

In vitro maturation and fertilization of buffalo oocytes: the effect of recovery and maturation methods

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Abstract: Conditions for in vitro maturation (IVM) of primary oocytes without conventionally used fetal calf serum and hormones were optimized in order to reduce the cost of laboratory produced buffalo embryos. Comparisons were made between oocyte recovery methods (aspiration vs. slicing) and IVM in medium 199 (static culture method vs. flux culture method) supplemented with $4-5 \times 10^6$ granulosa cells mL⁻¹ that contained either estrus buffalo serum (EBS) or fetal calf serum (FCS). Recovery methods were compared according to yield, i.e. cumulus oocyte complexes per ovary (COCs/ovary), the expansion rate (% of COCs that expanded), and nuclear maturation rate (% of germinal vesicle breakdown [GVBD]), following IVM for 22-24 h. In vitro maturation methods (static culture with EBS or FCS and Flux culture with EBS or FCS) were compared on the basis of the expansion rate and in vitro fertilization rate (cleavage rate). COC recovery with the slicing method (2.2 COCs/ovary) was better ($P < 0.05$) than with aspiration (0.9 COCs/ovary). However, the IVM rate was better ($P < 0.05$) based on expansion (86% vs. 63%) and GVBD (85% vs. 62%) with aspiration than with the slicing method. The cleavage rate (37%) was significantly better with the static culture containing EBS than with the static culture with FCS or the flux culture with either EBS or FCS. It was concluded that aspiration of oocytes and subsequent IVM with static culture containing EBS would be a potential method to reduce the cost of laboratory produced buffalo embryos.

Key words: Buffalo, oocyte, aspiration, slicing, serum

Introduction

Ever since the birth of the first buffalo calf following in vitro fertilization (1), in vitro production of buffalo embryos has been gaining attention for its research and commercial applications. Progress in the use of embryo transfer in the buffalo is slow due to poor superovulatory response. In vitro embryo

production (IVEP) would be an effective technique to improve the efficacy of transferable embryo production (2). However, the high cost of IVEP makes the use of in vitro production of buffalo embryos questionable.

Aspiration and slicing methods were mostly used for the recovery of buffalo oocytes from abattoir

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ovaries (3). The efficacy of this method was compared on the basis of cumulus oocyte complexes per ovary (COCs/ovary) (4). Buffalo oocyte IVM rates were low, but improved with the addition of buffalo serum (5) or hormones (6) in the culture medium. However, addition of cumulus cells alone did not improve the oocyte maturation rate (7). In cattle, a higher percentage of morula formation (73.5%) was achieved via the flux method with granulosa cell co-culture (8). This method needs to be tested for IVM of buffalo oocytes. Expensive components of in vitro maturation (IVM) medium, such as fetal calf serum and hormones, were successfully replaced by steer serum and follicular fluid (3). Estrus buffalo serum (EBS), a rich source of hormones and growth factors, could be used to improve the developmental competence of buffalo oocyte culture in vitro.

The present study was undertaken to improve the number of COCs/ovary worth culturing and replace such expensive components as fetal calf serum (FCS) and hormones used for IVM in order to reduce the cost of laboratory produced buffalo embryos.

Materials and methods

All chemicals used in the study were reagent grade (Sigma-Aldrich Chemie GmbH, Germany) or as otherwise indicated.

Experiment 1

Aspiration and slicing methods were compared for the recovery of oocytes from buffalo ovaries ($n = 257$) collected from a local abattoir (in triplicate) at a weekly interval over a period of 3 weeks. Pregnancy status and stage of the estrous cycle of the animals were not considered. Ovaries were transported to the laboratory in phosphate buffered saline at 37 °C within 2-3 h of slaughter. Upon arrival, the ovaries were washed twice in PBS, transferred to fresh PBS at 37 °C in a water bath, and further processed for COC recovery, either with aspiration ($n = 175$) or the slicing method ($n = 82$). The IVM method used was the same as reported earlier for buffalo (9). Medium 199 with 2% FCS (Flow Lab) was used as the wash medium (WM) and with 10% FCS as the culture medium (CM). Comparisons were made on the basis of (i) the number of good quality COCs/ovary, (ii) cumulus mass expansion, and (iii) nuclear maturation.

Aspiration

COCs were recovered from 2-6-mm follicles with a 5-mL syringe (attached to an 18 G needle) that contained 0.5 mL of WM. Follicular contents were pooled in 15-mL polystyrene conical tubes. After collection, pooled follicular fluid was allowed to sediment for 10-15 min. Supernatant was decanted and sediment was collected in a 60-mm petri dish (tissue culture tested). COCs were located within the fluid using a stereomicroscope (200×) and collected in a 35-mm petri dish with 2 mL of CM for grading.

Slicing

Excess tissue, corpora lutea, and large follicles (>10 mm) were removed. Ovaries were partially bisected and laid flat in a 120-mm petri dish containing 5 mL of WM. Oocytes were recovered by slicing the surface of the ovary with a single sterile surgical blade. After slicing, the ovary was thoroughly rinsed in the same petri dish. Then 2-4 ovaries per petri dish were sliced and examined under a stereomicroscope to search for COCs. The COCs were placed in CM for grading.

COC grading

COCs were classified into 3 categories, as per Chauhan et al. (5). Category 1 included oocytes surrounded by compact multi-layers of follicle cells (corona radiata and cumulus cells) with homogenous ooplasm. Category 2 oocytes were enclosed by multiple layers of compact granulosa cells, with only homogeneous ooplasm. COCs with expanded follicle cells or with granulated ooplasm, and denuded oocytes (category 3) were discarded. Good quality COCs (categories 1 and 2) were counted for COC recovery (COCs/ovary) and were further used for IVM.

Cumulus expansion and nuclear maturation

After 22-24 h of incubation, the maturation of oocytes was assessed based on the degree of cumulus expansion, as previously reported in cattle (10). Expansion of COCs was characterized by its sticky nature and enlargement of the cumulus mass to at least 2-3 diameters from the zona pellucida. Expansion of COCs was calculated as follows:

COCs expanded (%) = $\frac{\text{the number of COCs expanded}}{\text{total oocytes cultured}} \times 100$

A representative number of expanded COCs from each group (n = 60) were stained in order to examine nuclear maturation. Cumulus cells were removed from expanded COCs by gently shaking in a 5-mL vial containing 1-2 mL of 3% sodium citrate solution (pH 7.4). Denuded oocytes were washed twice with WM (2% FCS) and mounted on slides in a microdroplet (10-20 μ L) between 2 parallel lines of wax:vaseline (1:20). A cover slip was placed on the lines and pressed gently using a needle until it touched the microdroplet containing the oocytes. Gentle pressure was applied to anchor the oocytes between the slide and cover slip. Fixative (acetic acid:ethanol 1:3 v/v) was passed twice via introduction from one side of the cover slip and removal from the other by absorbing it with a piece of filter paper. Staining solution (1% aceto-orcein in acetic acid:ethanol 1:3) was then passed once, albeit in a similar manner. After 3-5 min, destaining solution (acetic acid:distilled water:glycerol: 1:3:1) was passed thoroughly to remove excess stain, making sure that the oocytes were not washed away. The cover slip was sealed with DPX mountant (BDH) and stained oocytes were examined under a light microscope (400 \times) to determine the stages of nuclear maturation, i.e. germinal vesicle (GV) and germinal vesicle breakdown. Oocytes with a distinct nuclear membrane and no detection of chromatin material were categorized as GV stage. Germinal vesicle breakdown (GVBD) was detected by either deeply stained chromatin at the mitotic plate (M I) or deeply stained chromatin with the presence of 1 or 2 polar bodies (M II). Nuclear maturation was calculated on the basis of the percent of GVBD as follows:

$$\text{GVBD (\%)} = \frac{\text{number of GVBD}}{\text{total oocytes stained}} \times 100$$

Experiment 2

IVM of oocytes was conducted using 4 different culture methods. Maturation of oocytes was assessed on the basis of the in vitro fertilization rate (cleavage rate).

IVM of COCs

Buffalo ovaries were collected from the slaughterhouse once a week over a period of 3 weeks. COCs were recovered from the ovaries via the aspiration method, as described above. The COCs

(good quality) were randomly assigned to receive 1 of the following 4 treatments for IVM:

- i. Static culture with EBS (static EBS).
- ii. Static culture with FCS (static FCS).
- iii. Flux culture with EBS (flux EBS).
- iv. Flux culture with FCS (flux FCS).

The CM was prepared as described in experiment 1, using either 10% FCS or 10% EBS. EBS was obtained from the blood collected from buffaloes in standing heat. Then it was heat inactivated at 56 °C for 30 min, filtered through a 0.22- μ m Millipore filter, and preserved at -20 °C until used. The static culture method was same as described for IVM of oocytes in experiment 1. With the flux method, the culture dishes were placed on a shaker (20-30 oscillations per minute) and incubated along with the static culture dishes for 22-24 h.

Sperm capacitation and in vitro fertilization

Semen from 3 buffalo bulls frozen in Tris, citric acid, fructose egg yolk, and glycerol extender (11) was thawed and pooled (three 0.5-mL straws per bull; each straw containing approximately 30 million live sperm at the time of freezing). Motile sperm were separated with swim-up in sperm-TALP (modified Tyrode's medium with lactate and pyruvate, pH 7.3-7.4), as described for buffalo (12). The final pellet of spermatozoa obtained after swim-up was measured and diluted with a heparin solution (200 μ g/mL in sperm-TALP) to obtain a final sperm concentration of 2-4 million/mL.

Following incubation for 22-24 h with 1 of the 4 culture methods, expanded COCs were washed 3 times in respective CM and then once in Fert-TALP (pH 7.7-7.8). Then, 20-25 COCs were placed in a 250- μ L droplet of Fert-TALP covered with mineral oil, and then 250 μ L of capacitated spermatozoa was added to each fertilization droplet to obtain a final concentration of 1-2 million/mL with heparin (100 μ g/mL). The fertilization droplets were incubated for 48 h at 39 °C in a CO₂ incubator. After incubation, presumptive zygotes were examined under a stereomicroscope to record the number of cleaved oocytes. The cleavage rate was calculated as follows:

$$\text{cleavage rate (CR)} = \frac{\text{number of cleaved eggs}}{\text{100/total oocytes inseminated}} \times 100$$

Statistical analysis

Results were analyzed using the chi-square test for significant differences between the aspiration and slicing methods on the basis of good quality COCs/ovary, the expansion rate (%), and the nuclear maturation rate (%). A 2 (serum) × 2 (culture method) experimental design was used to compare in the vitro culture method in experiment 2. The chi-square test was used to analyze data for significant differences between treatment effects on the incidence of oocyte expansion and the cleavage rate. Data were analyzed using Minitab v.12.22 (13).

Results

Oocyte recovery

Data on the recovery of COCs with aspiration and slicing are presented in Table 1. In total, 335 good quality COCs were recovered from 257 ovaries (1.3 COCs/ovary), with an overall expansion rate of

74.0%. The COC recovery rate was better with slicing than with the aspiration method ($P < 0.05$). However, the expansion rate was better in COCs harvested with aspiration than with the slicing method, after IVM ($P < 0.05$).

Table 2 shows the effect of the recovery method on subsequent IVM of buffalo oocytes. The oocytes recovered via the aspiration method had better nuclear maturation (% GVBD) than those recovered via the slicing method.

IVM

The effect of static and flux culture methods, with either EBS or FCS, on IVM (expansion) and in vitro fertilization (cleavage rate) of oocytes is shown in Table 3. IVM, as determined by cumulus mass expansion, was similar among the 4 treatment groups. The cleavage rate (37%) was significantly better in COCs matured with the static EBS method than those matured with static methods with FCS, and with flux methods with either EBS or FCS.

Table 1. Recovery of cumulus oocyte complexes (COCs) from buffalo ovaries with aspiration and slicing methods.

Method	No. of ovaries	COCs recovered (COCs/ovary)*	COCs expanded (%)
Aspiration	175	155 (0.9) ^a	134/155 (86.4) ^a
Slicing	82	180 (2.2) ^b	114/180 (63.3) ^b
Overall	257	335 (1.3)	248/335 (74.0)

* Good quality (multi-layered compact granulosa cells)
 Data are cumulative value of 3 replicates
 Values within a column with different superscript differ ($P < 0.05$)

Table 2. Effect of recovery method on nuclear maturation of in vitro mature buffalo oocyte.

Method	No. stained	No. at GV stage (%)	No. at GVBD stage (%)		
			M I	M II	Total
Aspiration	60	9 (15.0) ^a	12 (20.0)	39 (65.0) ^a	51 (85.0) ^a
Slicing	60	23 (38.3) ^b	13 (21.7)	24 (40.0) ^b	37 (61.7) ^b

Data are cumulative value of 3 replicates
 Values within a column with different superscript differ ($P < 0.05$)
 M I: metaphase I
 M II: metaphase II
 GV: germinal vesicle
 GVBD: germinal vesicle breakdown

Table 3. Effect of static and flux culture method with estrus buffalo serum (EBS) or fetal calf serum (FCS) on in vitro maturation (expansion rate) and in vitro fertilization (cleavage rate) of buffalo oocytes.

Treatment	Maturation rate	Cleavage rate
	No. expanded/No. set up (%)	No. cleaved/No. inseminated (%)
Static culture method		
EBS	54/56 (96.4)	20/54 (37.0) ^a
FCS	49/51 (96.1)	13/49 (26.5) ^b
Flux culture method		
EBS	47/56 (83.9)	15/47 (31.9) ^b
FCS	49/54 (90.7)	14/49 (28.6) ^b

Values within a column with different superscript differ ($P < 0.05$)

Discussion

The overall recovery of good quality COCs with the 2 methods in the present study was 1.3 COCs/ovary. Different studies have reported 0.4-3.85 good COCs/ovary in buffalo (3). This variation is due to the different methods used for COC recovery, seasonal effects, and variation in the reproductive status of the slaughtered buffaloes. However, as compared to temperate cattle (10 COCs/ovary) (14) the number of good COCs/ovary in buffalo is lower, which may be due to an inherently smaller number of primordial follicles and a higher frequency of atresia in buffalo (2). The present study shows that the COC recovery rate from slaughterhouse ovaries was better with slicing than with aspiration, which is in agreement with earlier findings reporting greater numbers of COCs/ovary with the slicing method than with aspiration (4,15). Lower COC recovery via the aspiration method might have been because oocytes were recovered from selected follicles (2-3 mm) on the ovarian surface, and were limited in number. Slicing the ovarian surface recovered COCs from follicles of every size, even from the follicles deep in the ovarian cortex (16). Khan et al. (4) reported that oocytes were recovered via aspiration from 55% of follicles, as compared to the slicing method, which recovered oocytes from 78% of follicles from buffalo ovaries.

Data obtained in the present study show that the IVM rate in buffalo oocytes recovered via the

aspiration method (expansion = 86.4% and GVBD = 85%) was better, as compared to the slicing method (expansion: 63.3%; GVBD: 61.7%). It is possible that slicing may release less developmentally competent oocytes from follicles deep in the cortex. It has been shown that oocytes released from embedded follicles in the ovarian cortex via slicing are not as meiotically competent as those from similarly sized follicles located on the bovine ovarian surface (16). In vitro fertilization has been carried out in buffalo (3) and in cattle (14) mostly with oocytes recovered via the aspiration method. Buffalo oocytes recovered via the aspiration method had an 85% oocyte maturation rate based on GVBD (5), and 81%-88% based on COC expansion (6). It is known that the ability of buffalo oocytes to undergo IVM is affected by biological and environmental factors (17). The present study shows that the oocyte recovery method is another factor that affects the ability of buffalo oocytes to undergo IVM.

The highest cleavage rate (37%) was achieved with the static culture method supplemented with EBS, as compared to static methods with FCS and flux culture methods with either EBS or FCS. Earlier, Mehmood et al. (9) reported a higher cleavage rate (75%) in buffalo with the same heparin concentration by adding penicillamine, hypotaurine, and epinephrine (PHE) in the fertilization droplet. PHE syrup acts as a sperm motility enhancer and increased the IVF rate in cattle (8). Inhibition of attachment and premature luteinizing of cumulus cells with a granulosa cell co-

culture method was achieved with the addition of hormones (18) or with the flux culture method (8) in cattle. In buffalo, the static culture method with the addition of hormones with sera has been used in most IVF studies (3). Chauhan et al. (5) reported a lower cleavage rate in buffalo oocytes with FCS than with EBS. Samad et al. (6) noted that EBS and pro-estrous buffalo serum resulted in better maturation and cleavage than with post-estrous buffalo serum. Madan et al. (19) reported that supplementation of EBS and co-culture with cumulus cells and oviductal epithelial cells improved the cleavage rate of buffalo oocytes, as compared to EBS and cumulus cells only. This might

have been due to the higher concentrations of LH, oestradiol, and TSH in EBS than in FCS, as reported for ECS (18).

It is concluded that buffalo oocytes recovered from ovaries excised via the aspiration method had a better IVM rate, as compared to the slicing method. A better cleavage rate was achieved with the static culture method supplemented with EBS, as compared to static methods with FCS and flux culture methods with either EBS or FCS. Therefore, aspiration of oocytes and subsequent IVM with the static culture method containing EBS cost-effectively improved the IVM rate of buffalo oocytes.

References

1. Madan, M.L., Singla, S.K., Jaikhavi, S., Ambrose, J.D.: In vitro fertilization in buffalo and birth of first ever IVF buffalo calf. In: Proceedings of Third World Buffalo Congress 1991; Varna, Bulgaria, Volume 7: 11-17.
2. Drost, M.: Advanced reproductive technology in the water buffalo. *Theriogenology*, 2007; 68: 450-453.
3. Nandi, S., Raghu, H.M., Ravindranatha, B.M., Chauhan, M.S.: Production of buffalo (*Bubalus bubalis*) embryos in vitro: premises and promises. *Reprod. Domest. Anim.*, 2002; 37: 65-74.
4. Khan, I.Q., Samad, H.A., Rehman, N.U.: Quantity and quality of buffalo follicular oocytes recovered by aspiration and scoring methods for in vitro studies. *Pak. Vet. J.*, 1997; 17: 187-189.
5. Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Madan, M.L.: In vitro maturation and fertilization, and subsequent development of buffalo (*Bubalus bubalis*) embryos: effects of oocyte quality and type of serum. *Reprod. Fertil. Dev.*, 1998; 10: 173-177.
6. Samad, H.A., Khan, I.Q., Rehman, N.U., Ahmad, N.: The recovery, in vitro maturation and fertilization of Nili-Ravi buffalo follicular oocytes. *Asian Aust. J. Anim. Sci.*, 1998; 11: 491-497.
7. Das, S.K., Chauhan, M.S., Palta, P., Tomer, O.S.: Influence of cumulus cells on in vitro maturation of denuded buffalo oocytes. *Vet. Rec.*, 1997; 141: 522-523.
8. Lu, K.H., Gordon, I., Gallagher, M., McGovern, H.: Pregnancy established in cattle by transfer of embryos derived from in vitro fertilization of oocytes matured in vitro. *Vet. Rec.*, 1987; 121: 259-260.
9. Mehmood, A., Anwar, M., Saqlan Naqvi, S.M.: Capacitation of frozen thawed buffalo bull (*Bubalus bubalis*) spermatozoa with higher heparin concentration. *Reprod. Domest. Anim.*, 2007; 42: 376-379.
10. de Loos, F., van Maurik, P., van Beneden, T., Kruip, T.A.M.: Structural aspects of bovine oocyte maturation in vitro. *Mol. Reprod. Dev.*, 1992; 31: 208-214.
11. Rasul, Z., Anzar, M., Jalali, S., Ahmad, N.: Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. *Anim. Reprod. Sci.*, 2000; 59: 31-41.
12. Mehmood, A., Anwar, M., Saqlan Naqvi, M.: Motility, acrosome integrity, membrane integrity and oocyte cleavage rate of sperm separated by swim-up or Percoll gradient method from frozen-thawed buffalo semen. *Anim. Reprod. Sci.*, 2009; 111: 141-148.
13. Minitab 12.22.: Minitab Inc., State College, Pennsylvania, USA, 1996.
14. Greve, T., Madison, V.: In vitro fertilization in cattle: a review. *Reprod. Nutr. Dev.*, 1991; 31: 147-157.
15. Das, G.K., Jain, G.C., Solanki, V.S., Tripathi, V.N.: Efficacy of various collection methods for oocyte retrieval in buffalo. *Theriogenology*, 1996; 46: 1403-1411.
16. Arlotto, T.M., Leibfried-Rutledge, M.L., First, N.L.: Size distribution and meiotic competence of bovine primary oocytes from two locations in the ovary. *Theriogenology*, 1990; 33: 188.
17. Gasparrini, B.: In vitro embryo production in buffalo species: state of the art. *Theriogenology*, 2002; 57: 237-256.
18. Younis, A.I., Brackett, B.G., Fayrer-Hosken, R.A.: Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. *Gamete Res.*, 1989; 23: 189-201.
19. Madan, M.L., Singla, S.K., Chauhan, M.B., Manik, R.S.: In vitro production and transfer of embryos in buffaloes. *Theriogenology*, 1994; 41: 139-143.