In vitro production of zygote from slaughterhouse driven buffalo oocyte

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Abstract

Buffalo is a highly potential animal species in terms of milk and meat production but traditionally they are regarded as poor breeder. *In vitro* embryos production technology has been introduced in many countries to improve reproductive efficiency of buffalo. Considering the above fact, the present study was undertaken aiming to produce *in vitro* buffalo embryo in the laboratory. Ovaries of slaughtered buffaloes were collected from abattoir and transported to the laboratory within 4 to 5 hr of slaughter. Cumulus-oocyte-complexes (COCs) possessing an even cytoplasm and covered with minimum 3 layers of compact cumulus cells was selected for *in vitro* maturation (IVM) for 24 hr (5% CO_2 in air at 38.5°C with maximum humidity). After IVM, the presumptive matured COCs were co-cultured with capacitated fresh spermatozoa for 18 hr After IVF, the presumptive zygote were denuded, washed and transferred in to *in vitro* culture medium (IVC 1) for 3 days. After three days cleavage were recorded and 4 cell embryos were transferred in to *in vitro* culture media II for next 2 days. The development of embryos was evaluated on day 6. A total of 227 buffalo ovaries were collected from the slaughterhouse and categorized into 2 groups based on presence (n=83) or absence (n=144) of corpus luteum (CL). A total of 1464 follicles were counted on the ovarian surface, 1066 being from CL absent and 398 from CL-containing ovaries. A significantly higher ($P<0.01$) number of follicles, aspirated follicles, normal COCs and total COCs (7.4 ± 0.21, 5 ± 0.00, 1.98 ± 0.77 and 2.98 ± 0.16 respectively) were observed in CL-absent ovaries than those aspirated from CL-containing ovaries (4.80 ± 0.17, 3.92 ± 0.95, 0.88 ± 0.60 and 1.88 ± 0.16 respectively). Total 358 normal COCs were set for *in vitro* maturation and underwent for IVF and IVC. Results showed that cleavage rates were 56.42%. Among the cleaved embryos, 137 were at 2-cell stage and 65 were at 4-cell stage. Therefore, development rate to 2 cell and 4-cell stage was 38.27% and 18.15% respectively. No embryo developed beyond 4-cell stage. This result indicates that follicle and oocyte numbers and oocyte quality are associated with CL of ovaries and current culture system support *in vitro* embryo production upto 4-cell stage. The *in vitro* culture condition may be improved for increasing efficiency of embryo production.

(**Key words:** Buffalo, oocyte, *in vitro* maturation, Cumulus oocyte complexes, cleavage)

Introduction

Buffalo is an economically important livestock species in Bangladesh. The total buffalo population is about 1.464 million in Bangladesh (DLS, 2015), with about 40% of the population in the coastal regions (Faruque *et al*., 1990). Buffaloes are reported to have low reproductive performance with several inherent reproductive problems, such as silent estrus, seasonal anestrous, delayed puberty, delayed first calving, late post-partum conception and a long calving interval (Nandi *et al*., 2002). In order to improve reproductive efficiency of buffalo, assisted reproductive technologies such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and in vitro production of embryos have been introduced (Nandi *et al*., 2002).

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In vitro embryo production (IVEP) technology is used for rapid multiplication and distribution of high yielding buffaloes in many developed and developing countries. Application of these technologies in assisted reproduction of buffalo is necessary to rescue the precious germplasm due to wastage by indiscriminate slaughter of this animal. IVEP by means of IVF has drawn the interest of innumerable researchers as it can salvage the genetic potential from infertile female and can yield large number of embryos from the ovaries of slaughtered females.

Substantial improvement to the *in vitro* embryo production systems available for buffalos is required, which still remain in sub-optimal levels, compared to those applied in bovines. Therefore, considerable basic developmental work still has to be undertaken in order to standardize IVEP techniques for buffalos.

Research on buffalo IVEP is remained at very preliminary stages including evaluation of follicular statistics, COCs recovery rate, grading of COCs and *in vitro* maturation of COCs (Khandoker *et al*., 2011 and 2012). So, adoption of culture system for development of embryos up to blastocyst stage is essential. From this point of view, the present study was undertaken aiming to produce *in vitro* buffalo embryo in the laboratory.

Materials and Methods

Collection, grading and aspiration of oocytes

Ovaries of slaughtered buffaloes was collected from abattoir located at Kaptan bazar, City Corporation Slaughterhouse, Gulistan, Dhaka in physiological saline (0.9% sodium chloride supplemented with 100 IU/mL penicillin and 0.1 g/mL streptomycin sulfate) at ambient temperature and transported to the laboratory within 4 to 5 hr of slaughter. In the laboratory, extraneous tissue was removed and ovaries were washed with phosphate buffer saline (PBS). The collected ovaries were divided into two groups according to presence and absence of CL. The cumulus-oocyte-complexes (COCs) were aspirated using a 10-mL disposable syringe attached with a 21G needle. The collected cumulus-oocyte-complexes (COCs) were graded as normal and abnormal grade for *in vitro* maturation (IVM) based on their size, diameter and quality of cumulus cell.

In vitro maturation (IVM)

The aspirated material were poured onto a 100-mm petridish containing TL-HEPES (114-mM sodium chloride, 3.2-mM potassium chloride, 2-mM sodium bicarbonate, 0.34-mM sodium biphosphate, 10-mM sodium lactate, 0.5-mM magnesium chloride, 2.0-mM calcium chloride, 10-mM hepes, 1 µL/mL phenol red, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin) solution and the cumulus-oocyte-complexes (COCs) were searched under a microscope at low magnification (4x). The 'normal' cumulus-oocyte-complexes (COC) possessing an even cytoplasm and covered with minimum 3 layers of compact cumulus cells were selected for *in vitro* maturation (Stojkovic *et al*., 2001). The selected COCs (50 to 70 per well) were washed 2-3 times in TL-HEPES and 2-3 times in IVM medium (TCM199 + 10% FBS, 1 µg/mL β-estradiol, 10 µg/mL FSH, 0.6-mM cystein, and 0.2-mM sodium pyruvate) before placing them into a well of 4-well dish containing 500 to 700 µL IVM medium for 22 to 24 hr.

In vitro fertilization (IVF)

The matured COCs were fertilized *in vitro* by fresh semen collected from buffalo bulls of BLRI buffalo farm using artificial vagina method. 10 µL semen was placed in a 15-mL conical tube containing 10 mL D-PBS and pelleted by centrifugation at 750×g for 5 min. The supernatant was removed carefully and 10 mL D-PBS was added in the tube. The sperm was washed for 2-3 times accordingly. Then spermatozoa was capacitated through incubation with 500 µL IVF medium (Tyrode's lactate solution supplemented with 6 mg/mL BSA, 22 µg/mL sodium pyruvate, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin) containing heparin sodium salt (20 µg/mL) for 15 min. After capacitation, the spermatozoa diluted at approximately 1×106 spermatozoa/mL with IVF medium. The matured COC were co-cultured with capacitated spermatozoa for 18 to 20 h through placing them into a well of 4-well dish (500 to 700 µL).

In vitro culture (IVC)

The cumulus cells was removed by gentle pipetting into TL-HEPES and the denuded presumed zygotes were placed in the well of a four-well dish containing 500-700 µL modified CR1-aa medium (Rosenkrans *et al*., 1993) supplemented with Na-pyruvate, glutamine, penicillin/streptomycin, BSA and glutathione for 3 days (IVC-I). These were cultured until day 8 of embryonic development in a medium of the same composition, except that the BSA was replaced with FBS (IVC II). The incubation conditions during IVM, IVF and IVC was 5% CO_2 in air at 38.5°C with maximum humidity.

Statistical analysis

All values relating to follicut or statistics were expressed as Mean±SE. The statistical analysis were done using SPSS IMB 20.0 version software programme.

Results and Discussion

Quantitative evaluation of ovarian follicles

A total of 227 buffalo ovaries were collected from the slaughterhouse and categorized into 2 groups based on presence (n=83) or absence (n=144) of corpus luteum (CL). A total of 1464 follicles were counted on the

Table 1. Effects of CL on follicular statistics and COCs collection in buffalo ovaries

Ovarian type	Follicles per ovary (Mean ± SE)	Aspirated follicles per ovary (Mean ± SE)	Collected COCs per ovary (Mean ± SE)		Total COCs per ovary (Mean ± SE)
			Normal	Abnormal	
CL absent ovary (144)	7.4 ^{a}± 0.21 (1066)	5.00^{a}± 0.00 (720)	1.98^{a} ±0.77 (285)	1.1 ± 0.53 (144)	2.98^{a}± 0.16 (429)
CL present ovary (83)	4.80^{b} ± 0.17 (398)	3.92^{b} ± 0.95 (325)	0.88^{b}± 0.60 (73)	1.1 ± 0.5 (83)	1.88^{b} ±0.16 (156)
Total (227)	6.45±0.21(1464)	4.60 ± 0.9 (1045)	1.58±0.10 (358)	1.1 ± 0.06 (227)	2.58± 0.13 (585)

Means bearing different superscripts (a, b)differ significantly ($p<0.05$)

ovarian surface, 1066 being from CL absent and 398 from CL-containing ovaries. Not all these follicles were suitable for aspiration. The total number of aspirated follicles was 1045, from which 720 were aspirated from CL-absent ovaries, while 325 were aspirated from CL containing ovaries. A significantly higher ($P<0.01$) number of follicles was observed in CL-absent ovaries than in CL-containing ovaries (Table 1). Consequently, the number of aspirated follicles from CL-absent ovaries was significantly higher ($P<0.01$) than those aspirated from CL-containing ovaries (Table 1).

The higher number of follicles found in without CL ovaries than those of with CL ovaries fits with the endocrinological explanation. The presence of corpus luteum in cyclic ovary cause a higher level of progesterone hormone production in which giving a negative response to anterior pituitary gland for the restriction of gonadotrophin secretion and leads to follicular degeneration and inhibition of the development of large follicles (Webb *et al*., 1999). In noncyclic female, the absence of corpus luteum cause no negative effect of progesterone on anterior pituitary and thus estrogen-progesterone levels remains balanced which allows the growth of follicles. Ginther *et al.* (1996) stated that ovaries without CL, the decrease in progesterone leads to increase in GnRH which stimulates the release of follicle stimulating hormone (FSH) and this hormone causes the rapid growth of ovarian follicles.

The results strongly supported by the previous finding of Khandoker *et al.* (2011) who reported that significantly higher number of follicles were found in ovaries without CL (6.78±0.18) than in CL containing ovaries (4.09±0.26). Similar findings also found by Asad (2015), who reported that higher number of follicles aspirated per ovary in without CL group (2.92±0.08) than those of the with CL group (2.52±0.11) in goat.

Quantitative and qualitative evaluation of COCs

Both the number and quality of the COCs are important to initiate the embryo culture experiment. Significantly higher ($P<0.01$) number of normal COCs was found in CL-absent ovaries compared to CL-containing ovaries. The mean retrieved COCs per ovary was higher in CL-absent ovaries than in CL-present ovaries (2.98 vs. 1.88 COCs). The cause for the low number of oocytes in ovaries containing a CL is likely because of the restricted follicular development, as lutein cells occupy a great portion of the ovary; furthermore, CL may inhibit the follicular growth and foster their atresia.

Nandi *et al*. (2000) stated that when ovaries had a corpus luteum, the oocyte recovery rate decreases. This is because there will be restriction of follicular development as lutein cells occupy most of the ovary (Kumar *et al*., 2004). Hafez (1993) mentioned that in the presence of CL in ovary, the growth of follicle is inhibited while atresia is increased. These statements can be the physiological explanation for lower number of COCs in the with CL ovaries compared to without CL ovaries. Similarly, the higher number of normal COCs in CL absent group ovaries may be due to the hormonal effect of CL. When CL is absent in the ovary, progesterone which has role in follicular degeneration could not be produced (Hafez, 1993). Thus, folliculogenesis can occur successfully and

further there is more chance to produce high quality of COCs. Therefore, the types of ovary at the time of COCs collection have affected the quantity and quality of COCs recovered as well as usable oocytes in animals for use in IVEP program.

In vitro embryo production

Collected total 358 normal COCs were allowed for *in vitro* maturation (5% CO2 in air at 38.5°C with maximum humidity) in which 285 oocytes showed cumulus expansion at the rate of 79.61%. All the expanded COCs were allowed for *in vitro* fertilization using fresh semen and they were cultured subsequently (IVC 1 and IVC 2) for 8 days. Following culture, 202 out of 358 COCs were undergone for cleavage step. Among the cleaved embryos, 65 were at 4-cell stage and 137 were at 2-cell stage at the rate of 18.15% and 38.27% respectively. No embryo developed beyond 4-cell stage. Deb *et al*., (2016) found 74.16+5.49% maturation rate, 62.05+7.07% cleavage and 14.95= 4.39% blastocyst rate for slaughterhouse driven cattle oocytes. During IVM, 60.00 to 85.00% of the immature oocyte normally develops to metaphase II stage (Dey *et al*., 2011). Maturation rates of COCs found in the current study are within the range of normal standard culture system. Development rates to cleavage and blastocyst was lower in the current study than standard culture system (Dey *et al*., 2011). However, some reports showed similar and/or lower cleavage rates compared to present study (Livingston *et al*., 2014). The efficiency of *in vitro* embryo development depends on oocyte donor, semen quality, and culture media and culture conditions employed during each phase of IVP (Brum *et al*., 2005). Beside these factors, the IVP efficiency is also affected by osmolality of media (Gordon, 1994), water quality used in different steps of IVP.

Table 2. Status of *in vitro* embryo production of buffalo ovary collected from slaughterhouse

Total COCs for IVM	COCs with cumulus expansion (%)	Cleavage (%)	*In vitro* culture		
			Two cell (%)	Four cell (%)	Blastocyst (%)
358	285 (79.61%)	202 (56.42%)	137 (38.27%)	65 (18.15 %)	0

* Percentage was calculated on the basis of total no. of COCs placed for IVM

Conclusion

Comparatively higher numbers of ovaries were found without corpus luteum compared to ovaries with corpus luteum confirms that usually noncyclic buffaloes are slaughtered in the slaughterhouse and that is may be due to economic reason. Ovaries without CL contributing larger number of follicles aspirated per ovary compared to ovaries with CL. Furthermore, comparatively higher number of total COCs and superior quality of COCs were possible to obtain from without CL ovaries, suggested to be suitable for collecting COCs for initiating *in vitro* embryo production experiment in buffaloes. *In vitro* embryo production experiment concluded that current culture system support

in vitro embryo production upto 4-cell stage. Improvement of culture condition may improve the efficiency of embryo production.

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