



Comparative Efficiency of Different Culture Media on *in vitro* Embryonic Development of Goat Oocytes

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ABSTRACT

Background: The study was conducted to compare the development of goat embryos in simple media with BSA and complex culture media supplemented with serum on the development of goat embryos. Also, studied the effect of oviductal cell co-culture on the developmental competence of goat oocytes.

Methods: The A and B quality goat oocytes subjected to *in vitro* maturation in TCM 199 + 10% FCS + FSH + Estradiol for 24 hours in a CO₂ incubator (6% CO₂) at 38.5°C. The oocytes that exhibited cumulus expansion subjected to *in vitro* fertilization in 1) BO - IVF and 2) TALP-fertilization media supplemented with heparin. The cleavage percent found significantly higher in BO-IVF (66.66%) compared to TALP- fertilization media (42.07%). After 18 hours of sperm-oocyte incubation, the presumptive zygotes from BO-IVF media divided randomly into 3 groups and cultured in one of the following 3 media, group 1) CR1aa media; group 2) BO-IVC media and group 3) TCM-199 + 10% FBS.

Result: The cleavage percentage was significantly higher ($P>0.05$) in BO-IVC media than CR1aa media and TCM-199 media (72.32, 64.35 and 35.23 percent respectively). The blastocyst development observed significantly higher ($P>0.05$) in CR1aa compared to commercial BO-IVC media and TCM-199 media (9.23, 5.55, and 4.05 percent, respectively). Among the different media compared, CR1aa and BO-IVC media were effective for supporting the development of goat zygotes to the morula and blastocyst stages than those obtained when a complex TCM-199 medium used with serum supplementation. The effect of oviductal cell co-culture on the developmental competence of goat oocytes studied. The blastocyst development rate found higher in CR1aa + oviductal and TCM-199 + oviductal cells as compared to CR1aa and TCM-199 alone.

Key words: Cleavage, Embryo, Goat, *In vitro* culture media, *In vitro* fertilization, *In vitro* maturation.

INTRODUCTION

Goat is important meat animal in India, the goat meat demand is expanding and gaining popularity on a commercial level hence there is a need to produce elite goat breeds for meat production. Hence, simplification and refinement in *in vitro* embryo production protocol could be useful for faster multiplication of superior germplasm (Kumar *et al.*, 2007).

The use of slaughter goat ovaries for optimising *in vitro* embryo production (IVEP) protocol provides an excellent source of low-cost embryos for basic research in developmental biology and physiology. The combination of *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of oocytes successfully used for the *in vitro* embryo production (IVEP), but complicated culture condition leads to 8-16 cell block and further exceptionally low blastocyst yield has limited the applicability of this technology (Kharche *et al.*, 2009).

In most studies of goat IVEP, the presumptive zygotes subjected to IVC in a system that involves the use of (i) a complex medium and (ii) serum supplementation in media yields low blastocysts and retards the growth rate of embryo. Simple media like Charles Rosenkrans (CR) medium, synthetic oviductal fluid (SOF), and potassium simplex optimized medium (KSOM) found capable of supporting the development of cattle zygotes to the blastocyst stage even in the absence of co-culture with somatic cells (Westberg *et al.* 2002). Nandi *et al.* (2006) used simple

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media like CR, but a limited reports are available in which the efficacy of these media to support the development of goat zygotes to the morula and blastocyst stages compared. The present study was, therefore, taken up to (i) study the effect of fertilization media on *in vitro* fertilization and subsequent embryonic development. (ii) compare the embryonic development of goat embryos in complex culture media supplemented with serum and simple media with BSA, and BME, MEM amino acid supplementation. (iii) and the effect of oviductal epithelial cells in culture media on embryonic development of goat oocytes.

MATERIALS AND METHODS

The present study conducted at IVEP laboratory facility, Department of Animal Reproduction Gynaecology and Obstetrics, Post Graduate Institute of Veterinary and Animal Sciences, Akola, Maharashtra from March-2021 to January-2022.

Collection and processing of ovaries

Goat ovaries obtained within 1-2 hours of slaughter from the local abattoir and transported to laboratory in sterile warm normal saline solution supplemented with broad spectrum antibiotic. All the ovaries then subjected to washings (9-10 times) with warm saline fortified with broad spectrum antibiotic and then transferred for further processing.

Recovery of COC's and *in vitro* maturation

The oocytes recovered from ovaries by slicing method in a TL-HEPES medium. From the recovered oocytes only A and B grade oocytes selected and washed 3-4 times using maturation media TCM-199 supplemented with 10% FCS, Sodium Pyruvate (0.25 mM), FSH (10 µg/ml), Gentamycin (50 µg/ml) and Oestradiol (1 µg/ml). The 50 µl droplets of maturation media in a 35 mm petri dish were covered with sterile mineral oil and equilibrated in a CO₂ incubator (6% CO₂) at 38.5°C for 4 hrs before the transfer of the immature oocyte.

Sperm capacitation and fertilization

The spermatozoa used for IVF throughout the study was frozen Beetal buck semen straw from the same donor and batch and evaluated for IVF earlier. The spermatozoa were prepared for insemination by washing in laboratory-prepared semen preparation media (SPTL) (SPTL has HEPES 2.5 mg/ml, BSA (fraction V) 6 mg/ml and NaHCO₃ 2 mg/ml) by the swim-up method. The motility of semen evaluated under a microscope and sperm concentration calculated to make the final concentration of 2.0×10^6 sperms/ml in the IVF medium. The *in vitro* matured oocytes were washed twice with the oocyte washing media and transferred to the 50 µl droplets of the BO-IVF and TALP- fertilization medium (10 oocytes/droplet).

The spermatozoa were then transferred to 50 µl droplets of BO-IVF and TALP- fertilization media in a 35 mm petri-dish, covered with sterile mineral oil, and placed in a CO₂ incubator (6% CO₂ in air) for 18 -20 hr. at 38.5°C. After 18 hr. of sperm-oocyte co-incubation, the zygotes were separated from the sperm and cumulus cell mass by repeated pipetting, divided into 3 groups and washed with the respective IVC medium.

In vitro culture

After 18 hours of sperm-egg interaction the zygotes were transferred into three separate groups: 1) simple media Charles Rosenkrans 1aa (CR1aa) was prepared in the laboratory. The composition of CR1aa media was sodium chloride 114.7 mM, potassium chloride 3.1 mM, sodium bicarbonate 26.2 mM with Hemi calcium lactate 5 mM,

sodium pyruvate 0.4 mM, BSA 3 mg/mL, BME essential amino acids (EAA), MEM non-essential amino acids (NEA), 1 mM L-glutamine (Rosenkrans and First, 1994) 2), commercially available Brackett and Oliphant (BO-IVC) media supplemented with BSA, vitamins, amino acids, antioxidants, sodium bicarbonate, and hyaluronate 3) complex tissue culture media TCM -199 supplemented with 10% FCS, Sodium Pyruvate (0.25 mM) and Gentamycin (50 µg/ml). During incubation, culture media replace 50 % on 2nd and 5th day with fresh media. The cleavage was recorded on day 2 and day 5 post-insemination. The embryos were examined on days 7 and 8 post-insemination for recording the stages of development under inverted phase-contrast microscope (Olympus, NY,USA; model: CKX53).

Oviductal epithelial cell preparation for co-culture

Goat oviducts collected from a slaughterhouse and transported to the laboratory on ice. Oviducts separated from connective tissue and mucous membrane, washed thoroughly with normal saline containing 50 mg/ ml gentamicin. The individual oviducts placed in a petri dish and oviductal cells squeezed out by using sterile glass slides from the infundibulum end to the isthmus. The oviducts rinsed well in a searching petri dish with aspiration media TCM-199 containing 10% fetal bovine serum (FBS) and gentamicin. They separated by gravity sedimentation and finally, the pellet suspended in TCM 199. A 20 µl drop of the oviductal cells was placed on a glass slide and checked under the microscope. According to the ciliary motility, the oviductal cells selected for culture. 100 µl drops of TCM-199 and CR1aa respectively containing approximately 100 - 150 oviductal cells were prepared. Cover drops with sterile mineral oil and incubated at 38.5°C temperature (6% CO₂) for 24 hours before putting them with zygotes.

Experiment 1

The effect of fertilization media and different capacitating agents on *in vitro* fertilization of goat oocytes examined. The oocytes matured in TCM 199 + 10% FCS + FSH + Estradiol maturation media and for fertilization, two different fertilization media i) TALP- fertilization with Heparin ii) Brackett and Oliphant (BO)- IVF media and further fertilized zygote cultured in BO-IVC media to observed embryo development.

Experiment 2

The oocytes matured in TCM 199 + 10% FCS + FSH + Oestradiol. After 24 hours of *in vitro* maturation, the matured oocytes fertilized in BO- IVF medium for 18 to 20 hours. For culture fertilized zygotes transferred in the 1) simple media CR1aa 2) Brackett and Oliphant (BO-IVC) media and 3) complex tissue culture media (TCM-199) supplemented with 10% FBS and further embryonic development examined.

Experiment 3

The effect of goat oviductal epithelial cell (GOEC) co-culture on the developmental competence of goat oocytes studied.

the oocytes matured in TCM 199 + 10% FCS + FSH + Oestradiol. After 24 hours of *in vitro* maturation, the matured oocytes fertilized in BO-IVF media and fertilized zygotes transferred in culture media for embryonic development. The oviductal cells collected from the oviduct and co-cultured with zygotes in - 199 and CR1aa development media. All chemicals and plasticware used were from sigma Aldrich, USA.

Statistical analysis

In vitro fertilization rate, cleavage rate, and embryo development competence between the treatment groups compared using the Chi-square analysis test. The level of significance recorded at the 95% level of confidence (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

In the *in vitro* embryo development, inadequacy of culture condition leads to stage-specific block or a loss of viability in a morphologically normal embryo. The present research work directed more attention towards standardization of the goat embryo culture conditions.

In the present study, on comparing *in vitro* fertilization in BO-IVF media and TALP fertilization media + heparin, we found that cleavage percentage was significantly higher ($P>0.05$) in BO-IVF media than in TALP fertilization medium. The 2 cell, 4-8 cell, morula and blastocyst development was observed more in BO medium than in TALF + heparin medium. However, a difference was not significant between both groups (Table 1).

After 24 hours the matured oocytes inseminated with capacitated spermatozoa and placed in the development media. 48 hours post-insemination cleavage percentage observed significantly higher ($P>0.05$) in commercially available BO- IVC media than in CR1aa media and TCM-

199 media. On day 5 post-insemination, the per cent of cleaved embryos that developed to morula/ compact morula was significantly higher ($P>0.05$) in CR1aa and BO-IVC media compared to TCM-199 media. However, on the 7th and 8th day the percent of expanded blastocyst / hatched blastocyst observed significantly higher ($P>0.05$) in CR1aa compared to BO-IVC media and TCM-199 media (Table 2).

The cleavage per cent found significantly higher in media CR1aa co-cultured with oviductal epithelial cells and media TCM-199 co-cultured with oviductal epithelial cells compared to CR1aa and TCM-199 media without oviductal cell co-culture ($P<0.05$). The embryo development up to the morula stage found significantly higher in TCM-199 co-cultured with oviductal epithelial cells and in media CR1aa co-cultured with oviductal epithelial cells than TCM-199 and CR1aa media without oviductal cell ($P<0.05$). The blastocyst development rate was higher in TCM- 199 media co-cultured with oviductal cells however, there was no significant difference with other treatment groups (Table 3).

Present study reported an increase in embryo development to the blastocyst stage when *in vitro* fertilization happened in presence of heparin and PHE (Table 1). The principal function of mitochondria in cell is to perform oxidative phosphorylation to produce ATP. Ōura and Toshimori, (1990) reported that sperm hyperactivation generated by heparin leads to a state of active mitochondrial respiration (high ADP levels), thereby increasing oxygen consumption and increasing oxidative stress. Miller *et al.* (1994) reported that the harmful effect of atmospheric O₂ levels on early embryonic development attributed to the formation of O₂ free radicals that accelerate the process of lipid peroxidation and enzyme activation resulting in cell damage and loss of motility. Hypotaurine and epinephrine present in PHE inhibit lipid peroxidation and reduce cell damage. This oxidative stress is reduced

Table 1: Effect of fertilization media and capacitating agents on the *in vitro* embryo developmental competence of goat oocytes.

Media	Capacitating agent	Number of oocytes	Cleavage (%)	2- Cell (%)	4- 8 Cell (%)	Embryo development (%)	
						Morula (%)	Blastocyst (%)
BO	PHE + Heparin	219	146 (66.66) ^a	53 (36.30)	35 (23.97)	28 (19.17)	13 (8.90)
TALP	Heparin	202	85 (42.07) ^b	41 (48.23)	18 (21.17)	15 (17.64)	8 (9.41)

The values in the same column with different superscripts differ significantly (a,b) at both 5% and 1% levels of significance.

Table 2: Effect of different culture media on *in vitro* embryonic development of goat oocytes.

Culture media	Number of oocytes	Cleavage (%)	2-Cell (%)	4-8 Cell (%)	Embryo development (%)	
					Morula (%)	Blastocyst (%)
CR1aa	202	130 (64.35) ^a	75 (57.69) ^a	44 (33.84) ^a	29 (22.30) ^a	12 (9.23) ^a
BO – IVC media	224	162 (72.32) ^a	84 (51.85) ^a	59 (36.41) ^a	27 (16.66) ^a	9 (5.55) ^{ab}
TCM - 199	210	74 (35.23) ^b	30 (40.54) ^b	22 (29.72) ^b	11 (14.86) ^b	3 (4.05) ^b

The values carrying dissimilar superscripts (a, b, ab) within a column differ from each other significantly ($P<0.05$).

Table 3: Effect of goat oviductal cell co-culture in culture media on the developmental competence of goat oocytes.

Culture media	Number of oocytes	Cleavage (%)	2 – Cell (%)	4 – 8 Cell (%)	Embryo development (%)	
					Morula (%)	Blastocyst (%)
CR1aa	122	44 (36.06) ^{bc}	17 (38.63) ^{bc}	11 (25.00)	9 (20.45) ^b	6 (13.63)
CR1aa (with oviductal cells)	124	58 (46.77) ^a	27 (46.55) ^a	18 (13.72)	16 (27.58) ^a	9 (15.51)
TCM- 199	126	37 (29.36) ^c	12 (32.43) ^c	8 (27.27)	10 (27.02) ^b	7 (18.91)
TCM- 199 (with oviductal cells)	128	50 (39.06) ^{ab}	21 (42.00) ^{ab}	14 (12.00)	18 (36.00) ^a	10 (20.00)

The values in the same column with different superscripts (a, b, c) differ significantly at ($P < 0.05$)

by PHE which may serve as antioxidants in the fertilization medium (Numabe *et al.*, 2001). So, the combined effect of PHE + heparin was beneficial for *in vitro* fertilization and subsequent embryonic development. The results of the present study found in agreement with Younis *et al.* (1991) who compared mTALP, BO (Brackett and Oliphant, 1975) and mH-199 for fertilization and cleavage of goat oocytes and reported a higher cleavage rate in BO medium (60%) than mTALP medium (40%). Also, the higher percentage of cleavage rates (52-60%) in BO Medium was reported by Numabe *et al.* (2001).

In the present study, cleavage percent found lower in TALP + heparin group compared to BO-IVF media (Table1). The TALP- fertilization and sperm TALP media used for *in vitro* fertilization and sperm wash respectively are usually prepared in the laboratory fresh every day, which may give different results during different replicates. This difference may be due to variation in chemicals, water quality, unnoticed error in measurement of chemicals during media preparation, pH, temperature variation, preservation of stock solutions, fluctuation in osmolarity at different stage of embryo culture and other idiopathic factors. Nagao *et al.* (1995) reported that during *in vitro* embryo development, the embryo is extremely sensitive to water quality used in the preparation of medium and that early embryos may be seriously affected by the purification method and the storage period of that water. This problem is overcome by troubleshooting at every step of IVEP protocol and by using commercially available media for optimum results.

A significant difference in embryo production among all the 3-culture media suggests that the actual chemical composition of the medium and the protein supplementation is a more important consideration for optimizing embryo production *in vitro*. In the present study the percent of 16 cell stage embryo to blastocyst was higher in CR1aa media containing BSA, MEM, BME and glutamine. Thus, indicating that the present components might be beneficial for caprine embryos to develop from 16 cell to blastocyst stage (Pinyopummintr and Bavister, 1991). Bavister, (1995) reported that when a cleaved embryo is present in sheep oviduct, it contains those amino acids which have been shown to stimulate the development of the cleaved embryo

(i.e., the essential, non-essential amino acids and glutamine). The non-essential amino acid (NEAA) and essential amino acids (EAA) with glutamine (1 mM) increased the development of ruminant embryos *in vitro* (Rosenkrans and First, 1994).

In agreement with the present study, Pinyopummintr and Bavister (1996) and Lee *et al.* (2004) described that the use of amino acids in serum-free culture media improves embryo development, through an antioxidant action (Liu and Foote, 1995) and controlling pH and osmolarity (Gardner, 1998). Amino acids can also reduce the stresses and cell fragmentation caused by *in-vitro* embryo culture (Donnay *et al.* 1999).

The ability of simple media such as Charles Rosenkrans (CR1), SOF, KSOM, and commercial BO-IVC to provide equivalent or better yields of transferable quality ruminant embryos in the absence of serum offer a great advantage (Table 2). Since, with serum it has been reported to be responsible for high fetal loss, complications during pregnancy and production of abnormally large calves resulting in high neonatal losses in cattle (Jacobsen *et al.* 2000; Farin *et al.* 2001). Monson *et al.* (1992) stated that embryos developed in CR1aa established pregnancies after transfer in recipient ruminants. The increased morula to blastocysts ratio in CR1aa indicates a development rate more like *in vivo* conditions (Betteridge and Flechon, 1988).

In the present study, IVC of presumptive goat zygotes in CR1aa and BO-IVC medium gave a higher yield of embryos than when these were cultured in TCM-199 with serum. These results are in agreement with those of Abdoon *et al.* (2001) who obtained higher yields of transferable embryos in CR1aa or CR2 aa than in TCM-199 medium. A primary reason for the higher rate of development in the simple media could be the high glucose concentration of TCM-199 (5.5 mM) compared to no glucose in CR1aa respectively. Glucose has inhibitory effects on the development of ruminant embryos, which require pyruvate and/ or lactate throughout development (Bavister 1995). Whereas none of the embryos developed to the morula or blastocyst stage when CR1aa medium was supplemented with 5.5 mM glucose (Rosenkrans *et al.* 1993). TCM-199 gave optimum embryo development when co-culture with

somatic cells since these cells decrease the glucose and increase the pyruvate and lactate concentrations in the medium (Table 3). (Matsuyama *et al.* 1993; Edwards *et al.* 1997).

The use of a complex media like TCM 199, which is for cell culture work, rather than embryo culture, and needs to avoid since constituents present in this medium at such a concentrations that are not optimal for supporting embryo development (Martins *et al.* 2001). While conducting this study present problems were address. It was noticed that even though all media used supported the initial cleavage and development to the 4-8 cell stage, further development significantly got arrested in all media might be due to inadequacy of culture conditions (Srirattana *et al.* 2013), the inadequacy of maternal and paternal genome (Singh *et al.* 2014), the inadequate cytoplasmic and nuclear *in vitro* maturation of oocyte or bad quality of sperm DNA either leads to loss of viability in a morphologically normal embryo or developmental block which is more common at 8 to 16-cell stage result in lower blastocyst rate (Abdullah *et al.* 2011).

CONCLUSION

The BO-IVF media gave a significantly higher cleavage rate than TALP + heparin media. The IVC of presumptive goat zygotes in CR1aa and commercial BO-IVC medium gave higher yield of embryos than TCM-199 with 10% FCS. Fertilized zygotes developed in TCM-199 + oviductal epithelial cells and CR1aa + oviductal epithelial cells gave a higher blastocyst rate than TCM-199 and CR1aa media.

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Conflict of Interest

All authors declare that they have no conflict of interest.

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