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In Vitro Production of Sheep Embryos in CR1aa Medium Supplemented with *L*-Ascorbic Acid

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Abstract

In vitro embryo-production procedures developed for sheep have tremendously been improved from decades, but many factors influencing their efficiency still need to be investigated. The overall of this study showed the production of the sheep embryos till blastocyst stage from the ovaries of the slaughtered ewes for IVM, IVF of the oocytes and then IVC in the complex culture media like TCM-199, TCM-199-, TCM-199+ and CR1aa. The morula yield was significantly higher in the wheat peptone and BSA supplemented group. The nuclear maturation rates of ovine oocytes matured in FBS (69.15 ± 1.07) group was more compared to BSA (56.47 ± 0.73) and wheat peptone (35.26 ± 0.79). The nuclear maturation of oocytes was significantly higher in the FBS group when compared to BSA and wheat peptone supplemented groups. The lowest maturation rate was observed in wheat peptone supplemented group. The development rates in 2, 4, 8, 16 and morula stages of ovine embryos produced from BSA group were high (74.22 ± 2.56, 60.93 ± 2.55, 42.97 ± 2.20 and 24.21 ± 1.28) compared to FBS group (73.58 ± 2.92, 58.77 ± 2.01, 41.22 ± 1.68 and 20.17 ± 0.76) and wheat peptone (72.89 ± 1.09, 57.01 ± 0.76, 42.05 ± 0.36 and 24.29 ± 0.55). The cleavage, morula and blastocyst percent in CR1aa was found significantly high compared with TCM- 199, TCM-199- and TCM-199+. The cleavage, morula and blastocyst percent in L-Ascorbic acid at 100 µM with CR1aa is found more significant compared with other compositions used in the experimentation.

Keywords: *In vitro* production; Sheep; *L*-Ascorbic acid; CR1aa

Introduction

The *in vitro* production of embryos is a multi-step process applied through oocyte maturation, fertilization and embryo culture [1]. The techniques like *In Vitro* Maturation (IVM), *In Vitro* Fertilization (IVF) and *In Vitro* Culturing (IVC) have enormous potential for the generation of large number of domestic animal embryos for research and the application of technologies such as transgenic animal production and embryo cloning. Ova from slaughterhouse have provided an important source of embryos for a variety of research purposes. The first lamb produced by IVM, IVF and IVC of ova was done in 1991. Major advance has been made in the IVM, IVF and IVC of adult and pre-pubertal oocytes.

Ovine *in vitro* embryo production is one of the future sheep breeding strategies for the development of biotechnologies in sheep reproduction. Efficient systems for the production of embryos demand through IVM, IVF and IVC procedures that not only produce large numbers of good quality blastocysts for embryo transfer but also result in developmentally normal offspring [2]. The *in vitro* production of embryos for commercial purpose requires the system that is free of specific pathogens, reaches high sanitary standards and allows high and reproducible developmental rates, leading to viable blastocysts that can resist cryopreservation [3].

Serum and BSA (*Bovine Serum Albumin)* are widely used as a protein source in oocyte maturation and embryo culture media. Depending upon the source, the serum containing different concentrations of growth factors, hormones, amino acids and binding proteins were found to have a beneficial effect on oocyte maturation. Serum provides nutrition to cells in the COCs (Cumulus Oocyte Complexes) and prevented zona hardening [4]. The process enhances sanitary risks due to virus, prions or mycoplasma contamination, produces alterations in embryo morphology, ultra structure and kinetics of development, accumulation of cytoplasmic "lipid-like" inclusions increased birth weight and musculo-skeletal abnormalities [5-8]. This condition was referred to as large offspring syndrome [9]. Recently, serious ethical concerns were raised with regard to the welfare of the donor fetuses in the harvest, production and processing of FBS (*Fetal Bovine Serum).* Thus, any efforts to decrease the global demands for FBS and thus to decrease the number of bovine fetuses required should be welcomed and supported.

The normal birth weights following by the transfer of embryos cultured in media containing BSA and amino acids in place of sera and replacing serum by BSA in the culture medium led to a significant decrease in the lipid content of the morulae which were much more resistant to freezing than those cultured in FCS (*Fetal Calf Serum)* [10-12]. But purified BSA is not devoid of sanitary risks and involves high cost of production.

The levels of embryo development were generally lower following *in vitro* development in media supplemented with synthetic macromolecules. The vegetal peptones can replace BSA in freezing medium without affecting blastocyst survival and quality [13]. Usage of plant peptones as substitutes for animal proteins in embryo culture medium and the culture with plant peptones enables embryos to be

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S.No	Component	mg/500 ml
1	NaCl	3505
2	HCl	120
3	NaH_2PO_4 .$2H_20$	31
4	Phenol Red	5
5	Na Lactate	930 µl
6	$MgCl_2$.$6H_2O$	50
7	$CaCl_2$.$2H_20$	150
8	$NaHCO_3$	84
9	Gentamycin	500

Table 1: Preparation of TL- BASE (Modified Tyrode's Lactate solution).

Component	mg/100 ml of TL-BASE
HEPES	240
BSA V	600
Adjust pH 7.3 ± 0.1	

Table 2: Oocyte Collection and washing medium.

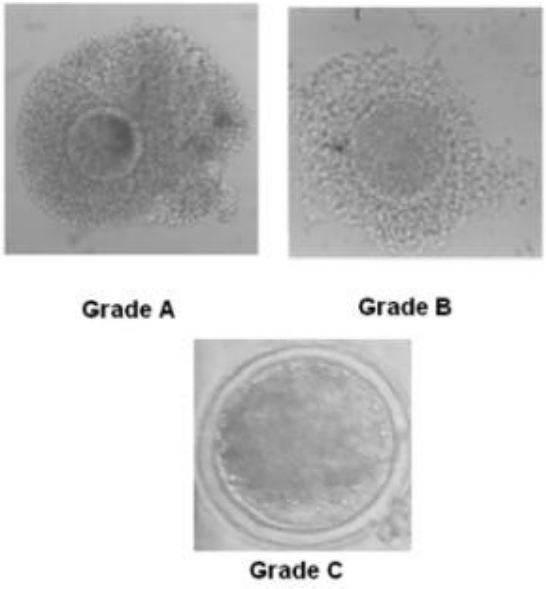

Figure 1: Grading of oocytes.

obtained at a similar rate and of similar quality to that seen following the use of BSA [3]. Further, the use of the plant peptones increased sanitary quality of the embryos and decreased the cost of embryo production. The higher mean number of cells was found in blastocysts cultured with plant proteins compared to blastocysts cultured with BSA shows anti-apoptotic activity of plant proteins [14].

A high incidence of polyspermic penetration still constitutes a major obstacle in the *in vitro* production of embryos, although historical problems like unsuccessful male pronuclear formation and low developmental competence have been overcome by ROS (*Reactive Oxygen Species)* with thiols during IVM and IVC for early development [15]. The early mammalian embryos are susceptible to damage from reactive oxygen species and they increase the production of oxygen free radicals when cultured *in vitro* [16].

In Vitro Production (IVP) of embryos is a multi step process and can be categorized mainly into three stages such as oocytes maturation, fertilization and embryo culture. The efficient collection and grading of oocytes is important in superovulation through IVP of embryos. The present study was carried out to study the effects of protein supplements on IVM and IVF, comparison of culture media. Effect of *L*-ascorbic acid during embryonic development of sheep oocytes, and protein-protein interaction studies provides better understanding of protein function that supports the interactions with cellular proliferation and division.

Materials and Methods

Normal ovaries from sheep, irrespective of body condition and age were collected immediately after slaughter from Chennai corporation abattoir. The ovaries were then transported to the laboratory in a thermos flask containing normal saline that is maintained at 37°C and supplemented with 10 µl/ml penicillin-streptomycin.

The ovarian tissues were trimmed off and the ovaries were washed thoroughly under running tap water, rinsed five times in normal saline and were kept in a sterile beaker containing normal saline at 37°C. The oocytes were retrieved by slicing technique and the COCs were screened using a stereo zoom microscope and transferred into a 35 mm petridish containing fresh oocyte collection medium (Table 1 and Table 2).

Add all the components (except for sodium lactate and Gentamycin) from 1 to 5 and allow complete dissolution; medium should be in acidic condition in this stage. Add $MgCl_2$ and dissolve completely. Add $CaCl_2$ and dissolve completely. Add $NaHCO_3$ and Gentamycin, where osmolarity should be between 260-270 mOsm. Finally sterilize by Millipore filtration and store at 4°C.

Oocytes grading is done on the basis of cumulus cells investment and homogeneity of cytoplasm**.** Those with more than three layers of cumulus cells surrounding the oocyte and uniform cytoplasm are categorized as Grade A (Good). Those with less than three layers of cumulus cells surrounding the oocyte and uniform cytoplasm are categorized as Grade B (Fair). Those with no cumulus cells surrounding the oocyte are categorized as Grade C (Poor). Oocytes of grade A and B were used for *in vitro* maturation (Figure 1).

All the chemicals were purchased from Sigma Aldrich (St. Louis, Mo, USA) unless otherwise indicated. HyPep 4601 (Wheat peptone) was contributed by Kerry group, Norwich, NY, USA. The disposable plastic wares used in this procedure were obtained from Nunclon, Denmark. All the stock solutions and media were prepared in sterile triple distilled Milli-Q water. The media that are prepared were sterilized by filtration method using 0.22µ membrane filters.

The experiment was designed to evaluate animal protein and plant peptones supplements during IVM, IVF and embryo development of ovine oocytes. In every group six replicates were maintained and tested. Group I contains 10% FBS, Group II contains 5mg/ml BSA-FAF (Bovine Serum Albumin-Fatty Acid-Free) and Group III contains 18mg/ml of wheat peptones in embryo development media.

This experiment was designed to evaluate animal protein supplements and its replacement with plant peptones during IVM in TCM 199 (Tissue Culture Medium-199) containing hormones (FSH, LH and estradiol) and Epidermal Growth Factor (EGF). In each group six replicates were carried out. Group IV contains COCs that were matured in maturation medium supplemented with 10% Fetal Bovine Serum (FBS). Group V contains COCs that were matured in maturation medium supplemented with 5 mg/ml fatty acid free bovine serum albumin (BSA-FAF). Group VI contains COCs that were matured in supplemented medium with 0.18 mg/ml of wheat peptones.

Oocytes were stained with Hoechst 33342 to determine the nuclear

Component	Volume
TCM – 199	9 ml
Fetal bovine serum	1 ml
Cysteamine	20 µl
FSH	10 µg
LH	0.2 units
17- β estradiol	10 µg
Gentamicin	500 µg

Table 3: Oocyte maturation medium.

maturation. The oocytes were then stained with 10 µg/ml Hoechst 33342 for 10 min, placed between slide and coverslip and examined under Leica microscope equipped with fluorescent illumination (UV 2-A of filter block, 420 nm emission and 330 to 380 nm excitation) for second metaphase stage (MII). Oocytes maturation was assessed by observing extruded first polar body. After maturation of COCs, the cumulus cells were removed by vortexing.

The culture medium TCM-199 is supplemented with 1 µg/ml of follitropin (FSH), 0.02 IU/ml of luteinizing hormone (LH), 1 µg/ml of estradiol, 100 ng/ml Epidermal Growth Factor (EGF) and 10 µl/ml penicillin-streptomycin for oocyte maturation medium. Nearly 10 per cent FB, 5 mg/ml BS and 0.18 mg/ml wheat peptones were supplemented separately in maturation medium to assess the maturation rate. About 50 µl of IVM droplets were prepared in a 35 mm Petri dish and overlaid with mineral oil and pre-equilibrated in a CO_2 incubator for a minimum of 2 h at 38.5˚C under 5 per cent CO_2.

The graded and selected oocytes were washed four times in maturation medium and 10 COCs were transferred to each droplet and allowed to mature at 38.5°C in a humidified atmosphere under 5 per cent CO_2 for 24 h on a static platform. About 10 ml of maturation medium (Table 3) was prepared freshly before each trial.

Maturation of oocytes was assessed based on the cumulus cell expansion by examination under stereo zoom microscope and the degree of Cumulus expansion was assessed. In Degree 2, full cumulus cell expansion observes where cumulus cells homogenously spread and clustered cells were no longer present. In Degree 1, Moderate cumulus cell expansion observed where cumulus cells non-homogenously spread and clustered cells were still present. In Degree 0, Slight or no expansion observed where cumulus cells highly adhering to the zona pellucida. Oocytes with degree 1 and 2 cumulus expansion were considered as matured and the maturation rate was expressed as percentage of the total number of oocyte cultured.

The Testis from slaughtered adult rams (one year and above) were washed thoroughly in tap water and transported in a thermos flask containing PBS supplemented with 50 µg/ml gentamicin to the laboratory within 30 min and used as source of semen.

The tunica albugenia was removed and the testis and was washed thoroughly with PBS (*Phosphate Buffered Saline)* and tap water. The cauda were cleaned with 70% ethanol. Then the cauda was incised deeply with Bard-Parker blade and the gushing fluid, rich in sperms was flushed into a 60 mm petridish containing sperm wash medium was assessed before processing.

The testis was trimmed free of covering tissues and the tail of the epididymis, presumed to contain mature sperm, was cut using a sterile blade. After cutting, the sperm-rich fluid that oozed out was directly placed on Bovine Serum Albumin-free Brackett and Oliphant (BSA free BO) medium (containing 10 mM caffeine sodium benzoate and 10 µg/ml Heparin) in a Petri plate. Selection of sperm was carried-out in Percoll density gradient (45/90%) placed in CO_2 incubator at 39°C for 2 h. Approximately 2 - 3 ml of BSA free BO medium containing the semen sample was layered over the pre-incubated gradient solution in sterile centrifuge tubes. The centrifuges tubes were then centrifuged at 1400 rpm for 10 min at room temperature. The supernatant was discarded. The sperm sediment was rewashed three times by centrifugation at 1400 rpm for 10 min in BSA-free BO medium as described above. The final pellet that is obtained was resuspended in 1 ml of BSA-free BO medium diluted with 1 ml BO medium containing 20 mg/ml BSA supplemented with 10 µg/ml heparin. A final sperm concentration of approximately 1-2 x 10^6/ml BO medium has been used for fertilization. The mature sheep COCs were washed in BO medium and distributed at a rate of 20/100 µl drop of fertilization medium under mineral oil.

The capacitated spermatozoa (2 µl) were added to these fertilization drops and incubated for 18 h at 38°C at 5% CO_2. All the experimental groups, cleavage rate was assessed at 48 h of culture and subsequent embryo development was assessed every 24 h post insemination for 5 to 6 days.

The in vitro developmental rates of ovine presumptive zygotes were compared in culture media supplemented separately with FBS, BSA and wheat peptones. The IVM was done in TCM-199 supplemented with 1 µg/ml of follitropin (FSH), 0.02 IU/ml of LH, 1 µg/ml of estradiol, 100ng/ml EGF and 10 per cent FBS and *in vitro* fertilization was done using Fertilization-Tyrode Albumin Lactate and Pyruvate (IVF-TALP) medium that is supplemented with BSA. From each group, six replicates were carried out for culture of embryos. Group VII supplied with 10% FBS in culture media. Group VIII supplied with 5mg/ml BSA-FAF in culture media. Group IX supplied with 0.18mg/ml wheat peptones in culture media.

In all the experimental groups, cleavage rate were also assessed at 48 h of culture and subsequent embryo development was observed every 24 h post insemination for 5 to 6 days.

Various media are prepared and the concentrations were standardized. Various media were added to oocyte maturation medium followed by culture in 20% O_2 environment. The media such as TCM-199, TCM-199-, TCM-199+ and CR1aa (Charles Rosenkran's 1 amino acid medium) has been prepared based on the compositions. The components act as the source of embryo development due to availability of vitamins like vitamin C (*L*-ascorbic acid) in lower levels may not be sufficient at normal conditions. The formulation of CR1aa media with *L*-ascorbic acid has been also experimented for better understanding of requirement in embryo development.

L-ascorbic acid (Sigma-Aldrich Chemicals Pvt Ltd., Bangalore, India) concentrations were standardized. Varying concentrations of L-ascorbic acid at 0 to 400 µM concentrations are added to the oocyte maturation medium, followed by culture of oocytes in 20% O_2 environment.

Statistical analysis of the data was done according to the Snedecor and Cochran method**.** The percentage mean value of standard error (mean % ± S.E) was calculated for fertilization rates of oocytes, cleavage and developmental rates of the embryonic stages.

Results

The number and recovery rate of ovine oocytes following slicing technique has been experimented. A total of 1,407 oocytes were recovered from 341 sheep ovaries with an average yield of 4.13 oocytes per ovary (Figure 2). Among the total of 1,407 oocytes, 546(38.81%),

A. ovaries with fallopian tubes

B. Ovaries after trimming

Figure 2: Collection of Ovaries for IVM.

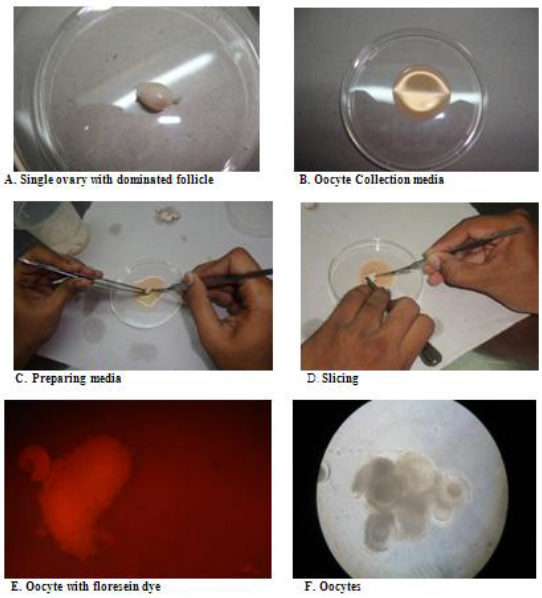

Figure 3: Preparation of Oocyte for IVM.

648(46.06%) and 213 (15.14%) oocytes were classified into three grades A, B and C respectively.

The effect of animal protein supplements and its replacement with plant peptones during IVM of ovine oocytes is presented in the present work. In FBS supplemented group (group I), out of 206 oocytes cultured, 184 oocytes matured with a mean maturation percentage of 89.32 ± 1.67. In BSA supplemented group (group II), out of 196 oocytes cultured, 157 oocytes matured with a mean maturation percentage of 80.10 ± 1.64. In wheat peptone supplemented group (group III), out of 199 oocytes cultured, 108 oocytes matured with a mean maturation percentage of 54.27 ± 1.81.

The maturation rate of oocyte was significantly higher in the FBS supplemented group when compared with BSA and wheat peptone supplemented groups. The collection of ovaries, preparation of oocytes, and the final observation of matures single polar body has been shown from Figure 3 and Figure 4.

The *in vitro* fertilization and developmental rates of ovine oocytes under three different protein supplements are presented. The number of oocytes produced from FBA, BSA and Wheat peptones from 6 replicates are 184, 157 and 108 respectively. The number of fertilized oocytes supplemented by FBS (138) is more compared with BSA (83) and Wheat peptones (48). In FBS supplemented group (group I), BSA-FAF group (group II), and wheat peptones group (group III), the fertilization rates (mean ± SE) are 75.00 ± 1.88, 52.87 ± 2.04, and 44.44 ± 0.77 respectively. Hence the fertilization rates in media supplemented with FBS is found significant compared to BSA+FAF or Wheat peptones.

The fertilized oocytes with six replicates has observed highest fertilization rate in FBS (75.00 ± 1.88) compared with the other two protein supplements rich as BSA (52.87 ± 2.04) and wheat peptones (44.44 ± 0.77).

The developmental rates of presumptive zygotes under different supplements are presented in Table 4. In the FBS supplemented group (group VII), the developmental rates of oocytes at 2-, 4, 8- , 16 cell and morula stages were 73.58 ± 2.92, 58.77 ± 2.01, 41.22 ± 1.68 and 20.17 ± 0.76 per cent (mean ± SE), respectively. In BSA-FAF group (group VIII), developmental rates of 2-, 4-, 8-, 16 cell and morula stages were 74.22 ± 2.56, 60.93 ± 2.55, 42.97 ± 2.20 and 24.21 ± 1.28 per cent (mean ± SE), respectively. In wheat peptones group (group IX), the developmental rates of 2-, 4-, 8-, 16 cell and morula stages were 72.89 ± 1.09, 57.01 ± 0.76, 42.05 ± 0.36 and 24.29 ± 0.55 per cent (mean ± SE), respectively.

The number of morula (mean% ± S.E.) developed was found to be higher in wheat peptone (or plant peptone) and BSA supplemented group followed by FBS.

The Cleavage, Morula and Blastocyst percent has shown high in CR1aa medium followed by TCM-199+, TCM-199 and TCM-199- (Table 5).

L-Ascorbic acid with CR1aa medium has shown higher Cleavage, Morula and Blastocyst percent at 100 µM concentration (Table 6). Figure 5 has shown various stages of embryo development.

Discussion

Collection of sheep ovaries has been done from slaughter house.

Figure 4: Matured oocyte with single polar body.

Supplement	No. of replicates	No. of oocytes	No. of two cells (mean% ±S.E.)	No. of four cells (mean% ± S.E.)	No. of 8-16 cells (mean% ± S.E.)	No. of morula (mean% ± S.E.)
Fetal bovine serum (Group VII)	6	228	168 (73.68±2.92)	134 (58.77±2.01)	94 (41.22±1.68)	46 (20.17±0.76)
Bovine serum albumin (Group VIII)	6	256	190 (74.22±2.56)	156 (60.93±2.55)	110 (42.97±2.20)	62 (24.21±1.28)
Wheat peptone (Group IX)	6	214	156 (72.89±1.09)	122 (57.01±0.76)	90 (42.05±0.36)	52 (24.29±0.55)

Table 4: Cleavage rate and morula development of ovine oocytes.

Medium	Number of oocytes insemination	Cleavage (mean% ± S.E.)	Morula (mean% ± S.E.)	Blastocyst (mean% ± S.E.)
TCM-199	280	62.3 ± 1.5	32.4 ± 1.8	10.4 ± 1.2
TCM-199+	268	63.4 ± 1.3	33.2 ± 1.3	10.9 ± 1.5
TCM-199-	260	62.2 ± 1.2	31.1 ± 1.5	9.4 ± 1.0
CR1aa	290	64.8 ± 1.6	33.4 ± 1.8	11.4 ± 1.4

Table 5: Effect of different media on development of sheep embryos.

Conc. (µM)	*L*-Ascorbic acid			
	Number of oocytes insemination	Cleavage (mean% ± S.E.)	Morula (mean% ± S.E.)	Blastocyst (mean% ± S.E.)
0	278	64.1 ± 1.2	33.7 ± 1.1	11.5 ± 1.3
50	275	65.2 ± 1.3	34.9 ± 1.2	12.5 ± 1.2
100	280	68.1 ± 1.0	39.7 ± 1.6	13.5 ± 1.7
200	278	63.2 ± 0.9	35.6 ± 1.1	11.4 ± 1.0
400	272	50.7 ± 1.5	31.9 ± 0.9	10 ± 1.4

Table 6: Effect of L-Ascorbic acid on CR1aa in development of sheep embryos.

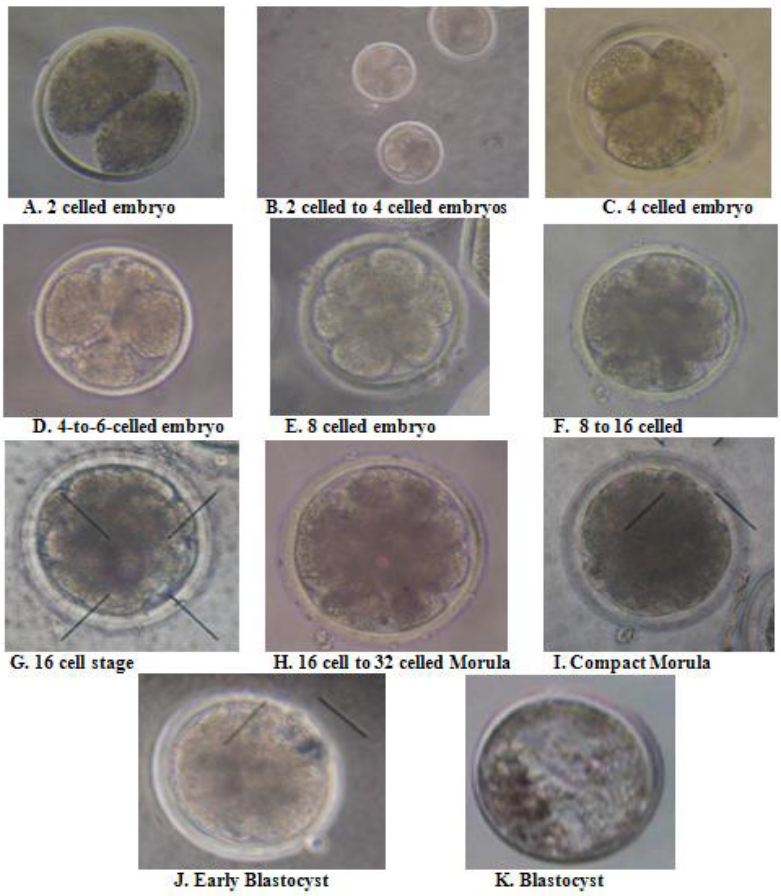

Figure 5: Different stages of embryo development (2 celled to Blastocyst stages).

Retrieval of Cumulus Oocyte Complexes (COCs) from antral follicles by slicing method and Grading of oocytes is done based on their morphological appearance. *In vitro* culture of superior grade oocytes is experimented in four different media with supplementation of *L*-ascorbic acid. *In vitro* fertilization of sheep embryos using fresh semen collected and capacitated from slaughtered ram testis in above said media. TCM-199 containing vitamin like *L*-Ascorbic acid is observed better results than the other culture media used for the *in vitro* development.

The exposure to *L*-ascorbic acid promotes the development of porcine denuded oocytes and cumulus-enclosed oocytes from MI to Mil and prevents cumulus cell DNA fragmentation [17]. Recommend supplementing porcine embryo culture medium with 100 µM L-ascorbic acid but not both as the developmental competence appeared to be reduced [18].

The quality of an embryo is influenced by the culture conditions such as media, temperature, pH etc., which affect in IVM, IVF and IVC rates [19]. FCS and BSA are complex mixtures of different proteins that contain small peptides, energy substrates and growth factors [20]. These proteins influence the growth and division of cells during IVM, IVF and IVC. The cell division and growth is mainly involved in MAP Kinase pathway [21,22].

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 [23]. The use of FCS and BSA during IVM+IVC was efficient for *in vitro* embryo production, regardless of the protein source used during IVF (BSA or OVA) [24,25].

Immature oocyte recovery followed by *in vitro* oocyte maturation and *in vitro* fertilization is a promising new technology for culture of oocytes, have progressed considerably in recent years [26]. Culture of oocytes in medium with FCS for the first 24 h and with BSA for the second 24 h decreases the incidence of polyspermy, without effecting nuclear maturation.

A basic culture system was established for the IVM of early preantral mouse ovarian follicles [27]. Up to 60% of sheep morulae produce normally during culture but previously cleavage stages undergo limited development and is suggested that there is a block to development *in vitro* at the eight- to- twelve-cell stage [28-33].

Supplementation of MEM (Minimum Essential Medium) vitamins during IVM enhanced subsequent parthenogenetic development in porcine oocytes [34]. However, the presence of Minimal Essential Medium vitamins (MEM vitamins) to semi-defined maturation medium enhanced subsequent developmental ability of caprine oocytes [35]. Nearly 0.5 and 1×MEM vitamins as compared to 1.5× had a positive effect during maturation resulting in an increased development of blastocyst from cleaved embryos [36]. Importance of biological components like proteins during cellular development can be also being understood using *in silico* procedures [37,38].

Conclusion

Based on the composition of the media and the availability of supplements, the growth and cell division varies in *in vitro* production of sheep embryos. TCM199-, TCM199+ and CR1aa have shown good results in stimulation of oocyte development. CR1aa had shown good results compared with other media of study. *L*-ascorbic acid in CR1aa at 100 µM has provided good influence on development of oocyte during cleavage, morula and blastocyte stages.

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