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Effect of follicular size on *in vitro* maturation, fertilization and culture of sheep embryos

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Summary

A study was conducted to investigate the effect of follicular size (small, <2 mm diameter and large, ≥2 mm) on *in vitro* maturation, fertilization and culture of ovine oocytes. The ovaries (n=80) were collected from abattoirs in Srinagar city, India and transported in normal saline solution containing 50 µg gentamicin sulphate per ml in a thermos flask. The oocytes were collected by puncture technique and good and fair quality oocytes obtained were subjected to maturation in TCM-199 supplemented with epidermal growth factor (EGF) for 24 h under 5% CO_2 and humidified atmosphere at 38.5°C. The matured oocytes were then subsequently subjected to fertilization and culturing was done for six days. The results revealed that the maturation rate of large follicle oocytes (85.20%) was higher than small follicle oocytes (52.44%, P=0.002). *In vitro* fertilization rates for small and large follicle oocytes were not significantly different (79.54 and 76.65%, respectively). However, cleavage and morula rates were higher in large follicle oocytes (58.69 and 34.13%) than small follicle oocytes (38.63 and 19.32%, respectively, P<0.05). In conclusion, there is higher developmental competence of oocytes from the large follicles than small follicles and the pre-maturation of oocyte occurring during follicular growth is essential for subsequent embryonic development in sheep.

Key words: Cleavage, Follicular size, *In vitro* maturation, *In vitro* fertilization, Sheep

Introduction

After artificial insemination and multiple ovulation and embryo transfer (MOET), *in vitro* production of embryos (IVP) represents the third generation of techniques aimed at a better control of animal reproduction. This technique involves four major steps: oocyte collection, oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* development (IVD) of the resulting embryos (Mermillod *et al*., 2006). These different steps are now well established in domestic ruminant species (cattle, sheep and goat) although the variability of the number and quality of the oocytes collected and the low viability of frozen-thawed *in vitro* produced embryos still limit the large-scale use of this promising technology (Mermillod *et al*., 2006). Beyond the potential use of IVP in breeding schemes, this technique is also required for the establishment of new biotechnologies such as cloning and animal transgenesis (Baldassarre *et al*., 2002). Additionally, the knowledge of oocyte and embryo physiology acquired through IVP techniques may stimulate the further development of other techniques such as MOET. Oocytes that are collected from growing follicles for IVP are blocked at the prophase stage of meiosis I. As soon as they are removed from the follicular inhibitory environment, meiotic resumption occurs and

progress to metaphase II (Gilchrist and Thompson, 2007) which is the basis of *in vitro* maturation. Although a large number of oocytes are present in the ovaries, only meiotically competent oocytes can be used for *in vitro* maturation and consequently the number of embryos that can be produced *in vitro* from a particular donor is limited. However, incompetent oocytes from early antral or pre-antral follicles might be used in the future for livestock production, if adequate systems to grow them *in vitro* are developed. Follicle size from which the oocyte is collected influences the oocytes ability to resume meiosis and reach optimum maturation during IVM of oocytes because as antral follicles progress towards ovulatory size, the synthetic activity of oocyte is gradually reduced until the oocyte reaches a quiescent state termed as "oocyte capacitation" (Hyttel *et al*., 1997). Hence, removing oocytes from mid-sized antral follicles for IVM interrupts the process of oocytes capacitation and spontaneous oocyte maturation *in vitro* then occurs in the absence of certain crucial oocyte cytoplasmic events and components that are required for complete developmental competence of oocytes (Gilchrist and Thompson, 2007). Follicles measuring >3 mm diameter have more cumulus cell layers and show better *in vitro* maturation results and, as follicle size increases, oocyte complete their growth, achieve meiotic competence and therefore give better *in vitro* maturation rate and embryo production yield (Martino *et al*., 1994). Many studies have shown that oocytes collected from large diameter follicles are more likely to develop to the blastocyst stage than oocytes collected from small diameter follicles (Hendriksen *et al*., 2000), hence pre-maturation of an oocyte occurring during follicular growth is essential for subsequent development. Although some oocytes collected from small and large follicles are already competent to develop *in vitro* into a blastocyst that can result in vital offspring after transfer, additional but essential development occurs *in vivo* during subsequent follicular growth and this final maturation process strongly affects the developmental competence of oocytes (Dieleman *et al*., 2002). Meiotic competence of oocytes is acquired in late pre-antral follicles in mice (Sorensen and Wasserman, 1976) and in antral follicles between 0.5 and 2-3 mm in diameter in goats (De Smedt *et al*., 1994). Therefore, the present study was conducted keeping in mind its importance besides having meager literature in this aspect of work in sheep.

Materials and Methods

Collection of ovaries

Sheep ovaries were collected from local abattoirs during December, 2010 to July, 2011 and transported within 1-2 h of slaughter to the laboratory in warm normal saline solution supplemented with 50 µg per ml gentamicin sulphate (Sigma, Steinheim, Germany) in a thermos flask. Upon arrival at the laboratory, extraneous tissues were removed from the ovaries and washed with 70% ethanol to control contamination.

Harvesting and grading of oocytes

All visible antral follicles on the surface of ovary were recorded for each ovary and the size was measured with Digital caliper. The oocytes were collected from small (<2 mm) and large (≥2 mm) diameter follicles separately in Dulbecco's phosphate buffer saline (DPBS; SAFC, Bioscience, Kansas, USA), containing 50 µg per ml gentamicin sulphate by puncture technique using 18 G needle and keeping the ovary completely dipped in the medium in a petri dish (35 mm). The petri dishes were kept undisturbed for 1-2 min; excess medium was taken out with a pipette gently and the oocytes were observed under stereo zoom microscope. Total number of oocytes were recorded for each ovary and their grading was done according to the appearance of cumulus cells (Pawshe *et al*., 1994). The oocytes were categorized into good (oocytes with many complete layers of cumulus cells and uniform cytoplasm), pair (oocytes with thin or incomplete layers of cumulus cells and uniform cytoplasm) and poor (oocytes with few or no cumulus cells). Only usable good and fair oocytes were used for IVM.

In vitro maturation (IVM) of the oocytes

Good and fair oocytes obtained were

subjected to maturation in TCM-199 (Invitrogen; NY, USA) supplemented with 10 percent FCS (CAMBREX Bio Science, USA) + 10 µg FSH (Sigma-Aldrich Co., St. Louis, USA) per ml + 20 ng EGF (Invitrogen, Oregon, USA) per ml and 50 µg gentamicin sulphate per ml. The usable oocytes obtained were washed twice in the maturation medium before they were subjected to maturation. The maturation medium was equilibrated for 2 h in a CO_2 incubator, before the oocytes were added. The usable oocytes were randomly distributed in maturation droplets of 100 µl (10-15 oocytes in 100 µl) and covered by sterile paraffin oil. The oocytes were cultured in 5% CO_2 in humidified air at 38.5°C in CO_2 incubator for 24 h.

Evaluation of maturation rate of the oocytes

At the end of the incubation period, the oocytes were evaluated for maturation status based on the degree of cumulus cell expansion as per Kobayashi *et al*. (1994) and categorized into the following classes:

Class 0 (D0): No expansion of cumulus cells was seen

Class 1 (D1): Cumulus cells homogenously spread and clustered cells were still present

Class 2 (D2): Cumulus cells homogenously spread and clustered cells were no longer present

The degree of cumulus cell (CC) expansion was evaluated using an inverted microscope (×200 magnification) and maturation rate of the oocytes in media was studied.

Sperm collection and capacitation and oocyte preparation

Sheep testes were obtained from the local abattoirs in warm normal saline supplemented with 50 µg per ml gentamicin sulphate and were taken to laboratory in a thermos flask at room temperature. After washing thoroughly the excess tissues were trimmed and the cauda epididymes isolated from testes. The spermatozoa were collected from two to three cauda epididymis by incision technique and were washed twice in capacitation medium by centrifugation at 2000 rpm for 10 min at room temperature. After discarding the supernatant the sperm pellet was resuspended in capacitation medium and washed again. About 100 µl of loose sperm pellet was overlaid with 2 ml of medium and kept in CO_2 incubator (38.5°C) at an angle of 45° for 1-2 h. The motile sperms were obtained by "swim up" method as described by Parrish *et al*. (1986). The capacitation medium consisted of TCM-199 supplemented with 50 IU heparin and 50 µg gentamicin sulphate per ml.

In vitro fertilization and culture

The matured oocytes with Class 2 (D2) cumulus cell expansion were washed twice in fertilization medium and then transferred to 1 ml of fertilization medium pre-equilibrated at 5% CO_2. The fertilization medium was the same as the capacitation medium. The capacitated spermatozoa were then added to the matured oocytes at the concentration of 1-2 × 10^6/ml. The mixture of gametes was incubated at 5% CO_2 level at 38.5°C in humidified air for 18 to 22 h under paraffin oil. The oocytes were removed from the fertilization medium after 18-22 h and cumulus cells were removed from the oocyte by vortexing for 1-2 min. After washing, the denuded oocytes were examined under inverted microscope for fertilization and fertilized oocytes were transferred into culture medium. The fertilization rate was evaluated based on pro-nucleus formation, presence of either sperm head or tail in the vitellus, emission of second polar body or cleavage.

The zygotes were washed three times before suspending them in the culture medium. Ten to fifteen presumptive zygotes in 1 ml of the culture medium were cultured for 6 days at 38.5°C and 5% CO_2 under humidified atmosphere in a 35 mm petri dish. The culture medium consisted of TCM-199 + 10% FCS (CAMBREX Bio Science, USA) + 50 µg gentamicin sulphate per ml. The medium was changed every 48 h and the embryos were evaluated for morphological stage of development under inverted microscope at 24 h interval.

Statistical analysis

The data obtained was analysed using Student's t-test and the arcsine transformed

data of the proportion of oocytes reaching D2 stage, fertilized or cleaved were compared by ANOVA using SPSS-17 software (SPSS Corporation, USA). The level of significance was set at P<0.05.

Results

A total of 80 ovaries were collected from the local slaughter houses and large (≥2 mm diameter) and small (<2 mm diameter) follicles present on ovarian surface were recorded for each ovary. The recovery rate of small and large follicle oocytes from the ovaries is given in Table 1. The number of large follicles per ovary was significantly (P<0.05) higher than the number of small follicles per ovary. The good quality and usable oocytes from large follicles were significantly (P<0.05) higher (1.62 ± 0.03 and 2.45 ± 0.04) than from the small follicles (1.21 ± 0.024 and 2.11 ± 0.04). However, the total and poor quality oocytes recovered from small follicles were significantly (P<0.05) higher (3.09 ± 0.04 and 0.98 ± 0.01, respectively) than the oocytes from large follicles (2.79 ± 0.04 and 0.34 ± 0.02, respectively). The usable oocytes from the small and large follicles were subjected to the maturation separately and the results were recorded. The maturation rates of oocytes from small and large follicles are presented in Table 2. The maturation percentage of large follicle oocytes (85.20%) was found to be significantly (P<0.05) higher than the small follicle oocytes (52.44%). The number of matured oocytes selected for fertilization was 88 and 167 from small and large follicles, respectively. The results of the fertilization, cleavage and morula development are presented in Table 3. The fertilization rates in both the groups were statistically non-significant. The cleavage percentage in small follicle oocytes was significantly lower (36.63%) than the large follicle oocytes (58.69%) and similar trend was observed in the morula development rates (Table 3).

Discussion

In vitro embryo development is known to be highly affected by oocytes quality. In the present study, the fertilization rates observed were 79.54 and 76.65% from small and large follicle oocytes, respectively which were not significantly different. After *in vitro* fertilization, the developmental competence of oocytes dependently increased with follicular size (Qian *et al*., 2001). The cleavage rates recorded were 38.63 and 58.69% in small and large follicle oocytes, respectively and were significantly different from each other. Similar trend was observed in morula percentages, which were observed as 19.32 and 34.13% and were significantly (P<0.05) varied between small and large follicle oocytes, respectively. Earlier studies have shown that the rate of embryonic development is greater for large follicle oocytes than for small follicle oocytes in bovine (Pavlok *et al*., 1992; Lonergan *et al*., 1994; Lequarre *et al*., 2005) and in camel (Khatir *et al*., 2007).

The present results support the hypothesis that there is higher develop-mental competence of oocytes from the

Table 1: Recovery rate of oocytes collected from small and large follicles of sheep ovaries (Mean±SEM)

Follicle size (n=80)	Follicles per ovary	No. of oocytes recovered per ovary				
		Good	Fair	Poor	Total	Usable
Small (<2 mm)	4.06±0.08[a]	1.21±0.02[a]	0.90±0.02[a]	0.98±0.01[a]	3.09±0.04[a]	2.11±0.04[a]
Large (≥2 mm)	3.40±0.06[b]	1.62±0.03[b]	0.83±0.02[a]	0.34±0.02[b]	2.79±0.04[b]	2.45±0.04[b]

Means within the columns with different superscripts differ significantly (P<0.05)

Table 2: IVM rate of oocytes collected from small and large follicles of sheep ovaries

Follicle size	Total oocytes (n)	Degree of cumulus cell expansion (%)		
		Degree 0	Degree 1	Degree 2 (mature)
Small follicle oocytes	169	16 (9.23[a])	65 (38.33[a])	88 (52.44[a])
Large follicle oocytes	196	6 (3.06[b])	23 (11.73[b])	167 (85.20[b])

Percentages with in same column with different superscript differ significantly (P<0.05)

Table 3: Effect of follicle size on *in vitro* fertilization and embryonic development in sheep

Follicle size	No. of IVM oocytes (n)	Fertilized (%)	Cleaved (%)	Morula (%)
Small follicles (<2 mm)	88	70 (79.54[a])	34 (38.63[a])	17 (19.32[a])
Large follicle (≥2 mm)	167	128 (76.65[a])	98 (58.69[b])	57 (34.13[b])

Percentages with-in the same column with different superscript differ significantly (P<0.05)

large follicles than small follicles in sheep. There is continued development and maturation of the oocytes within the ovarian follicle *in vivo*, which facilitates the production of oocytes of the highest developmental potential (Zheng *et al*., 2005). There are intricate temporal changes taking place inside and outside the cell *in vivo* which are unknown or poorly understood and *in vitro* conditions may not support this process as effectively due to differences in the extracellular milieu. The developmental competence of oocytes is acquired late during the final phase of follicular development (Khatir *et al*., 2007) and hence the small follicle oocytes might be showing poor results of embryo development. The results indicate that there is higher developmental competence of oocytes from the large follicles than small follicles.

The maturation rate of oocytes collected from small and large follicles based on cumulus cell expansion was 52.44 and 85.25%, respectively (Table 2). Our results showed higher maturation rate in oocytes from large follicles as compared with the small follicle oocytes. Similar findings were observed by De Matos *et al*. (2002) who found the maturation rate of 24 and 74% for oocytes collected from small (1-1.8 mm) and large (>2 mm) diameter follicles in sheep. The maturation rates were also in accordance with Qian *et al*. (2001) in pig, Khatir *et al*. (2007) in dromedary and Sofi (2010) in sheep. However, our results were in contrast to the observations of Iwata *et al*. (2004) who could not find any significant difference in the maturation rate of small and large follicle oocytes (87 and 88%, respectively) in bovine. Lesser yield of good quality and usable oocytes and low maturation rate in small follicle oocytes obtained in the present study indicate that essential development occurs in the oocytes *in-vivo* during subsequent follicular growth, which strongly affects the developmental competence of oocytes. So the pre-maturation of oocyte occurring during follicular growth is essential for subsequent embryonic development in sheep.

The ovaries contain a large number of oocytes but only meiotically competent oocytes can be used for IVM, which limits the number of embryos produced from a particular donor. Number of small follicles per ovary was higher than the number of large follicles. The recovery rates of good and usable oocytes were significantly higher for large follicles (1.62 and 2.45) than small follicles (1.21 and 2.11). The total and poor oocytes were higher for small follicles (3.09 and 0.98) than in large follicles (2.79 and 0.34). In accordance with the present results, Lonergan *et al*. (1994) reported that more oocytes with many layers of cumulus cells were obtained from follicles >6 mm diameter (70.2%) compared to 2-6 mm diameter follicles (46.8%) in bovines which was reported in the present study in terms of yield of usable and good quality oocytes. The higher number of good quality and usable oocytes recovered from the large follicles compared to small follicles might be attributed to the development of oocytes in large follicles.

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