Indian Journal of Animal Sciences **83** (10): 1042–1047, October 2013/Article

Effect of harvesting technique on recovery rate and *in vitro* development of caprine follicular oocytes for *in vitro* procedures*

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Received: 28 February 2013; Accepted: 14 May 2013

ABSTRACT

The present study was aimed at assessing efficacy of 3 harvesting methods on the quantity and quality of oocytes recovered for assisted reproduction procedures in goat. The average total number of oocytes recovered per ovary was significantly higher by slicing (6.54±0.39) and puncture (6.59±0.39) than by the aspiration method (4.09±0.19). However, the percentage of good quality oocytes was higher in the puncture method (71.56%), compared to the aspiration (71.27%) or slicing (61.61%) methods. The oocyte recovery was significantly lower in CL containing ovaries than that of ovaries without CL in aspiration (2.92 vs 4.57), puncture (5.89 vs 6.78) and slicing (5.40 vs 7.02) methods. However, the presence of CL did not affect the oocytes ability to reach the MII stage (75.31% vs 76.67%). The side (right or left) of the ovary not showed any significant effect on mean of total oocyte recovery and other grades of oocytes. However the large sized ovaries were yielded significantly higher number of oocytes than smaller ovaries. The results showed that the rates of COCs that reached the metaphase-II (M-II) stage were 79.88, 78.09 and 72.63% in aspiration, puncture and slicing techniques, respectively. The maturation rate was significantly lower in slicing method. The oocytes obtained were matured and parthenogenetically activated *in vitro* using ionomycin and 6-DMAP. There was no significant difference in the subsequent percentage of cleavage and development to blastocyst stage *in vitro* between the three methods of oocyte harvesting. It was concluded that though oocyte recovery, and *in vitro* developmental rates did not vary significantly between puncture and slicing methods, yet puncture method was found to be superior due to low debris content and recovery of more number of culture grade oocytes. Oocyte recovery by aspiration from small sized ovaries was difficult due to less number of visible follicles. Puncture method can be used as an alternative to slicing and aspiration for oocyte recovery in goat.

Key words: Aspiration, Goat, IVM, Oocytes, Parthenogenetic activation, Puncture, Slicing

The *in vitro* embryo production (IVEP) emerged as an alternative to the *in vivo* embryo production by superovulation, also known as conventional embryo transfer (ET). Conventional embryo production by superovulation and surgical collection has limited application in goat. Alternatively embryos can be produced by oocytes collected from slaughter house ovaries (Walker *et al.* 1994) or by laparoscopic aspiration (Tervit 1996) followed by *in vitro* maturation and fertilization (IVM-IVF) procedures. *In vitro* embryo production (IVEP) system has been successfully applied in a number of domestic species. The application of IVEP can be expected to bring about a significant increase in the population of superior genetic merit animals. The production of transgenic goats that produce milk containing proteins of pharmacological value is of special interest. The availability of a sufficient number of oocytes and their quality is the pre-requisite for IVEP procedure such as IVM-IVF, transgenics and somatic cell nuclear transfer (SCNT). Oocyte quality is one of the major factors determining the success of *in vitro* embryo production (Krisher 2004, Blanco *et al.* 2011). The reduced development potential of oocytes collected from the abattoir ovaries 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 surrounding the oocyte plays a key role in oocyte maturation, and they are known to supply nutrients, energy substrates (Krisher 2004) 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

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Brackett 1990). Cumulus cell investment is very much dependent on the efficiency of oocyte harvesting method. Ovaries collected from abattoir animals are the cheapest and the most abundant source of primary oocytes for *in vitro* culture (Agrawal 1992). However, the oocyte yield and quality varies with the harvesting technique employed and also depends on the reproductive status of the donor, season, species and breed (Nandi and Kumar 2008). Aspiration, puncture and dissection of ovaries were tried to harvest follicular oocytes with varying degree of success in sheep (Wani *et al.* 1999, Sofi *et al.* 2012), goat (Pawshe *et al.* 1994, Wang *et al.* 2007, Yadav *et al.* 2007, Majeed *et al.* 2011, Hoque *et al.* 2011), cattle (Katska 1984) and buffalo (Jamil *et al.* 2008, Mehmood *et al.* 2011). The present study was under taken to asses the comparative efficacy of different methods of oocyte collection, viz. follicular aspiration, puncture and slicing of ovarian surface and to evaluate retrospectively the influence of various factors like ovarian size, side of the ovary and presence or absence of corpus luteum on the quantity and quality of oocytes recovered per ovary. Also to study the influence of various oocyte collection methods on *in vitro* development of oocytes collected from goat ovaries.

MATERIALS AND METHODS

Preparation of media: HEPES-buffered tissue culture medium 199 supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) was used for washing and handling of cumulus oocyte complexes (COCs). IVM of oocytes was carried out using IVM medium that consisted of bicarbonate-buffered tissue culture medium 199 supplemented with 10% FBS, 0.22 mM sodium pyruvate, 10 µg/ml of follicle-stimulating hormone, 6 IU/ml luteinizing hormone, 1.0 µg/ml 17-ß estradiol, 100 IU/ml penicillin and 0.1mg/ml streptomycin. All media were sterilized by filtration through 0.22 µm filter and equilibrated in a humidified atmosphere with 5% carbon dioxide at 38.5°C for at least 2 h prior to use.

Oocyte collection and classification: Goat reproductive tracts were obtained from a local slaughter house and the right and left ovaries were collected and transported separately in a flask containing 0.9% normal saline at room temperature within 2 h to the laboratory. Each ovary was examined for the presence of visible follicles and corpora lutea. Ovarian dimensions (length, width and thickness) were measured with vernier calipers. Ovaries were rinsed once in 70% alcohol and thrice in pre-warmed D-PBS. The ovaries were then placed in oocyte collection medium (D-PBS) supplemented with 0.4% BSA, gentamycin sulphate 50mg/ml and 10 IU/ml heparin). Each ovary was processed individually and oocytes were collected by one of the following methods.

Aspiration of visible follicles (2–6 mm diameter) on the ovaries using a 18 gauze needle attached to sterile plastic syringe containing 2 ml oocyte collection medium (OCM). Aspirated follicular fluid was then transferred to a sterile 35 mm petridish.

Puncture of whole ovarian surface by a sterile 18 gauze hypodermic needle while the ovary is held completely sub merged in OCM in a 30 mm petridish.

Slicing of ovaries – in this method a hemostat was attached to the base of the ovary to hold it firmly in place and 2–3 mm deep incisions were made across the whole ovarian surface using sterile scalpel blade. The ovary was swirled vigorously in petridish containing OCM.

The petri dish was kept undisturbed for 5 min to allow the COCs to settle. The oocytes were separated into a 35 mm petridish for grading at 63× magnification and then graded as good, fair and poor quality based on their cumulus corona investment, compactness and homogeneity of ooplasm (Pawshe *et al.* 1994). The number and quality of oocytes were recorded for each ovary.

IVM of cumulus oocyte complexes (COCs): After isolation and classification of COCs, good and fair quality COCs was selected for IVM. All COCs were washed extensively in fresh droplets of handling medium followed by IVM medium. The washed oocytes were finally transferred into a 4-well plate containing 600 µl of pre-equilibrated IVM medium. The medium was overlaid with equilibrated mineral oil and cultured in an incubator with 5% CO_2 under humidified air at 38.5°C for 27 h.

Evaluation of oocytes following IVM: At the end of the IVM, COCs were examined for cumulus cell expansion. Subsequently, the oocytes were denuded of the cumulus cells by treating with hyaluronidase (100 IU/ml) for 10 min and repeatedly passing through a narrow bore glass pipette and examined for extrusion of the first polar body and were evaluated for nuclear status according to Otoi *et al.* (2002). The oocytes were then examined using a fluorescence microscope and classified according to chromatin configuration as germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase-I (MI), telophase-I (TI), and metaphase-II (MII). Those with diffusely stained cytoplasm characteristic of non-viable cells and those in which the chromatin was unidentifiable or not visible, were considered as degenerated. Meiotic maturation was defined as the number of oocytes that extruded a first PB and matured to the TI/MII stage relative to the total number of oocytes that were cultured *in vitro*. Oocytes that were arrested at the GV, GVBD stage or only progressed to MI were considered as immature.

Activation of oocytes: The oocytes that cultured *in vitro* for 27 h were activated using a combination of 5 µM ionomycin for 5 min and 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h in KSOM medium. Following six washes in KSOM medium oocytes were incubated in KSOM medium in a humidified atmosphere of 5% CO_2 incubator at 38.5 °C for 7days for further development.

Statistical analysis: Oocyte recovery rates using different collection techniques were analyzed by analysis of variance (ANOVA) and effect of various factors on oocytes recovery was analyzed using Student's t-test. For *in vitro* maturation, the percentage data were arcsine transformed and analyzed by ANOVA and followed by a least square difference (LSD) test using SPSS software version 16.0. Differences in means were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The mean length, with, thickness of the 620 abattoir derived goat ovaries were noted to be 1.06±0.01 cm, 0.76±0.00 cm and 0.58±0.00 cm respectively. No significant differences ($P > 0.05$) were observed in respect of these parameters between right and left ovaries. Similarly, the mean number of visible follicles does not vary between left and right ovaries (3.66 vs. 3.70). The mean number of visible follicles in goat ovaries was observed to be 3.68±0.12 (range 0 –13).

Effect of collection method on oocyte recovery: Ovaries (620) were individually processed for collection of oocytes by aspiration (n=205), puncture (n=206) and slicing (n=209) methods, with a mean total oocyte recovery of 4.09±0.19, 6.54±0.39 and 6.59±0.34 respectively (Table 1). The mean number of total oocytes (good, fair and poor quality) recovered was significantly ($P<0.05$) lower in aspiration than puncture and slicing methods. However, there was no significant difference between puncture and slicing methods in the mean number of oocytes collected. The number of good quality oocytes obtained per ovary was significantly higher in puncture method (3.06±0.19) than slicing (2.25±0.12) and aspiration (1.80±0.10). The proportion of culture grade (good and fair quality) oocytes recovered by aspiration and puncture method were 71.27% and 71.56% were significantly higher ($P<0.05$) than slicing method (61.61%) and poor oocyte recovery was lowest in aspiration and highest in slicing method of oocyte collection.

In present study puncture and slicing yielded similar numbers of oocytes, while being significantly ($P < 0.05$) more than the aspiration method. These findings were similar to earlier reports in goat (Wang *et al.* 2007, Hoque *et al*. 2011) and sheep (Wani *et al.* 1999, Sofi *et al.* 2012). In accordance with the results of the present study, Wang *et al.* (2007) reported that slicing (6.3) and puncture (5.8) techniques yielded significantly ($P<0.05$) more oocytes per ovary than follicle aspiration (2.9 and 3.1) in Boer goats. In contrast to the results of the present study, however, the number of oocytes per ovary for slicing (1.51) was significantly lower than aspiration (2.28) in Black Iraqui goats (Majeed *et al.* 2011). The number of oocytes retrieved in this study by puncture method was similar to the reports of Wang *et al.* (2007), higher than the reports of Sarkhel *et al.* (1997) and Hoque *et al.* (2011) in goats in which 2.54 and 4.22 oocytes recovered respectively. However, in present study the recovery of oocytes using puncture method was lower than that of Agrawal *et al.* (1992) in goats (12.4±4.6) and Wani *et al.* (1999) in sheep (9.5±0.4). The low oocyte recovery by aspiration compared to slicing and puncture methods might be due to small size of ovaries in local goats and less number of visible follicles and/or the presence of some follicles embedded deeply within the cortex, which cannot be recovered by the aspiration method. In present study, the proportion of poor quality oocytes (38.39%) was higher in slicing method than that of aspiration and puncture methods (28.72 and 28.43% respectively). Moreover, slicing technique produce more debris which might interfere with the searching of oocytes and also required more washing when compared to other methods. 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 (Hoque *et al*. 2011) when compared to aspiration and puncture. Further more it is known that the number of oocytes recovered depends on the species, age, season and reproductive status of the donor animal (Islam *et al.* 2007).

Influence of various factors on oocyte recovery: The effect of side and size of the ovary and the presence or absence of corpus luteum (CL) on the total, good, fair and poor quality oocytes recovered per ovary was given in Table 2. The oocyte recovery from right and left ovaries did not showed any significant differences among different collection techniques. The ovaries were divided into two groups based on their size: (a) ≥1.0 cm length (small) and (b) >1.0 cm (large). The data presented in Table 2 revealed that the small ovaries yielded significantly ($P<0.05$) lower number of oocytes/ovary than large ovaries by the aspiration and puncture method. No significant difference was observed in the number and type

Table 1. Effect of different harvesting techniques on the quantity and quality of oocytes recovered from goat ovaries

Method (number of ovaries)	Total number of oocytes	Oocytes per ovary (mean±SE)*				
		Total	Good (%)	Fair (%)	Poor (%)	Culture grade (%)
Aspiration (n=205)	839	4.09±0.19^{a}	1.80±0.10^{a} (44.10)	1.11±0.06^{a} (27.17)	1.18±0.07^{a} (28.72)	2.91±0.14^{a}(71.27)
Puncture (n=206)	1347	6.54±0.39^{b}	3.06±0.19^{b} (46.77)	1.62±0.12^{b} (24.79)	1.86±0.13^{b} (28.43)	4.68±0.29^{b}(71.56)
Slicing (n=209)	1378	6.59±0.34^{b}	2.25±0.12^{c} (34.10)	1.81±0.13^{b} (27.50)	2.53±0.16^{c}(38.39)	4.06±0.21^{b}(61.61)
Overall (n=620)	3564	5.74±0.19	2.37±0.08	1.52±0.06	1.86±0.07	3.89±0.13

*Mean values in the same column with different superscripts differ significantly at $P<0.05$.

Table 2. Effect of various factors on oocyte recovery from goat ovaries

Attributes	Oocytes per ovary (mean±SE)*										
	Aspiration				Puncture				Slicing			
	Total (no. of oocytes)	Good (%)	Fair (%)	Poor (%)	Total (no. of oocyte)	Good (%)	Fair (%)	Poor (%)	Total (no. of oocyte)	Good (%)	Fair (%)	Poor (%)
Side of the ovary												
Right	4.12±0.22^{a} (n = 425)	1.80±0.13^{a} (43.53)	1.14±0.08^{a} (27.76)	1.18±0.09^{a} (28.71)	6.59±0.52^{a} (n = 679)	2.87±0.21^{a} (43.59)	1.67±0.17^{a} (25.33)	2.05±0.20^{a} (31.08)	6.62±0.55^{a} (n = 688)	2.24±0.19^{a} (33.87)	1.79±0.20^{a} (27.03)	2.59±0.28^{a} (39.10)
Left	4.06±0.31^{a} (n = 414)	1.81±0.15^{a} (44.69)	1.08±0.10^{a} (26.57)	1.17±0.11^{a} (28.74)	6.48±0.58^{a} (n = 668)	3.24±0.32^{a} (50.00)	1.57±0.16^{a} (24.25)	1.67±0.15^{a} (25.75)	6.57±0.39^{a} (n = 690)	2.26±0.17^{a} (34.35)	1.84±0.16^{a} (27.97)	2.47±0.16^{a} (37.68)
Size of the ovary												
Length ≥1.0 cm	2.67±0.18^{a} (n = 200)	1.23±0.10^{a} (46.00)	0.64±0.07^{a} (24.00)	0.80±0.08^{a} (30.00)	4.02±0.32^{a} (n = 370)	1.91±0.15^{a} (47.57)	0.94±0.10^{a} (23.51)	1.16±0.12^{a} (28.92)	6.02±0.39^{a} (n = 530)	2.27±0.20^{a} (37.74)	1.44±0.12^{a} (23.96)	2.31±0.12^{a} (38.30)
Length >1.0 cm	4.91±0.27^{b} (n = 639)	2.14±0.14^{b} (43.51)	1.38±0.08^{b} (28.17)	1.39±0.10^{b} (28.32)	8.57±0.60^{b} (n = 977)	3.98±0.30^{b} (46.47)	2.17±0.18^{b} (25.28)	2.42±0.19^{b} (28.25)	7.00±0.50^{a} (n = 848)	2.23±0.16^{a} (31.84)	2.08±0.20^{a} (29.72)	2.69±0.24^{a} (38.44)
CL presence or absence												
With CL	2.92±0.22^{a} (n = 175)	1.35±0.12^{a} (46.29)	0.82±0.09^{a} (28.00)	0.75±0.08^{a} (25.71)	5.89±0.53^{a} (n = 330)	2.69±0.26^{a} (45.76)	1.50±0.17^{a} (25.45)	1.69±0.21^{a} (28.79)	5.40±0.42^{a} (n = 297)	1.87±0.16^{a} (34.68)	1.40±0.16^{a} (25.93)	2.12±0.17^{a} (39.39)
Without CL	4.57±0.25^{b} (n = 664)	1.99±0.13^{b} (43.52)	1.23±0.08^{b} (26.96)	1.35±0.09^{b} (27.94)	6.78±0.50^{b} (38.11)	3.19±0.25^{b} (29.52)	1.67±0.15^{a} (n = 1017)	1.92±0.15^{a} (47.10)	7.02±0.43^{b} (24.58)	2.38±0.16^{b} (28.32)	1.96±1.17^{b} (n = 1081)	2.67±0.21^{b} (33.95)

*Mean values within an attribute in the same column with different superscripts differ significantly at P<0.05.

of oocytes collected by the slicing technique of harvesting. Ovaries with a CL yielded significantly (P<0.05) lower number of oocytes than ovaries without a corpus luteum (Table 2) irrespective to collection method employed.

In present study, the side of the ovary did not influence oocyte recovery and it was in agreement to the earlier report in goat (Islam *et al.* 2007). The larger ovaries yielded significantly higher number of oocytes than smaller ovaries. It is known that large ovaries are expected to yield a higher number of oocytes because of large surface area which facilitates the presence of more follicles (Wani *et al.* 1999). In this study, it was observed that the presence of a CL adversely affected the number of oocytes recovered and in agreement with the finding of Islam *et al.* (2007) in goats and Wani *et al.* (1999) in sheep. The cause of a low number of oocytes per ovary with a CL may be attributed to the fact that a CL inhibits the growth of follicles and increases their atresia (Hafez 1993). However, in bovine species oocyte yield per ovary was higher for ovaries with a CL than without a CL (Varisanga *et al.* 1998). The reason could be that the regressing/regressed CL is likely to produce low level of progesterone and it may not be sufficient to inhibit growing follicles in the ovary (Rao *et al.* 2010). Although the influence of presence or absence of CL was reflected in mean number of oocytes collected, no significant difference was observed in maturation rates of oocytes recovered from ovaries with and without CL. These finding were in agreement with the reports of Singh *et al.* (2001) in buffalo, Vajta *et al.* (1992) in cattle indicating that pregnancy does not affect the meiotic competence of oocytes. However, our findings were in contrary to reports of Tasripoo and Kamonpatana (1997) recorded higher maturation rate of oocytes recovered from CL containing ovaries.

In vitro maturation and development of immature oocytes: Oocytes recovered by aspiration (n=360), puncture (n=411) and slicing (n=405) methods were matured *in vitro* for 27 h and their nuclear maturation evaluated. The cumulus expansion rate (CCE) of culture grade (good and fair quality) COCs collected by aspiration, puncture and slicing methods were 97.4±1.2%, 97.1±0.9% and 96.9±1.6% respectively. The CCE rate was similar among different collection methods. The oocytes showing extrusion of 1st polar body was significantly lower in slicing method (59.9±1.7%) than that of aspiration (64.1±0.7%) and puncture (63.6±1.0%) methods. The maturation rate of oocytes collected by aspiration, puncture and slicing was 79.88%, 78.09% and 72.63% respectively. There was a statistically lower (P < 0.05) proportion of MII stage after *in vitro* maturation of the oocytes collected by slicing method, compared to the other two collection techniques. No significant difference was observed between puncture, and aspiration in the percentage of MII oocytes. A total of 403 IVM oocytes from aspiration (126), puncture (121) and slicing (156) methods were parthenogenetically activated. The cleavage rate and the

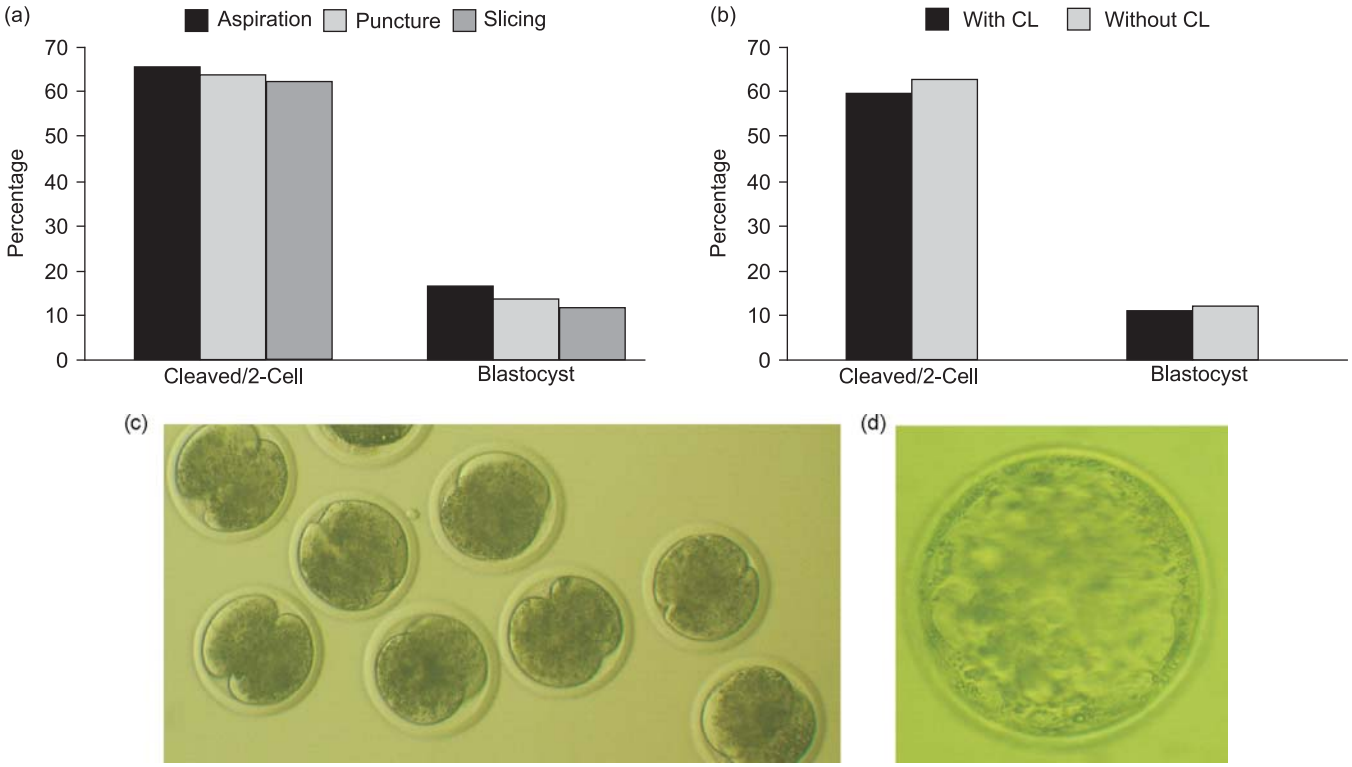

Fig. 1. *In vitro* development of goat oocytes after parthenogenetic activation; (a) effect of different oocyte collection methods (b) influence of presence or absence of CL on cleavage and blastocyst rate after activation, (c) cleaved oocytes/2-cell embryos, (d) blastocyst.

proportion of blastocysts (Fig 1) were not significantly different between aspiration (65.07 and 16.66%), puncture (63.63 and 14.05%) and slicing (62.17 and 12. 18%) groups respectively.

Oocytes (299) collected from ovaries with (n = 149) and without CL (n = 150) were subjected for IVM. Although the effect of presence or absence of CL was reflected in number of oocytes recovered, no significant difference (P>0.05) was observed in the ability to reach MII stage of oocytes in between luteal ovaries (75.31±1.82) and non-luteal ovaries (76.68±0.63). A non significant (P>0.05) variation was also observed among these groups for the oocytes that reached MI (11.7 vs. 14.3%) and GVBD stage (4.0 vs. 5.8%). A total of 64 and 74 oocytes from with and without CL groups respectively, were parthenogenetically activated. The cleavage (59.37 and 62.16%) and blastocyst rates (10.93 and 12.16%) were similar in with and without CL groups (Fig. 1) respectively.

The culture grade oocytes obtained by each method was kept for IVM/IVC procedure, there was a significantly lower (P < 0.05) rate of oocytes reaching the MII stage when using slicing techniques compared to the aspiration and puncture techniques. Wang *et al.* (2007) reported the maturation rate of 84.0 and 68.4% of oocytes in goats collected by puncture and slicing technique which is in close agreement with our results. In pre-pubertal goats (Martino *et al.* 1994), the maturation rate obtained for slicing (56.8%) was lower than that obtained for dissection and aspiration (69.3 and 72.0%, respectively), which was in accordance with the present results. However, our results are in contrast to the findings of Hoque *et al.* (2011) and Wani *et al.* (2000), who found no significant difference in the maturation rate of oocytes in goat and sheep collected by different collection methods. The lower maturation rate may be due to more pre-antral oocytes collected by slicing method than by puncture as pre-antral oocytes have low maturation rate compared with antral oocytes (Izquierdo *et al.* 2002). In present study, different collection methods had the similar cleavage rate and blastocyst yield of the embryos after parthenogenetic activation of the IVM oocytes. These findings were in agreement with the reports of Wang *et al.* (2007) where they reported 13.3, 13.5 and 13.8% blastocyst rate in IVM oocytes collected by aspiration, puncture and slicing techniques respectively.

In summary, present study concluded that slicing and puncture are better oocyte collection methods from ovaries of abattoir origin in caprine. Although, slicing method yields the highest number of oocytes recovered per ovary of abattoir origin but the puncture method is more preferable oocyte collection technique as it has the advantage that it produces less debris in the collection medium and makes oocyte collection and examination easy compared to slicing

technique and gives higher maturation rate compared to slicing technique. No significant difference was found in cleavage rates and blastocyst rates of oocytes obtained by the three different techniques. However, puncture of ovarian surface by an 18 gauge hypodermic needle was found to be simple practical and more efficient method for collecting oocytes in caprine, compared to the slicing method. Hence, puncture method can be used as an alternative to aspiration or slicing for harvesting of oocytes from goat ovaries.

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