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Collection grading and evaluation of cumulus-oocyte-complexes for *in vitro* maturation in sheep

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Abstract

An alternative to superovulation is *in-vitro* production (IVP) of embryos where the efficient collection and grading of oocytes is important. Ovaries from an abattoir were collected and categorized as type I with no corpus luteum (CL), and type II with CL. The length, width and weight of type I and type II ovary were 1.4 ± 0.03 and 1.5 ± 0.08 cm; 0.8 ± 0.04 and 1.0 ± 0.07 cm; 0.6 ± 0.07 and 0.7 ± 0.04 gm, respectively, each significantly ($P<0.05$) higher in type II ovaries. A total of 80 and 78 follicles were observed and 60 and 61 follicles aspirated from left and right ovaries, respectively, from each of 25 ovaries. Out of 133 follicles 100 were aspirated from 40 type-I ovaries, and 21 aspirated from 10 type-II ovaries. The differences in the number of normal, abnormal and total cumulus-oocyte-complex (COCs) per ovary between left and right ovaries were not significant ($P>0.05$). The number of normal (1.9 ± 0.11) and total (2.5 ± 0.14) COCs per ovary were significantly ($P<0.05$) higher in ovaries without than in those with CL (1.2 ± 0.36 and 2.0 ± 0.30, respectively). But the number (0.80 ± 0.13) of abnormal COCs per ovary was significantly ($P<0.05$) higher in ovaries with CL than in those without (0.7 ± 0.09). Significantly ($P<0.05$) higher percentage of COCs expansion was grade A (6.9 ± 2.05) than grade B (53.1 ± 1.27) COCs. It is suggested that type I (without CL) ovaries and follicles of 2-6 mm diameter are suitable to collect good quality COCs for *in-vitro* maturation (IVM) of oocytes and the culture condition for IVM of sheep COCs are reported. (*Bangl. vet.* 2011. Vol. 28, No. 1, 31 – 38)

Introduction

Oocyte maturation is traditionally defined as those events associated with the initiation of germinal vesicle breakdown (GVBD) and completion of the first meiotic division, referred to as nuclear maturation. The process of oocyte maturation, however, includes changes within the cytoplasm, which make the egg capable of fertilization and able to initiate the programme that directs pre-implantation embryonic development. Production of embryos can be based on three steps: *in vitro* maturation (IVM) of oocytes, then *in vitro* fertilization (IVF) and then *in vitro* culture (IVC) for cleavage up to blastocyst stage (Palta and Chauhan, 1998; Goswami *et al.*, 2004). Oocyte maturation is the most critical step. The culture medium and protein supplements and hormones for IVM play an important role in the maturation rate, and embryonic development (Bavister *et al.*, 1992). Since 1935 a lot of experiments on

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in-vitro embryo culture with slaughterhouse ovaries have been performed, to establish the best medium at different stages of development (Khandoker *et al.,* 2001; Tasripoo *et al.,* 2005; Jamil *et al.,* 2008). Actually, the improvement of culture media for *in-vitro* production (IVP) of embryos comes from follicular, oviductal and uterine fluid. Several experiments have been done on IVM, IVF and IVC of embryos in horses (Cognie *et al.,* 1992); buffaloes (Totey *et al.,* 1993); rats and mice (Khandoker and Tsujii, 1999; Ali, 2004; Khandoker *et al.,* 2005); cattle (Chanson *et al.,* 2001; Rahman *et al.,* 2003; Goswami *et al.,* 2004; Pervage, 2006), sheep and goats (Cognie *et al.,* 2003; Ferdous, 2006; Islam *et al.,* 2007; Mondal *et al.,* 2008; Hoque, 2009) but there are no comprehensive reports in Bangladesh. This study was undertaken with the following objectives:

To compare collection and grading procedures of cumulus-oocyte-complexes (COCs) in sheep obtained from ovaries at slaughter;

To establish the relationship between ovarian conditions and the quality of collected COCs; and

To establish suitable culture conditions for *in vitro* maturation (IVM) of sheep oocytes.

Materials and Methods

Physiological saline (0.9% NaCl) was prepared for washing. Dulbecco's Phosphate Buffered Saline (D-PBS) was prepared by adding one pack of PBS salt (Sigma, USA) in one litre of distilled water. It was autoclaved and refrigerated for further use. The ovaries were kept in collection vial containing 0.9% physiological saline in a vacuum flask at 25 - 30^{o}C and transported to the laboratory within 4 -5 hours of slaughter. Sheep ovaries were collected from Municipal slaughterhouse, Mymensingh and numbered. The ovaries were classified into type I, without CL, and type II with CL. After collection and trimming of ovaries, each ovary was weighed and measured with callipers. The visible follicles were counted. Syringe (10 ml) loaded with PBS (1.0 - 1.5 ml), with 19G needle was used for the aspiration of follicles of 2 to 6 mm diameter. The aspirated material was transferred slowly into a 90-mm Petri dish, and the COCs were graded under microscope at low magnification of x10. The COCs were classified as described by 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: degenerated oocytes and cumulus cells. Grade A and B were considered normal COCs and grade C and D abnormal (Fig. 1). Maturation medium, TCM-199 supplemented with 5% Fetal Calf Serum (FCS) was prepared and its pH adjusted to 7.4 on the day of aspiration and sterilized by filtration (22 µ Millipore filter). Normal graded COCs were washed 2-3 times in D-PBS, transferred into the maturation medium (TCM-199 + 5% FCS) and washed 2-3 times with the help of glass micro-pipette. About 2.5 - 3.5 ml of the medium was poured into each of two 35 mm culture dishes. In another culture dish 3 drops of 100 µl of maturation medium were poured

and covered with paraffin oil. Droplets containing normal graded COCs were kept in a carbon dioxide incubator at 38.5°C with 5% carbon dioxide in air for 24 hours. After 24 hour of IVM, cumulus expansion was determined by three levels in same magnification of x 10; 1: indicating less expansion of COCs; 2: indicates moderate expansion; and 3: indicating marked expansion of cumulus cells with a compact layer or corona radiate (Fig. 2).

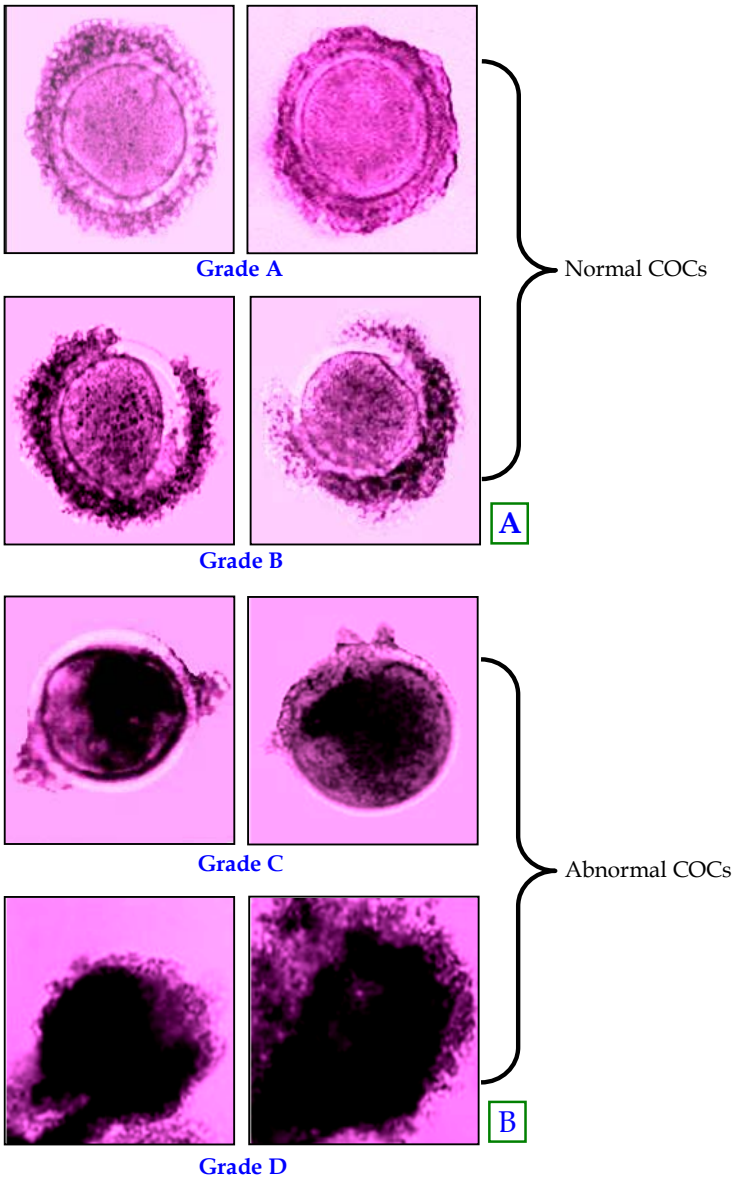

Fig. 1. Representative photograph showing: A) Normal COCs (Grade A and B); B) Abnormal COCs (Grade C and D)

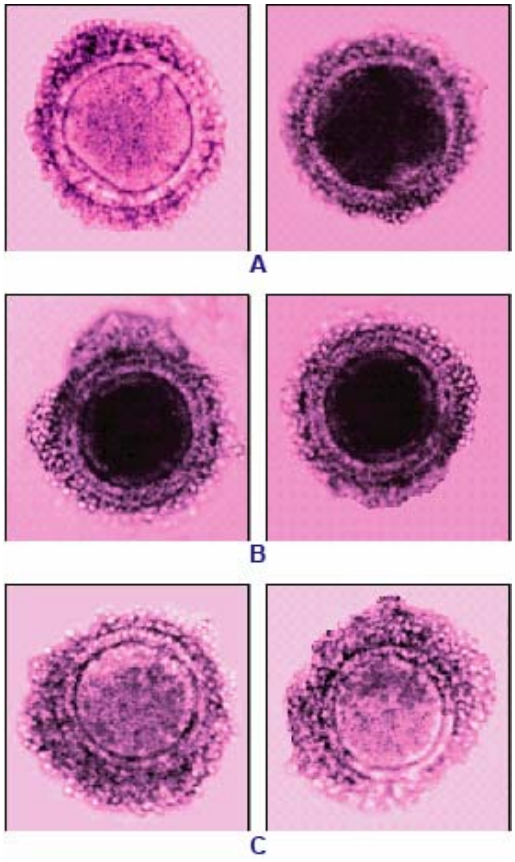

Fig. 2. Representative photograph showing: A. Cumulus cell expansion level-1 (less expansion); B. Cumulus cell expansion level-2 (moderate expansion); C. Cumulus cell expansion level-3 (marked expansion of cumulus cells with a compact layer or chorona radiata

Statistical analysis

The data were put in Microsoft Excel worksheet. Analysis was performed by analysis of variance (ANOVA) in completely randomized design (CRD), and for comparing means, Duncan's multiple range test (DMRT) was applied with the help of Statistical Analysis System (SAS).

Results and Discussion

Among 50 ovaries (25 left; 25 right), 40 were type-I and 10 type-II. CL was present on 6 right and 4 left ovaries. The number and dimensions of follicles observed and aspirated and COCs are summarized in Tables 1-2. There was no significant ($P>0.05$) difference in dimensions between left and right ovaries (Table 1). This observation

supports the studies of Islam *et al.* (2007). The length, width and weight of type I and type II ovaries were 1.4 ± 0.03 and 1.5 ± 0.08 cm; 0.8 ± 0.04 and 1.0 ± 0.07 cm; 0.6 ± 0.07 and 0.7 ± 0.04 gm, respectively: all were significantly (P<0.05) higher in type II than type I ovaries (Table 2). CL is situated within the ovary, which increase its size. A total of 80 and 78 follicles were observed and 60 and 61 follicles aspirated from left and right ovaries, respectively. A total of 133 follicles were observed and 100 aspirated from 40 type-I ovaries, and 25 follicles were observed and 21 aspirated from 10 type-II ovaries. The difference in the number of normal, abnormal and total COCs per ovary between left (1.7 ± 0.17, 0.6 ± 0.10, 2.3 ± 0.18, respectively) and right (1.6 ± 0.12, 0.7 ± 0.13 and 2.2 ± 0.19, respectively) was not significant (P>0.05: Table 1). The number of normal and total COCs per ovary were significantly (P<0.05) higher in ovaries without CL (1.9 ± 0.11 and 2.5 ± 0.14, respectively) than in those with CL (1.2 ± 0.36 and 2.0 ± 0.30, respectively). But the number of abnormal COCs per ovary was significantly (P<0.05) higher in ovaries with CL (0.8 ± 0.13) than those without (0.7 ± 0.09). The results are similar to the observation of Islam *et al*. (2007) in goats.

Table 1. Ovarian classification (left and right) and ovarian dimensions

Ovarian Type	Length (cm) (mean ± S.E)	Width (cm) (mean ± S.E)	Weight (gm) (mean ± S.E)	Total number of follicles (mean ± S.E)	Number of follicles aspirated (mean ± S.E)	Collected COCs per ovary (mean ± S.E)		
						Normal	Abnormal	Total
Left (25)	1.4 ± 0.04	0.9 ± 0.03	0.7 ± 0.05	3.1 ± 0.35	2.3 ± 0.27	1.7 ± 0.17	0.6 ± 0.10	2.3 ± 0.18
Right (25)	1.4 ± 0.05	1.0 ± 0.05	0.7 ± 0.07	3.0 ± 0.40	2.2 ± 0.31	1.6 ± 0.12	0.7 ± 0.13	2.2 ± 0.19

Means with different superscripts within the same column differ significantly (P<0.05); Parenthesis indicates the total number of ovaries

Table 2. Ovarian classification (Type I and Type II) and ovarian dimensions

Ovarian Type	Length (cm) (mean ± S.E)	Width (cm) (mean ± S.E)	Weight (gm) (mean ± S.E)	Total number of follicles (mean ± S.E)	Number of follicles aspirated (mean ± S.E)	Collected COCs per ovary (mean ± S.E)		
						Normal	Abnormal	Total
Type I (n = 40)	1.4^{b} ± 0.03	0.8^{b} ± 0.04	0.6^{b} ± 0.07	3.3^{a} ± 0.29	2.6^{a} ± 0.23	1.9^{a} ± 0.11	0.7^{b} ± 0.09	2.5^{a} ± 0.14
Type II (n = 10)	1.5^{a} ± 0.08	1.0^{a} ± 0.07	0.7^{a} ± 0.04	2.5^{b} ± 0.31	2.1^{b} ± 0.23	1.2^{b} ± 0.36	0.8^{a} ± 0.13	2.0^{b} ± 0.30

Means with different superscripts within the same column differ significantly (P<0.05); Parenthesis indicates the total number of ovaries; Type I: Ovaries without CL; Type II: Ovaries with CL

The *in vitro* maturation (IVM) of COCs after 24 h culture is shown in Table 3. Both A and B grades of COCs matured to some extent after 24 h culture. Significantly higher ($P<0.05$) percentage of COCs were expanded to level 3 (62.9 ± 2.05, 53.1 ± 1.27%), followed by level 2 (24.5 ± 2.24, 27.1 ± 1.31%) and level 1 (12.5 ± 1.96, 19.8 ± 1.98%) in grades A and B, respectively (Table 3). After 24 h culture significantly ($P<0.01$) higher percentage of COCs had matured in grade A (62.9) than in grade B (53.1), similar to the result of Suzuki *et al. (*1992); Warriach and Chohan *(*2004); Islam *et al. (*2007). The maturation rate of sheep COCs varied between 53.1 ± 1.27 and 62.9 ± 2.05% (Table 3), similar to results of Totey *et al.* (1992; 44.8 ± 25.6 - 47.4 + 17.8), Suzuki *et al. (*1992; 59 - 67.3%), Warriach and Chohan. (2004; 51.4 - 64.5%), Jamil *et al.* (2007; 62.3 - 77.4%), Mandol *et al.* (2008; 51-71%). Jamil *et al.* (2007) found 62.3 -77.4% maturation rate of buffalo oocytes when cultured in TCM-199 medium supplemented with oestrus buffalo serum. Warriach and Chohan (2004) found 64.5% and 51.4% maturation rate with oocytes with three or more layers of cumulus cells and one or two layers of cumulus cells, respectively. Similar maturation rate was recorded by Mondal *et al.* (2008) in goats using TCM-199 medium supplemented with fetal calf serum. Totey *et al.* (1992) found (44.8 ± 25.6 - 47.4 ± 17.8) maturation rate in TCM-199 medium supplemented with 20% buffalo oestrus serum.

Table 3. *In vitro* maturation (IVM) of COCs after 24 h culture

Grades of COCs	Maturation rate (%) ± S.E		
	Expansion levels		
	1	2	3
A (54)	$12.5^{b} \pm 1.96$	24.6 ± 2.24	$63.93^{a} \pm 2.05$
B (26)	$19.8^{a} \pm 1.98$	27.1 ± 1.31	$53.1^{b} \pm 1.27$

Means with different superscripts within the column differ significantly ($P<0.05$); Parenthesis indicates the total number of COCs; The expansion level; 1: Less expansion of COCs; 2: Moderate expansion of COCs and 3: Marked expansion of cumulus cells with a compact layer of corona radiata

The results indicate that cumulus cell expansion level might be considered as a good measure of oocyte maturation. Cumulus cell expansion during *in vitro* oocyte maturation was beneficial for completion of the maturation process. The role of the cumulus cells might revolve around their ability to produce pyruvate to provide energy during this period (Ball *et al.,* 2003). Moreover the culture condition of the present study might be optimum for *in vitro* maturation (IVM) of sheep oocytes in the context of Bangladesh.

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