic studies that the plasma membrane of buffalo spermatozoa is quite fragile particularly in the acrosome region (Anand, 1979). Buffalo spermatozoa have low survival rate and thus lower fertilizing ability when semen is either refrigerated or deep frozen (Rawat, 1979).

Preservation of buffalo semen is a long-standing problem. Various media have been used for storage and preservation of buffalo semen (Matharoo & Singh, 1980; Nath *et al.*, 1991). The motility diminished in all the media used with increase in number of hours of preservation (Vyawahare *et al.*, 1989; Sidhu & Guraya, 1979). Meur and Dhoble (1988) demonstrated cryogenic changes in acrosomal proteins of buffalo spermatozoa. They found that total sulphydryl group decreased owing to freezing and to irreversible damage to acrosome proteins during cryopreservation.

We could improve the fertilization rate when preparation of frozen-thawed spermatozoa was carried out in BO medium treated with caffeine and heparin. Increased stimulation could result from easy access of caffeine in the membrane-damaged spermatozoa. Motility of buffalo spermatozoa after freezing was reported to be increased by 25 to 100% on addition of 6 mmol caffeine l^{-1} (Gehlaut & Srivastava, 1987).

A high fertilization rate was observed when fresh ejaculated semen was used, whereas fertilization rate was reduced significantly when diluted semen, or frozen semen was used. These results confirm the observation that preservation and freezing of buffalo semen significantly reduced fertilization capacity.

Uterine, oviductal and follicular fluid contains glycosaminoglycans (Lee & Ax, 1984; Lee *et al.*, 1986; Ax & Bellin, 1988). Heparin was the glycosaminoglycan that was considered to be most potent and most consistent in stimulating the acrosome reaction and fertilization *in vitro* in cattle, but heparin was not effective in capacitating buffalo spermatozoa. However, Sidhu *et al.* (1990) demonstrated a 39.8% acrosome reaction of fresh ejaculated buffalo spermatozoa when treated with 2% deoxygenated uterine fluid. It is necessary to confirm that this results from the presence of glycosaminoglycans in the uterine fluid or the presence of some other proteins. Detailed studies will have to be carried out on the dose-dependent effect of heparin on frozen-thawed buffalo spermatozoa, isolation of glycosaminoglycans in the uterine fluid or oviductal fluid and effect of individual bull variation on buffalo IVF.

In our experiment fresh ejaculated semen was more effective when treated with caffeine and heparin in BO medium than frozen-thawed semen. A higher cleavage rate was observed when spermatozoa were treated with caffeine and heparin in BO medium than in untreated spermatozoa.

We compared culture systems for development of early buffalo embryos. Embryos were co-cultured either on buffalo oviductal cell monolayer or ligated rabbit oviduct. Developmental rate was significantly lower on oviductal cell monolayer (8.2%) than when co-cultured in ligated rabbit oviduct (28%). Most of the embryos cultured on oviductal cell monolayer did not develop beyond the four-cell stage. Bovine embryos are particularly sensitive to culture condition at the eight- to 16-cell stage (Eyestone & First, 1989) and pigs, at the four-cell stage (Mattoli *et al.*, 1989), since embryos block at this point in a variety of media. However, recent co-culture of bovine embryos on monolayer of oviductal cells improved viability and development beyond the 8–16-cell stage (Eyestone & First, 1989) and was efficient and cheap. However, similar results were not observed in buffalo. Lu *et al.*, (1989) derived as many as 60% of embryos using sheep oviduct. However, for the large-scale production of buffalo embryos the use of rabbits or sheep as an intermediate host would be unsuitable. More work is needed for the development of efficient *in vitro* culture system for buffalo embryos.

There are many limitations in buffalo IVM, IVF, *in vitro* culture, in terms of lower yield of usable oocyte, high atresia, low fertilization rate, seasonal effect on follicular recruitment and quality of oocytes, seasonal effect of quality of semen, and the quality of buffalo that are slaughtered, which will have to be taken into consideration while performing IVF in buffalo.

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