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Research Article

Production of Sheep Embryos *In Vitro*

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Abstract

In-Vitro Maturation (IVM) and In-Vitro Fertilization (IVF) are the potential methods for producing the early embryos in large numbers. Recently many producers have introduced IVF into their reproductive programs. Successful *in vitro* production of embryo depends upon the good oocyte maturation and correct sperm capacitation. Ovaries were collected from non-pregnant slaughtered sheep from a local slaughterhouse in normal saline. Ovaries having visible follicle and granular homogenous ooplasm were selected and cultured in maturation medium at 38.5°C, 5% CO_2 and 95% humidity for 22-24 hr. The degree of cumulus cell expansion was determined after 22-24 hr of IVM and oocyte with expanded cumulus cell mass to at least 2 diameters away from the zona pellucida were considered as cumulus expanded. The matured oocytes were inseminated with 1 to 2 million spermatozoa/mL in Brackett and Oliphant medium and the embryos were then cultured in CO_2 incubator at 38.5°C, 5% CO_2 and 95% humidity and the cleavage was checked after 42-48 hr post insemination. The embryos were then further cultured for 6-7 days in order to produce a complete developmental series from the 2 cell to blastula stage of embryonic development. Our results showed that the percentage of maturation was 81.81±1.10, cleavage rate was 66.03±1.33, 8-16 cells was 43.44±0.62, Morula was 20.01±0.29, and Blastula was 8.59±0.56. The maturation rate was significantly ($P<0.05$) higher in May as compared to January, February, March and April. The percentage of 8-16 cell stage was also significantly higher in May as compared to those during February. It is concluded that the cleavage rate was good with Brackett and Oliphant fertilization media and TCM 199 as culture media in sheep with high blastula percentage during January.

Keywords: Oocytes; Sheep; *In vitro* maturation; *In vitro* fertilization; Embryo

Introduction

Livestock sector is one of the growing industries which contribute major income to the dairy farmers of across the country. For the successful growth of this sector the animal must be productive in its life term in order to yield maximum returns to farmer. Failure of implantation can lead great economic loss in livestock industry. The majority of this loss occurs because of early embryonic mortality and this is more common during the early than the late embryonic period. The natural *in vivo* process of fertilization and early embryonic development involves innumerable factors, which interact to affect each event of the developmental process include the first cleavage division, the timing of which is known to be indicator of subsequent developmental potential of the embryo, activation of embryonic genome [1], compaction of morula, and formation of blastocyst. Some of these innumerable factors are embryonic intrinsic defects, deficient maternal environment, or failure of embryonic signals to the mother to respond appropriately. These morphological and physiological transitions in pre-implantation embryo development are known to be accompanied and regulated by differential expression of developmentally important genes [2-5]. A better knowledge about gene expression patterns during early embryo development would give us detail information in the molecular pathways controlling early development. It is necessary to have a better understanding of stage specific gene expression because each stage of embryo is characterized by temporal and spatial activation of a specific subset of genes [6]. Scanty information is available on expression profiling of developmentally important genes in sheep embryos. Quality of an embryo also plays a key role in its developmental potential and the assessment of quality using molecular tools has been viewed as a novel approach. Keeping this in view the aim of our study was to produce sheep embryos of different developmental stages *in vitro*.

Materials and Methods

Oocyte collection, evaluation and maturation of oocytes *in vitro*

Ovaries were collected from sexually mature non-pregnant slaughtered sheep within 30min of slaughter from the abattoir from a local slaughterhouse during the months of January to June, 2014 and then transported to the laboratory in a thermos flask containing normal saline. The ovarian tissues were trimmed off and the ovaries were washed thoroughly under running tap water, rinsed five times in normal saline and were finally washed with 70% ethanol to avoid contamination. Ovaries having visible follicles with a diameter of 2 to 6mm was aspirated using 18-G hypodermic needle attached with syringe having consisting of TCM-199, Dulbecco's phosphate buffered saline (PBS), 0.3% bovine serum albumin, heparin (10µg/ml) and gentamicin (10µg/ml). Cumulus oocytes complexes (COCs) were graded by morphological appearance of the cumulus cells investments and homogeneity of ooplasm under a stereo zoom microscope. Grade 1: compact COCs with an unexpanded cumulus mass having ≥5 layers of cumulus cells and with homogenous cytoplasm; grade 2: COCs with ≤4 layers of cumulus cells and with

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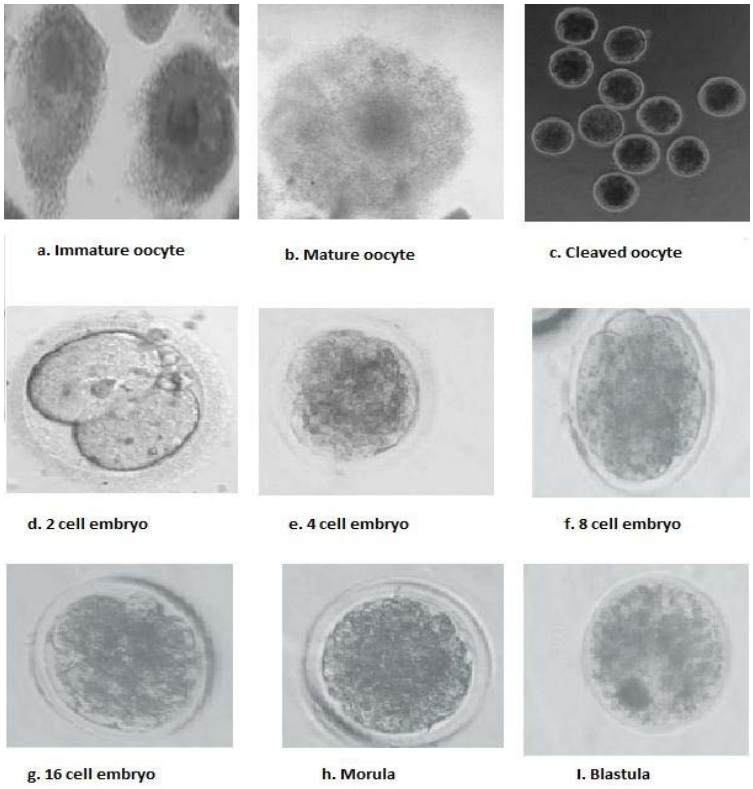

Figure 1: Different stages of embryo development: a. Immature oocyte; b. Mature Oocyte; c. Cleaved oocyte after *in vitro* fertilization; d. 2 cell embryo; e. 4 cell embryo; f. 8 cell embryo; g. 16 cell embryo; h. Morula; i. Blastula.

homogenous cytoplasm; and grade 3: oocytes without cumulus cells and with irregular (shrunken) cytoplasm. Only oocytes having more than 5 layers of unexpanded cumulus cells and granular homogenous ooplasm (grade 1) was recovered and selected for *in vitro* maturation [7,8]. All selected oocytes for IVM were washed once with aspiration media and three times with IVM medium (medium consisted of TCM-199 (Catalogue No.M7528, Sigma, USA) supplemented with 10% FBS (Catalogue No. F2442, Sigma, USA) and FSH (Catalogue No. F2293, Sigma, USA) 10µg/ml) and then 5-10 COCs were cultured in 50µl maturation medium drop in 35mm petri dish covered with mineral oil at 38.5°C, 5% CO_2 and 95% humidity for 22-24 hr. The degree of cumulus cell expansion was determined after 22-24 hr of IVM and oocyte with expanded cumulus cell mass was considered as matured. The maturation of oocytes was further evaluated by identifying the first polar body in the perivitelline space after denuding them with 0.1% Hyaluronidase and vortexing for 90 sec.

In vitro fertilization and *In vitro* culture of embryos

Fresh semen was collected from a ram with the use of electronic ejaculator from Experimental livestock unit (NIANP), Bangalore and spermatozoa were capacitated using Brackett and Oliphant (BO) medium [9]. Media was supplemented with caffeine (2.2mg/ml). Fertilization was carried out by co-incubation of sperm and oocytes at 38.5°C under 5% CO_2 for 2-4 hr as described previously [10]. Thereafter, oocytes were transferred to *in vitro* culture medium (medium consisted of TCM-199 supplemented with 10% FBS and Gentamicin 50µg/ml) and incubated for 18-20 hr. After 18hr oocytes were washed in IVC media in order to remove the attached sperms and kept it in IVC medium for further development inside CO_2 incubator and cleavage was checked approximately every 24hr for up to 6-7 days in order to confirm the fertilization and different stages of embryo development (Figure 1).

Table 1: *In vitro* maturation, *in vitro* fertilization and *in vitro* production of sheep embryos.

Parameters	Rate (%)
No. of ovaries	80.81±3.23
Number of oocytes	78.00±2.50
Maturation rate	81.81±1.10
Cleavage rate	66.03±1.33
8-16 cell	43.44±0.62
Morula	20.01±0.29
Blastula	8.59±0.56

Statistical analysis

The experiment was replicated thirty two times on different days. The maturation rate, cleavage rate and embryo development rates were analyzed by one-way ANOVA, followed by Tukey's test for comparisons of more than two groups using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). Differences between mean the values were considered significant when the probability values were < 0.05.

Result and Discussion

The overall maturation rate, cleavage rate, 8-16 cell stage, Morula and Blastula are presented in Table 1 and the maturation rate,

Table 2: *In vitro* maturation, *in vitro* fertilization and *in vitro* production of sheep embryos during different months.

Month	No. of ovaries (Mean±SEM)	No. of oocyte (Mean±SEM)	Maturation rate (Mean±SEM)	Cleavage rate (Mean±SEM)	% 8-16 cell (Mean±SEM)	Morula (Mean±SEM)	Blastula (Mean±SEM)
January	87.40±10.1	89.40±3.11	79.16±4.15[a]	63.26±3.06	42.02±1.23	19.30±0.93	8.82±1.17
February	76.80±6.58	76.40±6.04	76.51±3.90[a]	72.48±0.78	41.27±0.57[a]	20.08±0.66	7.22±0.84
March	74.20±4.33	72.40±3.04	82.54±0.86[a]	65.16±4.05	42.43±1.44	19.61±0.62	8.79±0.42
April	83.00±11.75	81.25±7.04	81.55±3.10[a]	66.75±2.59	42.27±1.24	20.43±0.43	8.01±0.50
May	77.29± 6.53	69.71±5.35	85.18±1.27[b]	62.10±3.95	47.15±1.72[b]	19.52±0.61	6.84±0.70
June	86.83±9.59	82.00±7.90	84.05±1.55[a]	68.05±1.75	43.71±0.80	21.16±0.68	7.22±0.47

[a-b] Different superscript in the same column differ significantly (P<0.05).

cleavage rate, 8-16 cell stage, Morula and Blastula during different months are presented in Table 2. The maturation rate, cleavage rate, embryo development rates for 8-16 cells, Morula and Blastula were 81.81±1.10, 66.03±1.33, 43.44±0.62, 20.01±0.29, 8.59±0.56 percent, respectively. Our results showed that the percentage of maturation rate in May (85.18±1.27) was significantly (P<0.05) higher in comparison to January (79.16±4.15), February (76.51±3.90), March (82.54±0.86) and April (81.55±3.10). No significant variation was observed in maturation rate between May and June. The percentage of 8-16 cell was also significantly (P<0.05) higher in May (47.15±1.72) in comparison to February (41.27±0.57). There was no significant effect of month in cleavage rate, Morula and Blastula. Our results are in agreement with the report of Crozet et al (1987) who reported that the cleavage rate was 62.6% in *in vitro* matured oocytes which is slightly lower than the ovulated oocyte (75.8%) [11]. Recent report showed that the cleavage rate was 62.3% in *in vitro* matured oocyte using TCM199 media [12]. IVM and IVF are the potential methods for producing early embryos in large numbers for research viz., sexing of embryo, transgenesis, and production of clones. Now a day's many producers have introduced IVF into their reproductive programs. Earlier IVF struggled to establish itself as a legitimate technology because embryo production and pregnancy rates were low, which made the cost of a live animal unacceptable to most. But the technology has now improved to such a level that it has become a very practical and competitive reproductive tool with numerous applications. Successful *in vitro* production of embryo depends upon the good oocyte maturation, correct sperm capacitation, sperm concentration, type of medium, sperm ova interaction and temperature. The *in vitro* method of oocyte maturation, sperm capacitation and *in vitro* fertilization ensure the *in vitro* production of embryos. In IVF oocytes and sperm could be obtained from animals even many hours after their death. Ovaries from slaughter house are the cheapest and most abundant source of primary oocytes for large scale production of embryos through IVM- IVF [13]. The quality of an embryo is influenced by the culture conditions such as media, temperature, pH etc., which affect IVM, IVF and IVC rates. Different media have been used by many workers for culture of sheep embryos. Improved culture conditions for the oocytes, particularly of small ruminants like sheep and goats, are a must to improve viability after handling. Culture media would be the ideal system for the IVC of oocytes and embryos; supplementation with protein sources of animal origin provides the best results from oocyte maturation to the final step of *in vitro* embryo development [14]. Sheep embryos are cultured normally in media containing BSA [15], Human serum [16] or fetal calf serum [17]. Inclusion of amino acids and growth factors in the culture medium facilitates embryo development. TCM-199 gave the best results in maturation and fertilization and the beneficial effect of TCM-199 media on IVM and IVF of oocytes may be related to some factors in its composition such as essential amino acids and glutamine that stimulate DNA and RNA synthesis and enhance cell division [18,19]. Culturing sheep embryos in groups, compared to culturing them individually, results in increased blastocyst showing that sheep embryos produce factors, which stimulate cleavage *in vitro* [20]. Oocytes fertilized *in vitro* with spermatozoa treated with calcium ionophore and caffeine in Brackett and Oliphant medium significantly improved oocyte fertilization and embryo development *in vitro* than with spermatozoa separated by swim-up in synthetic oviduct fluid containing estrus ewe serum [21].

Limitations of the Study

The maturation rate, cleavage rate and embryo development rate varied from month to month during our study period. To avoid this effect, we had pooled embryos of same developmental stage from different cultures to get more embryos of particular developmental stage. Therefore, future studies with a large sample size are warranted to avoid biasness of environmental effect.

Conclusion

We have produced sheep embryos of different developmental stages in our laboratory which will be helpful in future to investigate the expression profiling of different developmental genes as early embryonic losses occur due to aberration in expression of developmental genes in sheep.

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