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Comparative study on effect of different embryo culture media on *in vitro* blastocyst production in goats*

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ABSTRACT

The present study was aimed to assess the efficacy of different culture media for blastocyst development. Oocytes (2,539) from 1,059 ovaries recovered by follicle puncture technique were utilized for present investigation. In experiment 1, effect of different embryo culture media (Gr. 1; mKSOMaa; Gr. 2; TCM-199+OEC; Gr. 3; mSOFaa and Gr. 4; mCR2aa) and in experiment 2, effect of sequential embryo culture medium, Gr. 1: mKSOMaa containing 5% FBS (1–3 days) and 10% FBS (4–10 days); Gr. 2: 0.8% BSA (1–3 days) and continuous embryo culture medium, 10% FBS (4–10 days); Gr. 3: 0.8% BSA (1–10 days) and Gr. 4: 10% FBS (1–10 days) on *in vitro* blastocyst development potential was evaluated. Significant higher ($P<0.05$) blastocyst production rate was observed in TCM–199 co-culture with OEC and mKSOMaa medium compared to mSOFaa embryo culture medium. Similarly, significantly higher morula production rate was observed in mKSOMaa medium compared to mSOFaa and mCR2aa medium. However, numerically higher number of embryos cleaved in mSOFaa medium compared to TCM-199 co-culture with OEC. Results indicated that embryos cultured in mKSOMaa, TCM-199+OEC and mCR2aa embryo development media are equally effective in supporting pre-implantation development.While considering the risk factor associated with OEC co-culture, mKSOMaa is proved to be efficient medium for obtaining higher *in vitro* embryo development rate. In experiment 2, significantly higher morula and blastocyst formation was observed in sequential embryo culture medium compared to BSA supplemented continuous embryo culture medium. Further, the present findings indicated that the use of mKSOMaa+0.8% BSA or 5% FBS for first 3 days of embryo culture resulted in increased rate of blastocyst stage embryos, if initial 3 days of culture in mKSOMaa+0.8% BSA was followed by culture in mKSOMaa containing 10% FBS. However, no significant difference was observed in sequential embryo culture medium and continuous embryo culture medium supplemented with 10% FBS.

Key words: Blastocyst, Caprine, Culture media, *in vitro* fertilization, Sequential media

The *in vitro* embryo production (IVEP) has emerged as an alternative to the *in vivo* embryo production due to inconsistent super ovulatory response. Maintaining embryo viability during *in vitro* culture is a key to the application of IVEP. During *in vitro* embryo production, the culture system may deeply influence embryonic development of pre-implantation stage embryos (Cognie *et al.* 2003, Lonergan *et al.* 1999, Yadav *et al.* 2013). Major developmentally important events take place during development of embryos from post fertilization to the blastocyst stage (Kharche *et al.* 2011a). Pre-implantation stage embryos can be developed in different media comprising simple balanced salt solutions and carbohydrates to complex constituents, such as tissue culture medium (TCM-199) with further supplementation of serum, somatic cells and/or a feeder layer of somatic cells (Krisher *et al.* 1999, Summers and Biggers 2003). Further embryo-somatic cell co-culture is aimed at improving the development and viability of mammalian preimplantation embryos generated and cultured *in vitro*. Scanty information is available about the mechanisms underlying the beneficial effects of co-culture, although the production of embryotrophic compounds, modulation of nutrient profile, protection against culture-induced stress and/or toxin clearance are all contenders (Orsi and Reischl 2007). Although the use of media without co-culture is recommended for embryo culture to understand fully the requirements for embryo development, which are essential to know the precise composition of medium in which embryos are grown. It also negates the possible effects of unknown components, minimizes risk of contamination with pathogens and their metabolites, reduces undesirable variations among different lots of culture by eliminating the use of co-culture system. Use of sequential embryo culture media has also been established by various

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researchers to fulfill the requirement of embryo in early and later stages of development. Selection of embryo development medium and/or its supplement during different stages of embryonic development may markedly affect *in vitro* blastocyst production efficiency. Among the additives in media, the role of serum on embryonic development is still not fully understood. Retrospectively, FBS has been reported to be beneficial for blastocyst development and hatching rate (Wang *et al.* 1997, Kim *et al.* 2004, McElroy *et al.* 2008). However, the exposure of early embryos to FBS has been shown to be detrimental to the quality of blastocysts produced (Thompson *et al.* 1995, Rizos *et al.* 2002, 2003), as serum shows biphasic effects with marked inhibition of the first cleavage division and pronounced stimulatory effects later on (Pinyopummintr and Bavister 1994). The stepwise increase in serum supplementation and two-step culture method is routinely used in some laboratories for the production of bovine embryos for transfer, freezing and micromanipulation (Zhang *et al.* 1992, Bavister 1995). Researchers have recently used modified simple and complex embryo culture media but no such report is available on the efficacy of these media during the course of embryonic development from zygote to blastocyst stages. The present study was conducted (i) to compare the effect of 4 different embryo culture media and (ii) sequential embryo culture media along with FBS or BSA supplementation on *in vitro* blastocyst production.

MATERIALS AND METHODS

Collection and processing of ovaries: Goat ovaries were obtained within 3–4 h of slaughter from the local abattoir and transported to laboratory in a thermos flask containing sterile warm physiological normal saline solution supplemented with antibiotics. All the ovaries were then subjected to washings (5–6 times) with warm saline fortified with antibiotics and then transferred into laminar flow for further processing.

Recovery of oocytes and in vitro *maturation:* The oocytes were collected from each ovary in to a petri plate containing oocyte collection media (Dulbecco's phosphate-buffered saline with 3 mg/ml BSA) by follicle puncture method with the help of a B.P. blade. Each petri dish containing oocytes was evaluated under a stereo zoom microscope and grade A, B, C oocytes were chosen for *in vitro* maturation by the method described by Kharche *et al.* (2008). Selected oocytes were washed 4 to 5 times in Oocyte Holding Medium (OHM) containing TCM-199 with Hepes modification, FBS (10%), Sodium Pyruvate (0.25 mM), Gentamycin (50 µg/ml), Glutamine three (100 µg/ml), BSA (3 mg/ml). The oocytes were then washed times with Oocyte Maturation Medium (OMM) containing TCM-199 supplemented with 10% FBS, Sodium Pyruvate (0.25 mM), Glutamine (100 µg/ml), LH (10 µg/ml), FSH (5 µg/ml), EGF (10 ng/ml), BSA (3 mg/ml) and Gentamycin (50 µg/ml). COCs were assigned for *in vitro* maturation in maturation medium covered with sterile mineral oil for 27 h in humidified atmosphere of 5% CO_2 at 38.5°C in the presence of 5% CO_2.

Semen collection and in vitro capacitation: Fresh semen was collected using the artificial vagina from a fertile pure bred adult Sirohi buck. The first and second seminal ejaculates were examined for volume, color, consistency and gross sperm and progressive motility. A sample of 100 µl fresh semen was diluted with 5 ml of sperm TALP medium containing 4 mg/ml fatty acid free BSA and washed twice by centrifugation at 1200 rpm for 5 min. Final washing was done with 1 ml fert TALP medium and 100 µl of pellet was added to 900 µl of fert TALP medium. Sperms were then kept for incubation in a CO_2 incubator in humidified atmosphere of 5% CO_2 at 38.5º C for 60 min in order to obtain swimmed up sperms and finally the top layer was used for further use in *in-vitro* fertilization.

In vitro *fertilization (IVF): In vitro* fertilization was carried out as per the method described by Kharche *et al.* (2011b) with slight modifications. After 27 h of culture, oocytes were separated from the cumulus cells by treating the complex with 0.1% hyaluronidase enzyme and passing it repeatedly through a fine pipette. Denuded oocytes were washed several times with Fert-TALP medium containing 10% FBS, 8 mg/ml fatty acid free BSA and 20 µg/ml heparin. Approximately 15–20 oocytes were transferred in each 50 µl drop of Fert-TALP medium. Finally, Fert TALP medium drops containing the matured oocytes were coincubated with 25–50 µl of the diluted semen so as to obtain a sperm concentration of 4×10^6 sperm/ml. The sperms and oocytes were co-incubated for 18 h under humidified atmosphere at 38.5°C in the presence of 5% CO_2.

In vitro *culture (IVC):* After 18 h of post-incubation, fertilized oocytes were washed in order to separate adhering sperm cells. In experiment 1, presumptive zygotes were washed, grouped and cultured in different embryo culture medium viz.

Group 1: mKSOM aa medium.
Group 2: TCM-199 with oviductal epithelial cells.
Group 3: mSOF aa medium.
Group 4: mCR_2 aa medium for 10 days in humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator.

In experiment 2, mKSOMaa medium from experiment 1 was selected for embryo culture to assess the effect of sequential embryo culture medium. Presumptive zygotes were grouped, washed and cultured in different sequential embryo development medium in a humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator.

Group 1: mKSOM aa medium containing 5% FBS for 1–3 days and 10% FBS for 4–10 days.
Group 2: mKSOM aa medium containing 0.8% BSA for 1–3 days and 10% FBS for 4–10 days.
Group 3: mKSOM aa medium containing 0.8% BSA for 1–10 days.
Group 4: mKSOM aa medium containing 10% FBS for 1–10 days in a humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator.

Evaluation of oocytes: Oocytes were observed after every 48 h for embryo development competence and half

of the culture medium was replenished with fresh medium until 10 days. Embryos were morphologically evaluated under inverted phase contrast microscope.

Statistical analysis: Cleavage rates and embryo development competence between the treatment groups were compared using the Chi-square test (χ^2). The level of significance was recorded at the 95% level of confidence (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

Oocytes (2,539) from ovaries (1,059) were recovered by follicle puncture technique, resulting with an average recovery of 2.39±0.11 oocytes/ovary. Two experiments were conducted to assess the effect of culture system on *in vitro* embryo development potential. Under experiment 1, effect of different embryo culture medium was evaluated and summarized in Table 1. In the present study, there was no statistically significant difference (P>0.05) in blastocyst production rate among mKSOMaa, TCM-199 plus OEC and mCR2aa medium, while being significantly higher (P<0.05) in comparison to mSOFaa medium. However numerically, more blastocysts were formed in TCM-199 + OEC as compared to mKSOMaa and mCR2aa medium. Statistical analysis also revealed significantly higher (P<0.05) morula formation rate in mKSOMaa medium in comparison with mCR2aa and mSOFaa medium. Similarly, no significant difference (P>0.05) was observed in morula formation rate between TCM-199+OEC, mCR2aa and mSOFaa medium. However, it was significantly higher in mKSOMaa as compared to mSOFaa medium. Findings also revealed that significant number of embryos got arrested at 8 cell stage in mSOFaa medium. However, significantly higher (P<0.05) cleavage rate was observed in embryos cultured in mSOFaa medium compared to TCM-199. In experiment 2, effect of different sequential and continuous embryo culture medium was evaluated and summarized in Table 2. In this experiment, significantly higher number of embryos (P<0.05) got arrested at 8–16 cell stage in only BSA supplemented embryo culture medium. Significantly higher (P<0.05) blastocyst and morula formation was observed in sequential embryo culture medium in comparison to BSA supplemented continuous embryo culture medium. However, no significant difference (P>0.05) was observed between the sequential and serum supplemented continuous embryo culture medium.

Data analysis revealed a non-significant difference (P>0.05) between different sequential embryo culture medium. However numerically, more number of blastocyst formed in sequential embryo culture medium (supplemented with BSA for 1–3 days and then with serum for 4–10 days) in comparison to group 1 sequential medium (supplemented with 5% serum for 1–3 days and 10% serum for 4–10 days).

Data analysis also revealed a non-significant difference (P>0.05) between different continuous embryo culture medium used. Numerically, more number of blastocyst and morula formed in continuous embryo culture medium supplemented with FBS (10%) in comparison to BSA supplemented embryo culture medium. Similarly, a non significant difference (P>0.05) in cleavage rate, 2 cell stage and 4 cell stage embryos was found between the different groups tested in our study.

In the present study, 3 embryo culture media (TCM-199+OEC, mKSOMaa, and mCR_2aa) supported the development of caprine embryos to the blastocyst stage. However, TCM-199+OEC and mKSOMaa were found to be better than mSOFaa embryo culture medium (P<0.05). It appeared that TCM-199 with oviductal epithelial cell co-culture has got beneficial effect, since these cells decrease the glucose and increase the pyruvate and lactate concentrations in the medium (Edwards *et al.* 1997, Kumar *et al.* 2007). Beneficial effects of co-culture with oviductal epithelial cells on blastocyst rates were previously reported (Katska *et al.* 2004, Kharche *et al.* 2008, Pawar *et al.* 2009, Singh *et al.* 2010). Co-culture with various somatic cell types is also used for *in vitro* embryo production systems and believed to provide necessary growth factors and/or metabolizes or sequestrate embryo toxic factors inhibitory to development (Kane *et al.* 1992), and reduction of oxygen concentration (Volkel and Hu 1992, Goto *et al.* 1994). In present investigation, as far as blastocyst production is concerned, results obtained in TCM-199 with somatic cell co-culture were similar to those obtained with mKSOMaa medium. Similarly, significantly higher number of morula (P<0.05) were developed in mKSOMaa medium in comparison to mCR2aa and mSOFaa medium. These results are in agreement with those obtained by Kharche *et al.*

Table 1. Developmental competence of *in vitro* fertilized caprine embryos cultured in different semi-defined and complex media

Treatment groups	No. of Oocytes	Cleavage	2-Cell	4-Cell	8-16 Cell	Morula	Blastocyst
mKSOMaa	226	85^a (37.61%)	11^a (12.94%)	15^a (17.65%)	19^a (22.35%)	29^a (34.12%)	11^a (12.94%)
TCM-199+OEC	297	99^b (33.33%)	17^b (17.17%)	17^a (17.17%)	24^b (24.24%)	28^b (28.28%)	13^a (13.13%)
mSOFaa	252	103^c (40.87%)	19^b (18.45%)	21^b (20.39%)	39^c (37.86%)	19^c (18.45%)	5^b (4.85%)
mCR2aa	299	115^a (38.46%)	18^c (15.65%)	26^c (22.61%)	35^d (30.43%)	25^d (22.61%)	11^c (9.57%)

(2010). They also found KSOM and TCM-199 to be superior to mSOF embryo culture medium.

In the present study, culture of presumptive caprine zygotes in mKSOMaa, TCM-199 plus co-culture and mCR2aa embryo culture media resulted in a non-significant difference (P>0.05) in embryo development competence. Yield of developmentally competent embryos was lower, when cultured in mSOFaa medium. Similar to present findings, Westberg *et al.* (2002) also reported that KSOM was capable of supporting the development of cattle zygotes to the blastocyst stage even in the absence of co-culture with somatic cells. Embryos cultured in mKSOMaa medium,TCM-199 with OEC and mCR2aa medium, all have development potential to reach to blastocyst stage as no significant difference was observed between groups. Similarly, Lv *et al.* (2009) observed no difference in cleavage rates and rates of blastocyst development for embryos cultured in SOF, CR1 or TCM-199 media containing 10% FBS.

In our study, a non-significant effect in blastocyst production among most of the media suggests that serum or its derivatives might have a greater effect on *in vitro* embryo production than the actual chemical composition of the medium. Considering the effects of protein supplementation on *in vitro* embryo development (Table 2), and non-significant effect among most of the media (Table 2), the selection of protein supplementation is probably a more important consideration for optimizing embryo production *in vitro*. Due to changes in the requirements of embryo during growth, the use of culture media with formulations more similar to the secretions found at different sites of reproductive tract during pre-implantation seems to be promising and has to led the concept that media components and physical conditions should be altered during culture to achieve improved development. Protein effect is more evident in a latter stage of embryo development as FBS provided significant embryo promoting effects on post cleavage embryo development compared to BSA. We observed that the per cent of 8–16 cell stage embryos was higher in media containing BSA thus indicating that FBS contains some components which are beneficial for caprine embryos to develop from 16 cell to blastocysts. Pinyopummintr and Bavister (1991) also reported that serum markedly inhibits the first cleavage division of fertilized ova *in vitro* and does not appear to benefit development from the 2-cell to the morula stage. Similarly in the present study, serum supplementation of the mKSOMaa medium from day 4 increased the blastocyst yield as also observed for buffalo embryos with mSOFaa medium (Kumar *et al.* 2007) and cattle embryos with CR2 medium (Wang *et al.* 1997). Nedambale *et al.* (2006) also found that culturing bovine embryos in KSOM medium until day 3 post fertilization with the addition of 1 mg/ml BSA increased the blastocysts rate and the cell number.

Addition of 5% FBS during cleavage phase did not significantly decrease cleavage rate compared to those supplemented with BSA. However, there was a significant increase (P<0.05) in the yield of blastocysts in media containing FBS during post cleavage culture as compared to culture media containing BSA suggesting a possible lack of embryo-stimulating properties of BSA. Serum not only stimulates blastocyst development, but also increases the percentage of hatched blastocysts. The enhancement by serum for development of morula into blastocysts is further substantiated by a significantly higher proportion of embryos retarded at the 16 celled stage in medium containing BSA as compared to FBS (Table 2). The advantages of the two-step embryo culture method of Pinyopummintr and Bavister (1994) and step-wise method of Zhang *et al.* (1992) to increase the concentrations of FBS

Table 2. Effect of sequential and continuous embryo culture media on *in vitro* embryo development competence of caprine oocytes

Culture medium	Treatment groups	No. of oocytes	Cleavage	2-Cell	4-Cell	8-16 Cell	Morula	Blastocyst
1.Sequential embryo culture medium	mKSOMaa containing 5% FBS (1-3 days) + 10% FBS (4-10 days)	325	120^{a} (36.92%)	13^{a} (10.83%)	21^{a} (17.50%)	33^{a} (27.50%)	37^{a} (30.83%)	16^{a} (13.33%)
	mKSOMaa containing 0.8% BSA (1-3 days) + 10% FBS (4-10 days)	583	239^{b} (40.99%)	32^{b} (13.39%)	45^{a} (18.83%)	58^{b} (24.27%)	69^{b} (28.87%)	35^{a} (14.64%)
2. Continuous embryo culture medium	mKSOMaa containing 0.8% BSA (1-10 days)	334	133^{b} (39.82%)	20^{c} (15.04%)	27^{b} (20.30%)	53^{c} (39.85%)	26^{c} (19.55%)	7^{c} (5.26%)
	mKSOMaa containing 10% FBS (1-10 days)	223	84^{a} (37.67%)	11^{b} (13.10%)	18^{c} (21.43%)	21^{b} (25.00%)	24^{b} (28.57%)	10^{c} (11.90%)

*Values in the same column with different superscripts differ significantly at P<0.05.

during post cleavage IVC culture, is substantiated by our study. These methods reduce the adverse effects of serum on early embryonic development while taking advantage of its embryotrophic attributes during post cleavage development. The results of this study indicate that the adjustment of serum concentrations to 5% during pre-cleavage culture and higher concentrations (l0–20%) during post -cleavage culture stages is an effective way to overcome the detrimental effects of serum on early-stage embryos while taking advantage of the embryotrophic properties during later stages. In this study, culture of presumptive caprine zygotes in sequentially serum supplemented culture medium yielded lower number of blastocyst and morula than those of serum supplemented culture media. This indicated that when serum is added to culture medium at a low concentration for first three days, supplementation with serum may not offer any disadvantage for the *in vitro* culture of caprine embryos. Contradictory to the present study, Kumar *et al.* (2007) found comparable or marginally better yield of transferable embryos in the absence of serum. It could be suggested that sequential culture system might provide better nutritional environment to the embryos at specific stage of early and late embryo developmental stages. These sequential media would mimic the change in environment experienced by the developing embryos *in-vivo*, enabling them to biochemical and morphological changes such as maternal zygotic transition, compaction, blastocoel formation and expansion (Swain *et al.* 2001). Garcia *et al*. (2007) compared *in vitro* embryonic development of bovine oocytes cultured in G1.3/G2.3 sequential culture media and CR1aa medium. Their study indicated that sequential medium supported higher maturation rate as compared with conventionally used CR1 medium and suggested that the requirements of embryos during developmental stages keeps changing. Similarly, Xu *et al.* (2004) has demonstrated higher quality for G1.2/G2.2-cultured mouse embryos compared to those cultured in KSOM or in CZB medium. The present findings are well comparable to above reports and reconfirm their findings.

Present study lead to the conclusion that both simple and complex embryo culture medium supported *in vitro* embryo development to reach blastocyst stage. A comparison of the 4 media in this study indicated that mKSOMaa, TCM-199 with OEC co-culture and mCR2aa had equal development competence to support pre implantation embryos to reach blastocyst stage ($P>0.05$). However, while considering the risk factor of contaminations associated with OEC co-culture, mKSOMaa was efficient medium for supporting the development of caprine zygotes to reach morula and blastocyst stages. Between the sequential and continuous embryo culture media, sequential embryo culture medium supported the development of caprine zygotes to reach morula and blastocyst stages ($P<0.05$).

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