

***IN VITRO* PRODUCTION OF GOAT EMBRYOS IN BANGLADESH**

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Abstract

The present study was undertaken to collect the quality cumulus-oocyte-complexes (COCs) from ovaries of goat from slaughterhouse by aspiration to establish the suitable culture condition for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). Follicular COCs were collected from follicles of 2-6 mm diameter, categorized by microscopic observation and cultured for 22 h in TCM-199 medium supplemented with 5% fetal calf serum (FCS) to determine the success rate of *in vitro* maturation in a condition of 5% CO₂ in air at 38.5°C. The collected ovaries were classified as type-I (corpus luteum absent) and type-II (corpus luteum present). The average numbers of follicles aspirated per ovary were 3.15 and 2.57 in type-I and type-II, respectively. The collected COCs were classified into normal COCs (grade A and B) and abnormal COCs (grade C and D). The number of normal and abnormal COCs collected from two type of ovaries were significantly (P<0.01) differed. Average number of normal COCs per ovary obtained from type-I (1.30) was significantly (P<0.01) higher than that of type-II (0.68). Within the normal COCs significantly (P<0.01) higher maturation was obtained in grade A COCs (71.70%) than that of grade B (51.52%). The matured COCs were cultured for 5 h with fresh buck semen in Brackett and Oliphant (BO) medium and assumed that the COCs were fertilized successfully. In progress, IVC was practiced in TCM-199 supplemented with FCS and bovine serum albumen (BSA) at 38.5°C with 5% CO₂ for 6-7 days. The rate of development to compact morula was found significantly (P<0.01) higher in grade A (25.64%) compared to grade B COCs (6.89%) and similar trend of blastocyst was found in grade A COCs (12.82%) than that of grade of B (3.45%). The results suggested that culture condition for IVM, IVF and IVC was found optimum and grade A COCs might be suitable for *in vitro* production (IVP) of goat embryos.

Key words : Goat ovaries, Cumulus-oocyte-complexes (COCs), *In vitro* production

Introduction

In Bangladesh, *in vitro* production (IVP) of goat embryos is a very recent work but a great deal of work has been done regarding evaluation and grading of bovine ovaries, collection of COCs from slaughterhouse ovaries and grading of bovine oocytes followed by IVM, IVF of the oocytes and IVC of the resulting zygotes (Rahman *et al.*, 2003; Goswami *et al.*, 2004; Pervage *et al.*, 2007) and *in vitro* culture of mouse embryo (Khandoker *et al.*, 2005). Preliminary research on goat embryo culture

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(Islam *et al.*, 2007; Ferdous, 2006) have been done, but till now, there exists vast opportunity to conduct the research work on *in vitro* goat embryo production.

For IVP of embryos, the evaluation of ovaries, efficient collection and grading of oocytes followed by IVM, IVF and IVC of the resulting zygotes have allowed extensive research on modern reproduction techniques in farm animals. Goat ovaries from the slaughter house were found to be an economical source of oocytes for IVM, IVF and IVC research (Martino *et al.*, 1995). The average number of good quality oocytes recovered from ovaries without corpora lutea was more as compared to the ovaries with corpora lutea, which can be effectively used for IVF (Kumar *et al.*, 2004). In addition it has been shown that a comparable higher maturation rate could be reached within 24 hours of culture if the oocytes had a compact cumulus structural cell. Oocytes with intact cumulus or at least four layers of cumulus cells gave good result for IVM and IVF (Yang *et al.*, 1993). Whereas, denuded oocytes or oocytes with few cumulus cells are usually rejected because of their low capacity of fertilization and/or *in vitro* development (Criste *et al.*, 1986). Beside this, culture condition, hormone and protein supplementation also plays an important role for IVM, IVF and IVC (Bavister and Rose-Hellenkant, 1992; Fukui and Ono, 1989; Moor *et al.*, 1980; Tsafri and Thibault, 1975). Though, a lot of experiments in this context have been performed such as IVM, IVF and IVC of embryos in cattle (Fukuda *et al.*, 1990; Kotsuji *et al.*, 1996; Khandoker *et al.*, 2001; Chanson *et al.*, 2001), in buffalo (Totey *et al.*, 1993) in sheep and goat (Gardner *et al.*, 1994; Cognie *et al.*, 2003), horses (Cognie *et al.*, 1992) and in rat and mouse embryos (Khandoker and Tsujii, 1999). Similar experiments in Bangladesh have not been done in the past. Previously experiment on collection and evaluation of cumulus-oocyte-complexes (COCs) from slaughterhouse goat ovaries conducted and reported that the higher number of follicles was found in the ovaries without corpus luteum (Islam *et al.*, 2007). The number of follicles measuring 2-6 mm diameter was found to be higher in ovaries without CL than ovaries with functional and regressed CL (Ferdous, 2006). It was also suggested that ovaries having no CL might be the source of quality COCs (Islam *et al.*, 2007). With this view in mind, present research work was undertaken to collect the quality COCs by aspiration method.

Materials and Methods

Collection and processing of ovaries

Ovaries were collected from local slaughterhouse in collection vial containing 0.9% physiological saline kept in a thermos box at 25 to 30°C and transported to the laboratory within 4 to 5 hours of slaughter. Then, the ovaries were categorized as corpus luteum absent and present group and the number of both types of ovaries were recorded.

Collection and grading of cumulus-oocyte-complexes (COCs)

After washing 2-3 times in saline solution, the ovaries were placed in a beaker and kept in a water bath at 30°C. The 10 ml syringe was loaded with PBS (1-1.5ml), and the needle (19G) was put in the ovarian parenchyma near the vesicular follicles (2 to 6 mm diameter) and follicles aspirated near the point at the same time. The aspirated follicular materials were transferred slowly into a 90mm petridish, avoiding damage to the cumulus cells and the COCs were searched and graded under microscope at low magnification. The COCs was then classified into 4 grades as described Khandoker *et al.* (2001). Briefly, grade A : oocytes completely surrounded by cumulus cells; grade B : oocytes partially surrounded by cumulus cells; grade C : oocytes not surrounded by cumulus cells and grade D

: degeneration observed both in oocytes and cumulus cells. Grade A and B were considered as normal COCs and grade C and D will be considered as abnormal. The number of different grades of COCs in each category was recorded. The representative photograph of two categories of COCs is shown in Figure 1. In the meantime another petridish with D-PBS was prepared for pooling COCs and the COCs were picked up with an appropriate glass micropipette. The total work was performed in aseptic condition.

The tip diameter of the pipette was checked under the microscope to ensure COCs, which could be easily aspirated without damaging the cumulus cells. Basically the glass micropipettes were prepared slowly stretching the tip of pasteur pipette above burners flame. Then the COCs were washed 2-3 times into D-PBS before initiating the maturation experiment.

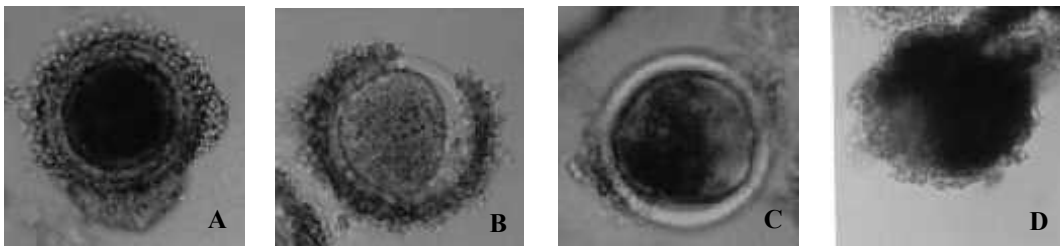


Fig. 1 : Representative photograph showing A, B, C and D grade COCs where A and B considered as normal and C and D Considered as abnormal COCs

***In vitro* maturation (IVM) of oocytes**

The maturation medium, Tissue Culture Medium-199 (TCM-199) supplemented with 5% fetal calf serum (FCS) was prepared and its pH was adjusted at 7.2 and sterilized by passage through a 20 μ m sartorius Minisart filter. In a culture dish 4 drops of each about 100 μ l of maturation medium were poured and covered with paraffin oil. Then it was kept in an incubator at 38.5 $^{\circ}$ C with 5% CO₂ in air. Normal COCs (A and B grade) were washed 2-3 times separately in PBS and then transferred into the maturation medium (TCM-199 supplemented with 5% FCS) and washed 2 or 3 times in maturation medium. Droplets containing graded oocytes were kept in a CO₂ incubator at 38.5 $^{\circ}$ C with 5% carbondioxide in air for 22 hours. After 22 hours of IVM, cumulus expansion was determined according to Rahman *et al.* (2003) by three levels in same magnification under microscope as i. indicating less expansion of COCs; ii. indicating moderate expansion and iii. indicating marked expansion of cumulus cells with a compact layer. The number of COCs classified on the basis of expansion rate of COCs was recorded.

Semen collection and sperm capacitation

The fertilization medium (BO medium) was prepared and its pH was adjusted to 7.8 on the day of use. Finally it was sterilized by filtration. Semen was collected from the buck of departmental Artificial Insemination Center by artificial vagina (AV) method and was brought to the laboratory in icebox within a short period. The concentration of raw semen was calculated by haemocytometer. Fifty micro liter (μ l) of raw semen were taken in 10 ml sterilized pipette and 3 ml to 4.2 ml (depending on the sperm concentration) of semen washing solution were added to adjust the sperm concentration to 25 \times 10⁶ per ml. The semen washing with washing solution (BO with 1% BSA) was taken in a

centrifuge tube and centrifuged at 800 rpm at room temperature for 5 minutes and then top liquid portion was discarded. After that the same amount of sperm washing solution was added to the centrifuge tube and repeated the procedure twice. Finally the sperm concentration was adjusted at 12.5×10^6 per ml by adding semen dilution solution (BO with 2% BSA). Four insemination droplets (100 μ l) of BO medium were prepared in a 35 mm culture dish, covered with paraffin oil and were kept in the incubator for preincubation.

***In vitro* fertilization**

The matured oocytes were washed 3 times in the oocyte washing solution (BO with 1% BSA). About 15-20 oocytes with minimum volume of medium were transferred to each of the sperm drops prepared previously and incubated for 5 hours at 38.5°C with 5% of CO₂.

***In vitro* culture (IVC) and observation**

After 5 hours incubation, the fertilized ova were taken from the semen drops with cumulus cells by using the appropriate micropipette. After that they were washed three times in pre-incubated medium (TCM-199) and were transferred to other culture drop (600 μ l) of TCM-199 with 5% FCS. The dish was then kept in the CO₂ incubator at 38.5°C with 5% of CO₂ in air. The development was checked every 48 hrs and the culture were continued for 6 to 7 days. The number of compact morula and early blastocysts were recorded on day seven (Fig. 2).

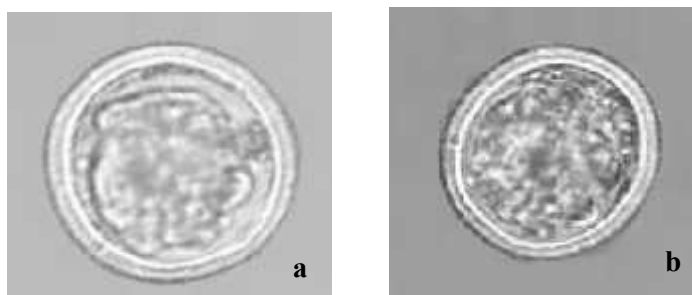


Fig. 2 : Representative photograph showing a) compact morula and b) early blastocyst

Statistical analysis

Data were analyzed using SAS (Statistical Analysis System, 1998) package program in accordance with the principles of CRD (Steel and Torrie, 1980) and Duncun's Multiple Range Test (DMRT) was also done to identify the significant differences between the mean values (Snedecor and Cochran, 1980).

Results and Discussion

Ovarian types, number of follicles aspirated and collected COCs per ovary

Goat ovaries were collected from local slaughterhouses and classified into two types. The ovaries without corpus luteum (CL) considered as type-I and with CL considered as type-II. Among 138 ovaries, 103 were found as type-I and 35 as type-II. The result of the number of follicles aspirated and collected COCs from two types of ovaries is summarized in Table 1. A totals of 516 follicles were aspirated from both types of ovary and among them 432 were obtained from type-I and 84 from

type-II ovaries. Significantly ($P<0.05$) higher number of follicles were aspirated per ovary in type-I than in type-II with the mean of 3.15 and 2.57 follicles per ovary, respectively. When compared the collected COCs per ovary between two types, higher number was found in type-I than type-II with the mean of 1.96 and 1.54 follicles per ovary, respectively. Again, when the COCs were classified in normal and abnormal groups, significantly ($P<0.01$) higher number of normal COCs was found than that of abnormal in type-I and the same trend also obtained in type-II ovaries. Similarly, when comparing the result between types significantly ($P<0.01$) higher number of normal COCs was obtained in type-I than that of type-II with the mean of 1.30 and 0.68 follicles per ovary respectively and the reverse trend was found in abnormal group (with the mean of 0.66 and 0.86 follicles per ovary, respectively). These results strongly supports the result of Islam *et al.* (2007), who reported that higher number follicles aspirated (2.55 ± 0.10 vs 2.48 ± 0.21 per ovary) and normal COCs (1.12 ± 0.07 vs 0.76 ± 0.14 per ovary) were found in CL-absent group than those of CL-present group of ovaries. The female goats destined for slaughter were usually less reproductive performer and most of them might be non-cyclic. So there was a possibility to get more non-cyclic ovaries from the slaughterhouse during random sampling. The less number of CL group ovaries obtained in this experiment supports the above statement.

Table 1. Ovarian types and number of follicles and cumulus-oocyte-complexes (COCs) per ovary

Ovarian type	Total number of visible follicle (mean \pm SE)	Number of follicle aspirated (mean \pm SE)	Collected COCs per ovary (mean \pm SE)		
			Normal	Abnormal	Total
Type I (103)	5.25 ^a \pm 0.20	3.15 ^a \pm 0.12	1.30 ^a \pm 0.07	0.66 \pm 0.06	1.96 ^a \pm 0.09
Type II (35)	3.94 ^b \pm 0.34	2.57 ^b \pm 0.20	0.68 ^b \pm 0.12	0.86 \pm 0.11	1.54 ^b \pm 0.15

^{ab} Means with different superscripts within the same column differ significantly ($P<0.05$)

Figure in the parenthesis indicates the total number

In the present study significantly greater number of oocytes per ovary were recovered from ovaries without a corpus luteum than from ovaries with a corpus luteum. According to Nandi *et al.* (2000) the oocyte recovery rate decreased when ovaries had a corpus luteum. This was due to restriction of follicular development as lutein cells occupy most of the ovary (Kumar *et al.*, 1997). The dominant follicle was usually observed in the corpus luteum-bearing ovary, and the other follicles were very small and remained mostly inaccessible. Agrawal (1992) reported goat ovaries containing a corpus luteum yielded a lower number of oocytes and also a lower proportion of usable oocytes than ovaries without a corpus luteum. So, status of ovaries at the time of oocyte collection significantly might have affected the recovery of quality as well as usable oocytes in goat for use in IVP program.

Macroscopic observation of *in vitro* maturation (IVM) of COCs after 22 h culture

The result of maturation rate of these two groups of COCs after 22 h culture is presented in Table 2. Both grade A and B COCs were matured to some extent after 22 h culture. Significantly ($P<0.01$) higher percentage of COCs were expanded in level 3 (71.70, 51.52), followed by level 2 (26.41, 44.44) and level 1 (1.89, 4.04) in either grades. Significantly higher percentage of ($P<0.01$) COCs was found to be matured in grade A (71.70) than that of grade B (51.52).

The maturation rate of goat COCs found in this experiment varied between 52 and 73% (Table 2) which was similar to the result of Naruse *et al.* (2004). Who found 69.1-73.8% maturation rate of goat oocytes when cultured in TCM-199 medium supplemented with penicillin, streptomycin and gentamycin. Islam *et al.* (2007) found 70-75% maturation rate of goat oocytes when cultured for 22 h at 38.5°C under 5% CO₂ in TCM-199 medium supplemented with 5% fetal calf serum (FCS). While Harper *et al.* (1993) found 79-92% maturation rate of bovine oocytes when cultured for 26 h at 39°C in TCM-199 medium supplemented with epidermal growth factor (EGF). The result indicated that cumulus cells expansion level might be considered as the tool of oocyte maturation. Cumulus cells expansion during *in vitro* oocyte maturation was beneficial for completion of the maturation process. The role of the cumulus cells might revolved around their ability to produce pyruvate to provide energy substrate during this period (Ball *et al.*, 1984). Moreover culture condition of the present experiment appeared to be optimum for *in vitro* maturation (IVM) of goat oocytes in the context of Bangladesh.

Table 2. Macroscopic *in vitro* maturation (IVM) of COCs after 22 h culture

Grade of COCs	Maturation rate (%)		
	Expansion level		
	1	2	3
A (159)	1.89 ^b ±0.94 (3)	26.41 ^b ±2.15 (42)	71.70 ^a ±2.59 (114)
B (99)	4.04 ^a ±1.12 (4)	44.44 ^a ±5.36 (44)	51.52 ^b ±3.46 (51)

^{ab} Means with different superscripts within the same column differ significantly (P<0.01)
Figure in the parenthesis indicates the total number

***In vitro* fertilization (IVF) of goat oocyte**

After 5 hours of culture with goat semen the COCs were assumed to be fertilized successfully and removed from the incubator and washed several times to remove the sperm so as to initiate the further culture of zygotes for development.

Rate of development (%) of goat embryo after 7 days of *in vitro* culture (IVC)

Macroscopic observations of rate of development (%) to compact morula and early blastocysts were observed after 7 days of culture and the total number of compact morula and blastocyst of two different grades have been shown in Table 3.

Table 3. Rate of development (%) of goat embryo after 7 days of *in vitro* culture

Number of zygote	Development of embryos	
	Number of morula (%)	Number of blastocysts (%)
39 (A grade)	25.64 ^a (10)	12.82 ^a (5)
29 (B grade)	6.89 ^b (2)	3.45 ^b (1)

^{ab} Means with different superscripts within the same column differ significantly (P<0.05)
Figure in the parenthesis indicates the total number

Significantly ($P < 0.05$) higher number of compact morula and early blastocysts were obtained from grade A (25.64% and 12.82%, respectively) as compared to grade B (6.89% and 3.44%, respectively). Keskinetepe *et al.* (1998) found 31.4% and 18.6% morula and blastocyst stages of goat oocytes which were very much similar to the present result. The results observed in the present research was contradicted with the findings of Katska *et al.* (2004) who obtained blastocyst yield upto 37.3% after IVF with fresh sperm capacitated without heparin and also with John *et al.* (2000) (observed differences of blastocyst formation from 23 to 31%). Gardner *et al.* (1994) studied sheep oocyte and found 29% rate of development to compact morula. On the other hand, 25.9% oocytes developed upto morula stage in case of goat oocytes (Keskinetepe *et al.*, 1994), which was also similar to that of the results found in the present study.

The result on the rate of development obtained in grade A or in grade B could be due to some physiological condition of the ovaries. The marked development was observed from A grade COCs which were mostly obtained from CL-absent group ovaries (Kumar *et al.*, 2004). CL-absent group ovaries did not contain the CL and the negative effect of progesterone on anterior pituitary might not be functional in this category of ovaries. Therefore, the highest number of grade A COCs was observed from CL-absent group than that of CL functional group. The good quality COCs (A-grade) might be larger in size (Kumar *et al.*, 2004) and with a homogenous evenly granulated cytoplasm possessing at least three layers of compact cumulus cells while that of poor quality COCs (B-grade) have less than three layers of cumulus cells. There were partially denuded possessing a homogenous evenly granulated cytoplasm (Echert and Niemann, 1995), and for this, may better result led to with the former category of COCs.

It was concluded that grade A COCs were important criteria for production of goat embryo in IVP experiment than that of grade B COCs. The results demonstrated to be efficacious for the study of *in vitro* production of goat embryos and for the application of embryonic manipulation procedure in Bangladesh.

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