

Full Length Research Paper

Effect of collection techniques on cumulus oocyte complexes (COCs) recovery, *in vitro* maturation and fertilization of goat oocytes

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The experiment was undertaken to study the effect of collection techniques on cumulus oocyte complexes (COCs) recovery, *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of goat oocytes. COCs were collected by three techniques viz. puncture, slicing and aspiration of goat ovaries obtained at slaughterhouse. It was observed that, the total number of COCs/ovary as well as the number of abnormal COCs/ovary were significantly higher ($p < 0.01$) in puncture (4.22 and 2.38, respectively) and slicing (4.14 and 2.22, respectively) followed by aspiration (3.28 and 0.80, respectively) technique. In contrast, the number of normal COCs/ovary was significantly higher ($p < 0.01$) in aspiration (2.48) followed by slicing (1.91) and puncture (1.85) techniques. Only normal quality COCs were cultured in TCM-199 supplemented with 2.5% bovine serum albumin (BSA) plus 10% goat follicular fluid (gFF). The matured COCs were then fertilized in BO medium with fresh buck semen. The results showed that the rates of COCs that reached the maximum cumulus cell expansion (level-3) were 64.14, 65.93 and 65.73%; metaphase-II (M-II) stage were 57.75, 58.23 and 58.57%; normal fertilization (formation of male and female pronuclei) were 34.43, 35.03 and 34.65% in puncture, slicing and aspiration techniques, respectively; there were no significant differences among these rates. The results revealed that the collection techniques have no significant effect ($p > 0.05$) on *in vitro* maturation and fertilization of goat oocytes as long as normal quality COCs was used.

Key words: Collection techniques, cumulus oocyte complexes (COCs), *in vitro* maturation (IVM), *in vitro* fertilization (IVF).

INTRODUCTION

Over the last ten to fifteen years, after a dramatic development of cellular biology, a lot of research efforts have been moved towards the implementation of embryo

technologies involving multiple ovulation and embryo transfer (MOET), *in vitro* production (IVP) of embryos, cloning and transgenesis to transfer a targeted number of embryos. Among all, IVP of embryos has become a routine method of producing embryos from abattoir-derived ovaries with minimal cost. Although, goat oocytes can be recovered in relatively large numbers from abattoir ovaries, the oocytes frequently have reduced development potential when compared to *in vivo* matured or immature oocytes collected after gonadotropin

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Abbreviations: COCs, cumulus oocyte complexes; IVM, *in vitro* maturation; IVF, *in vitro* fertilization.

treatment (Cognie et al., 2003). Poor development potential starts with maturation that limits the suitability of these oocytes for research biotechnology and slows the application of *in vitro* embryo production to commercial embryo transfer. The cumulus cells (CCs) surrounding the oocyte plays a key role in oocyte maturation, and they are known to supply nutrients, energy substrates (Sutton et al., 2003) and/or messenger molecules for the development of oocytes (Buccione et al., 1990) and to mediate the effects of hormones on the cumulus oocyte complexes (COCs) (Zuelke and Brackett, 1990). Cumulus cell concentration is very much dependent on the efficiency of oocyte harvesting. Several methods have been used for harvesting oocytes from slaughterhouse ovaries of farm animals. A number of research works have been conducted to compare the efficiency of the oocyte collection techniques in cattle (Katska, 1984; Lonergan et al., 1991), sheep (Wahid et al., 1992; Wani et al., 2000) and goat (Mogas et al., 1992; Wang et al., 2007). In Bangladesh, few researches have performed IVP of goat embryos where COCs were collected only by aspiration of 2 to 6 mm diameter follicles (Ferdous, 2006; Islam et al., 2007; Mondal et al., 2008). However, no other technique was used for this purpose. Keeping the aforesaid reality in mind, the present research was undertaken to compare the effects of three oocyte collection techniques of puncture, slicing and aspiration on the recovery of efficiency, *in vitro* maturation and fertilization of goat oocytes.

MATERIALS AND METHODS

Collection and processing of ovaries and COCs

Ovaries of goats with unknown reproductive history were collected at local slaughterhouse and kept in collection vial containing 0.9% physiological saline in a thermo flask at 25 to 30°C. The ovaries were trimmed to remove the surrounding tissues and overlying bursa. Each ovary was treated with three washings in Dulbecco's phosphate-buffered saline (DPBS) and two washings in COCs collection medium (DPBS + 4 mg/ml BSA + 50 IU/ml penicillin) as developed by Wani et al. (2000) for ewe. Each ovary was processed individually and the COCs were harvested by one of the following three techniques:

Puncture

Ovaries were placed in a Petri dish (Esbee Biotech, Maharashtra, India) containing 5 ml of COCs collection medium, held with the help of forceps and the whole ovarian surface was punctured with an 18 gauge hypodermic needle.

Slicing

Ovaries were placed in a Petri dish containing 5 ml of the COCs collection medium, held with the help of forceps. Incisions were given along the whole ovarian surface using a scalpel blade.

Aspiration

Visible follicles were aspirated using an 18 gauge hypodermic

needle attached with a sterile 5 ml disposable syringe containing 2 ml harvesting medium. The media along with the collected COCs was then transferred to a 35 mm Petri dish. When collecting the COCs by puncture and slicing methods, the ovary was kept completely dipped in the medium. In all the three techniques, the Petri dishes were kept undisturbed for 5 min, allowing the COCs to settle down. Excess media were taken out by a syringe without disturbing the oocytes at the bottom of the Petri dish. The Petri dishes were then examined under an inverted microscope, and the total number of COCs harvested was counted. The COCs were graded into 4 grades on the basis of cumulus cells and nucleus as described by Khandoker et al. (2001): 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. The grade A and B were considered as normal and grade C and D as abnormal COCs.

Follicular fluid collection and preparation

Follicular fluid was collected from all categories of morphologically healthy surface follicles by aspiration using 10 ml syringe with 19 G needle. Criteria for assessment of follicular health established by Kruip and Dieleman (1982) for bovine ovaries were applied in this experiment to assess goat follicles: (i) Non atretic: uniform bright appearance, extensive and very fine vascularization and no free floating particles in the follicular fluid and (ii) Atretic: loss of translucency, slightly or dull grayish and/or opaque appearance and free-floating particles in follicular fluid. At each collection, fluid from each surface follicle was pooled, centrifuged 2 times at 3000 rpm for 30 min. The supernatant was collected and filtered through a 45 µm millipore filter and heat inactivated at 65°C for 1 h in a water bath, and then stored at -20°C until use.

In vitro maturation (IVM) of COCs

The normal quality COCs were cultured in Tissue Culture Medium-199 (TCM-199: Sigma Chemicals Co., St Louis, MO, USA) supplemented with 2.5% Bovine serum albumin (BSA) (Sigma Chemicals (St Louis, MO, USA) plus 10% goat follicular fluid (gFF) for 27 h. After maturation, the degree of cumulus cell expansion was determined according to Rahman et al. (2004) under microscope at 10x magnification as level-1: indicating less expansion of COCs; level-2: indicating moderate expansion and level-3: indicating marked expansion of cumulus cells with a compact layer or corona radiata. Thereafter, half of the matured COCs from each droplet were taken and denuded from cumulus cells by repeated pipetting. Oocytes were then placed on a glass slide, covered with cover slip, fixed with aceto-ethanol (acetic acid : ethanol, 1:3, V/V), stained with 1% aceto-orcin and examined under an inverted microscope (Olympus, Germany) at high magnification (100x) with emersion oil for germinal vesicle break down (GVBD), metaphase-I (M-I) and metaphase-II (M-II) stage.

In vitro fertilization

The fertilization medium, Brackett and Oliphant (BO) (Crozet et al., 1995) was prepared and its pH was adjusted to 7.8 on the day of use. Semen was collected and the sperm concentration was adjusted at 10⁶/ml. Then, insemination droplets were prepared, covered with paraffin oil (Labo America, Inc., California, USA) and kept in the CO₂ incubator for 4 to 5 h for preincubation. Then the remaining half of the matured COCs (other half was used for nuclear maturation) were transferred to each of the sperm drops and incubated for 5 h in the incubator at 38.5°C with 5% of CO₂ in

Table 1. Effects of collection techniques on number of cumulus-oocyte-complexes (COCs) and types of COCs harvested.

Collection technique	Total number of ovaries	Total number of COCs per ovary	Number of normal COCs per ovary			Number of abnormal COCs per ovary		
			Grade A	Grade B	Total	Grade C	Grade D	Total
Puncture	88	4.22 ^a ±0.14 (431)	1.20 ^b ±0.09 (131)	0.64±0.08 (64)	1.85 ^b ±0.09 (195)	0.83 ^a ±0.08 (82)	1.55 ^a ±0.13 (154)	2.38 ^a ±0.12 (236)
Slicing	88	4.14 ^a ±0.15 (442)	1.24 ^b ±0.07 (139)	0.67±0.09 (67)	1.91 ^b ±0.10 (206)	0.94 ^a ±0.07 (82)	1.28 ^a ±0.13 (154)	2.22 ^a ±0.13 (236)
Aspiration	88	3.28 ^b ±0.13 (354)	2.29 ^a ±0.08 (205)	0.57±0.08 (50)	2.48 ^a ±0.10 (255)	0.63 ^b ±0.08 (65)	0.17 ^b ±0.12 (34)	0.80 ^b ±0.10 (99)

Values are shown in mean±SE; Means with different superscripts within the column differ significantly ($p < 0.05$); Figure in the parenthesis indicates the total number.

humidified air. After 5 h of incubation, all the COCs from each drop were denuded from cumulus cells by repeated pipetting and fixed on a glass slide with aceto-ethanol (acetic acid : ethanol, 1:3 v/v) and stained with 1% aceto-orcein. After drying, the slides were examined at high magnification (100x) with emersion oil to observe pronuclear (PN) formation as- (i) Oocyte with male and female PN, normal fertilization; (ii) Oocyte with one PN, asynchronous PN development/parthenogenetic activation or one PN was obscured by lipid droplets and (iii) Oocyte with more than two PN, polyspermy.

Statistical analysis

Simple analysis of variance (ANOVA) in completely randomized design (CRD) was performed and comparison of means Duncan's multiple range test (DMRT) was applied with the help of statistical analysis system (SAS, 1998).

RESULTS AND DISCUSSION

Effect of collection techniques on COCs recovery

The result of COCs recovery per ovary by three different techniques of puncture, slicing and

aspiration is summarized in Table 1. Total number of 429, 427 and 349 COCs were collected by puncture, slicing and aspiration techniques, respectively, from each of 88 ovaries (Table 1). The results indicate that puncture and slicing yielded a significantly higher ($p < 0.01$) number of total COCs per ovary (4.22 and 4.14, respectively) than that of aspiration (3.28), however, a significantly higher ($p < 0.01$) number of normal COCs per ovary was observed in aspiration (2.48) than those of puncture (1.85) and slicing (1.91) techniques (Table 1). The most commonly practiced methods of COCs recovery in goat are puncture and aspiration of visible follicles and follicular dissection (Wang et al., 2007). In the aspiration technique, COCs were collected from 2 to 6 mm diameter of surface follicles using a hypodermic needle with 10 ml syringe. However, in the case of puncture, the whole ovarian surfaces were punctured by hypodermic needle, and in the case of slicing, incisions were given along the whole ovarian surface using a scalpel blade that is, all sizes of surface follicles were harvested. Thus, the lower number of COCs recovered by the aspiration method in this experiment may be attributed to the presence of some follicles

embedded deeply within the cortex, which can be released by puncture or slicing of the ovary. Ferdous (2006) reported that the numbers of normal COCs were found to be significantly higher ($p < 0.05$) in 2 to 6 mm diameter follicles than others. Moreover, puncture and slicing techniques produce more debris which might interfere with the searching of oocytes under the microscope and also required more washing when compared to aspiration. As a result, a number of COCs were denuded from cumulus cells due to repeated washing and ultimately resulted in a lower number of normal COCs when compared to aspiration at the final observation. The result of this study was comparable with the observation of Wang et al. (2007) who harvested oocytes from ovary of Boer goat by one of the four collection techniques (slicing, puncture, aspiration I and aspiration II). They reported that, slicing and puncture of the ovaries yielded a higher ($p < 0.05$) number of oocytes per ovary (6.3 and 5.8, respectively) when compared to aspiration I (2.9) and aspiration II (3.1). Furthermore, Wani et al. (2000) reported that slicing (9.5 ± 0.4) and puncture (9.5 ± 0.4) yielded significantly ($p < 0.05$) more COCs per ovary than aspiration (6.8 ± 0.3) in sheep but the

Table 2. Macroscopic observation of *in vitro* matured COCs collected by three techniques.

Collection technique	Number of COCs taken into maturation	Cumulus expansion level (%)			Number of COCs subjected to nuclear maturation	Nuclear maturation rate (%)			
		Level-3	Level-2	Level-1		M-II	M-I	GVBD	Others
Puncture	195	64.14±0.51 (124)	23.30±1.12(45)	13.56±1.36(26)	97	57.75±0.87(56)	19.09±0.68(18)	12.11±0.72(12)	11.24±1.19(11)
Slicing	206	65.93±0.49(133)	22.89±1.10(46)	13.21±1.31(27)	103	58.23±0.78(60)	20.29±0.87(21)	10.11±0.69(10)	11.57±1.13(12)
Aspiration	255	65.73±0.52(98)	22.46±1.13(34)	15.61±1.34(23)	127	58.57±0.72(74)	20.17±0.74(25)	12.06±0.76(16)	9.20±1.17(12)

Values are shown in mean±SE; Means with different superscripts within the column differ significantly ($p < 0.05$); Figure in the parenthesis indicates the total number. M-I, metaphase I; M-II, metaphase II; GVBD, germinal break down vesicle.

percentage of good quality oocytes was higher in the aspiration method (64.4%), when compared with the puncture (54.7%) or slicing (54.3%) in ewe lamb which was also in accordance with the results of the present study. In contrast, Shirazi et al. (2005) reported that the number of COCs per ovary for slicing (4.0) and aspiration (3.7) did not differ significantly. This discrepancy might be due to species and size variation of Bengal goat and Iranian ewe. The final observation of this study is that, aspiration of 2 to 6 mm diameter vesicular follicles by an 18 gauge hypodermic needle is the best, simple and efficient way of recovering morphologically normal COCs from slaughterhouse goat ovaries.

Effect of collection techniques on IVM and IVF of COCs

The maturation of COCs was initially measured by

macroscopic observation of cumulus cell expansion level and then confirmed by checking the nuclear maturation and the results were summarized in Table 2. The percentages of marked expansion (level 3) of COCs after 27 h of culture were almost similar ($p > 0.05$) for puncture, slicing and aspiration technique (Table 2). The differences of expansion level 2 and 1 among different collection techniques were also insignificant ($p > 0.05$) (Table 2). In the case of nuclear maturation, it was observed that the maturation rates (metaphase-II stage) of goat oocytes for puncture, slicing and aspiration have no significant variation ($p > 0.05$) (Table 2). An insignificant ($p > 0.05$) variation was also observed among the collection techniques for the oocytes that reached metaphase-I and GVBD stage. The result of IVF (the pronuclear formation) of oocytes collected by three different techniques is presented in Table 3. There was no significant ($p > 0.05$) difference among the collection techniques in the formation

of 2 pronuclei (34.43, 35.03 and 34.65%, respectively); 1 pronucleus (1.98, 0 and 2.31%, respectively) and more than 2 pronuclei (2.06, 0 and 0% for puncture, slicing and aspiration techniques, respectively) as shown in Table 3. It is well established that *in vitro* maturation of oocyte is divided into nuclear and cytoplasmic processes. Nuclear maturation involves resumption of meiosis and progression to the metaphase-II stage. Cytoplasmic maturation encompasses a variety of cellular processes that must be completed for the oocytes to be fertilized and developed into a normal embryo and offspring (Eppig, 1996). The maturation and fertilization rates depend on oocyte quality, sufficiency and efficacy of the media and optimization of incubation condition. In this experiment, only normal quality COCs (Grade A and B) collected by each technique were taken and similar media and condition were used for each technique for maturation and further proceeded to fertilization.

Table 3. *In vitro* fertilization of oocytes collected by different techniques.

Collection technique	Total number of oocyte taken	Pronuclei (PN) formation (%)			
		2 PN	1 PN	>2 PN	No PN
Puncture	98	34.43±0.70 (34)	1.98±0.11 (2)	2.06±0.12 (2)	61.24±0.70 (60)
Slicing	103	35.03±0.89 (36)	0.00 (0)	0.00 (0)	64.92±0.68 (67)
Aspiration	128	34.65±0.97 (44)	2.31±0.10 (3)	0.00 (0)	63.38±0.89 (81)

Values are shown in mean±SE; Means with different superscripts within the column differ significantly ($p < 0.05$); Figure in the parenthesis indicates the total number.

The fact that the maturation and fertilization rates in different collection techniques showed a similar trend (insignificant difference among collection techniques) might be due to this reason. The findings of the present study are comparable with those of cattle (Carolon et al. 1992), sheep (Wani et al., 2000) and goats (Pawshe et al., 1994) and that the maturation and fertilization rate does not depend on the collection techniques.

Conclusion

It could be concluded from the present study that aspiration of 2 to 6 mm diameter follicles is the effective technique for oocyte recovery from slaughterhouse goat ovaries, however, there was no significant ($p > 0.05$) effect of COCs collection techniques on *in vitro* maturation and fertilization in goats.

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