Effect of different culture media and supplementation of oxidative uncoupler on the development of ovine embryos *in vitro*

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Abstract

In vitro embryo production (IVEP) in livestock species is important for breed conservation, rapid propagation of superior germplasm and many other potential downstream applications. Nevertheless, the efficiency of IVEP is substantially low in domestic animals, especially in ovine. In vitro culture (IVC) media is one of the most crucial factors that determines the success of IVEP. The current study aimed to assess the effect of different IVC media and supplementation of oxidative uncoupler 2, 4-dinitrophenol (DNP) on the in vitro development of ovine embryos. In the experiment 1, in vitro derived ovine embryos were cultured until 192 h in research vitro cleave (RVCL) and synthetic oviductal fluid (SOF-IVC) media or in early SOF (ESOF-IVC) medium until 96 h and then in late SOF (LSOF-IVC) medium until 192 h. In the experiment 2, in vitro derived ovine embryos were cultured in SOF-IVC medium until 96 h and then in SOF-IVC medium without or with supplementation of 5 and 10 μM of DNP until 192 h or embryos were cultured in SOF-IVC medium until 120 h and then in SOF-IVC medium supplemented with 5 μM of DNP until 192 h.

In the experiment 3, in vitro derived ovine embryos were cultured in RVCL medium until 120 h and then in SOF-IVC medium supplemented with 5 μM of DNP until 192 h. It was evident that RVCL medium yielded significantly ($p<0.05$) greater rate (%) of cleavage and the formation of 4-8 cell embryos (73.3±2.66 and 64.7±1.73 respectively) as compared to SOF (56.9±7.30 and 44.4±7.45 respectively) and ESOF-LSOF (43.9±3.26 and 39.4±3.18 respectively) media. In contrast, significantly ($p<0.05$) greater formation (%) of blastocysts was observed with the use of SOF (7.08±0.79) than RVCL (4.32±0.07) or ESOF-LSOF (3.67±0.61) media. In conclusion, RVCL resulted in better cleavage and formation of 4-8 cell embryos, but SOF supported better embryo development to blastocyst stage. DNP supplementation in SOF medium significantly improved blastocyst rate. However, sequential culturing of embryos in RVCL and DNP supplemented SOF media did not have any additional benefit towards blastocyst development.

Keywords: Culture media, oxidative uncoupler, embryo, development, ovine.

Introduction

The *in vitro* embryo production (IVEP) in domesticated livestock species is important and is crucial for many potential downstream applications like breed conservation, rapid propagation of the animals with superior genetic merits, genetic engineering for producing transgenic animals, establishing embryonic stem cell lines, conducting research on different aspects of developmental biology and treating infertility[11, 15, 20]. The design and preparation of *in vitro* culture (IVC) media is crucial in supporting the development of embryos for the success of IVEP[10].

Culture conditions influence the kinetics of development, development of inner cell mass and trophectoderm, metabolism and embryonic gene expression during the *in vitro* development[4]. Since the introduction of IVEP, several strategies have been designed to imitate the female uterine tract in the laboratory by supplementing crucial elements of female oviduct and uterus into the IVC media[10, 21].

Synthetic oviductal fluid (SOF) medium was developed based on the composition of sheep oviductal fluid nearly four decades ago and could be used successfully for culturing sheep and cattle embryos *in vitro*[29]. Later, it was widely adopted as such or with modified composition for IVEP in different species including cattle, pigs and goats[21]. During the *in vivo* development, embryos move from oviduct to uterus where the secretions and surrounding environment differ.

Therefore, to cater such requirements for embryo development *in vitro*, a two-step culture system with sequential media was developed for cattle[31] and human[2]. It is reported that the commercially available research vitro cleave (RVCL) medium supports better *in vitro* embryo development to blastocyst stage in goat and buffalo[18].

Production of excessive free radicals during *in vitro* embryo development causes oxidative damage and embryonic arrest which is the major problem of IVEP[1, 17]. Ruminant embryos experience developmental block at the 8-16 cells-stage during IVC, which is an irreversible phenomenon that may not results in immediate death of the embryos, but inhibits the overall outcome of IVEP[30]. It is suggested that the supplementation of metabolic inhibitors and oxidative uncoupler such as sodium azide and 2,4-dinitrophenol

(DNP) into culture media during the day-5 to day-7 of IVC can increase the proportion of embryos developing to compact morula and blastocysts[31].

With the standard protocols available currently, the efficiency of IVEP is substantially low in domestic animals especially in ovine. The rate of blastocyst formation following *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and IVC mostly varies from 10 to 40% in ovine[24, 25]. Therefore, it is necessary to find out the suitable conditions for efficient culturing of *in vitro* produced ovine embryos with better success. At present, meagre information is available on the comparative evaluation of different IVC media in supporting the *in vitro* development of ovine embryos. The current study aimed to assess the effect of different IVC media and supplementation of oxidative uncoupler (DNP) in IVC medium on the development of ovine embryos *in vitro*.

Material and Methods

All the chemicals used in the experiments were procured from Sigma-Aldrich Co., St. Louis, MO, USA, unless mentioned otherwise. All experiments including the use of ram for semen collection met the guidelines and regulations of the Institutional Animal Ethics Committee (IAEC) of the ICAR-National Institute of Animal Nutrition and Physiology (ICAR-NIANP), Bengaluru, India.

IVM of ovine cumulus-oocyte complexes (COC): Ovine COC were matured *in vitro* as described previously[8]. Briefly, ovine ovaries were collected from a local abattoir within 3 to 4 h of slaughter and transported to the laboratory in a thermos containing warm (~37 °C) normal saline supplemented with strepto-penicillin (1.6 g/l; Cadila Healthcare Ltd., Vadodara, India). COC were aspirated from the 2 to 6 mm follicles. The COC with more than five layers of compact cumulus cells and homogeneous cytoplasm were collected, washed and subjected to IVM in B199 medium supplemented with 0.01 U/ml of FSH, 0.02 U/ml of LH, 1 µg/ml of 17b-estradiol and 10% fetal bovine serum (FBS) for 24 h at 38.5 °C in a CO_2 incubator (5% CO_2 in a humidified environment).

IVF of matured ovine COC: IVF of matured ovine COC was performed according to the procedures described previously[8]. Briefly, following the 24 h of IVM, COC were washed and 10 to 12 COC in 10 µl of SOF-IVF medium were transferred into 38 µl fertilization drops of SOF-IVF medium. Subsequently, 2 µl of prepared spermatozoa was added into each fertilization drop for a final volume of 50 µl and final spermatozoa concentration of 1.5×10^6/ml and incubated for 24 h at 38.5 °C in a CO_2 incubator (5% CO_2 in a humidified environment).

To prepare spermatozoa for fertilization, semen was collected from a healthy adult ram on the day of IVF using an electro ejaculator for small ruminants[8]. Following collection, neat semen was diluted with milk-egg yolk extender as described previously[8]. The diluted sample was stored in a refrigerator (4 °C) for 4 h and spermatozoa were prepared for fertilization by percoll density gradient method[28].

IVC of ovine embryos: IVC of ovine embryos was performed according to the previously described method[8]. Briefly, at the end of the 24 h of sperm-oocyte incubation, cumulus cells were removed from COC. Presumptive zygotes were washed and 8-10 zygotes were cultured in 20 µl drops of RVCL (Cook®, Queensland, Australia), SOF-IVC, early SOF-IVC (ESOF-IVC) and late SOF-IVC (LSOF-IVC) media at 38.5 °C until 192 h in a CO_2 incubator (5 % CO_2 in a humidified environment). FBS (10 %) was supplemented into the culture drops from the 24 h of culture onwards.

The embryos were evaluated at 24, 120 and 192 h post-culture and different development stages were recorded. The commercially available RVCL medium was procured and the SOF-IVC, ESOF-IVC and LSOF-IVC media were prepared in-house for performing the experiments[7,22].

Experimental design

Experiment 1: In this experiment, the effect of RVCL, SOF-IVC and ESOF-IVC/LSOF-IVC media on the *in vitro* development of ovine embryos was assessed. Embryos were cultured until 192 h in RVCL (transferred in fresh medium at 96 h post-culture) and SOF-IVC (transferred in fresh medium at 96 h post-culture) or they were cultured in ESOF-IVC until 96 h and then in LSOF-IVC until 192 h.

The experiment was conducted in triplicate (N=115 in RVCL group; N=145 in SOF-IVC group; N=135 in ESOF-IVC/LSOF-IVC group).

Experiment 2: In this experiment, the effect of supplementation of DNP in SOF-IVC medium on the *in vitro* development of ovine embryos was assessed. Embryos were cultured in SOF-IVC until 96 h and then in SOF-IVC without (control) or with supplementation of 5 (group 1) and 10 µM (group 2) of DNP until 192 h. Additionally, embryos were cultured in SOF-IVC until 120 h and then in SOF-IVC supplemented with 5 µM (group 3) of DNP until 192 h. The experiment was conducted in triplicate (N=148 in control group; N=174 in group 1; N=136 in group 2; N=152 in group 3).

Experiment 3: In this experiment, the effect of combination of RVCL and SOF-IVC media on the *in vitro* development of ovine embryos was assessed. Embryos were cultured in RVCL medium until 120 h and then in SOF-IVC medium supplemented with 5 µM of DNP until 192 h. In the control group, embryos were cultured in SOF-IVC medium until 120 h and then in SOF-IVC medium supplemented with 5 µM of DNP until 192 h. The experiment was conducted in triplicate (N=120 in control group; N=133 in RVCL/SOF-IVC group)

Statistical Analysis: Statistical analyses were performed using the PASW 18.0.0 software package (SPSS/IBM, IL, USA). The values expressed in percentage for the rates of embryo developments were subjected to arcsine transformation before analyses to maintain homogeneity of variances[8]. Student's t-test was performed to analyse the variations in embryo development rates between the experimental groups. The data were presented as mean ± SE and differences were considered significant if the p value was less than 0.05.

Results and Discussion

The effect of different culture media on the development of ovine embryos was investigated in the experiment 1 to suggest a suitable culture system for the *in vitro* production of ovine embryos. Three different media were used for embryo culture and the results are depicted in figure 1. The use of RVCL medium yielded significantly ($p<0.05$) greater cleavage rate (%) and the formation (%) of 4-8 cell embryos (73.3±2.66 and 64.7±1.73 respectively) as compared to that of the SOF (56.9±7.30 and 44.4±7.45 respectively) and ESOF-LSOF (43.9±3.26 and 39.4±3.18 respectively) media.

In contrast, the use of ESOF-LSOF medium resulted significantly ($p<0.05$) lesser formation (%) morula (9.65±1.36) than SOF (31.9±5.60) and RVCL (34.4±1.85) media. On the other hand, significantly ($p<0.05$) greater formation (%) of blastocysts was observed with the use of SOF (7.08±0.79) than RVCL (4.32±0.07) or ESOF-LSOF (3.67±0.61) media.

The improvement of *in vitro* culture system is crucial for achieving better success of the IVEP[14]. The composition of culture media can significantly influence the *in vitro* development potential of embryos[5]. Previous reports indicate that RVCL is capable to support better cleavage and blastocyst formation as compared to SOF in buffalo and goat[16,18,27]. However, the current study revealed that RVCL medium supported better embryo development until the stage of 4-8 cells, but SOF supported better blastocyst formation. In contrast, among the three media evaluated, ESOF-LSOF was found most inferior in supporting *in vitro* development of ovine embryos.

Bovine embryos exhibit metabolic changes and increased ROS production at the morula stage[6]. Further, the formation of ROS depends on the composition of the embryo culture media and is associated with the rate of blastocyst formation[23].

Therefore, supplementing oxidative uncouplers in embryo culture medium to control the formation of ROS could be a possible option for improving the success of IVEP. DNP is an oxidative uncoupler and supplementation of 10 µM of DNP in culture media has been shown to improve embryo development to blastocyst stage in bovine[26,31].

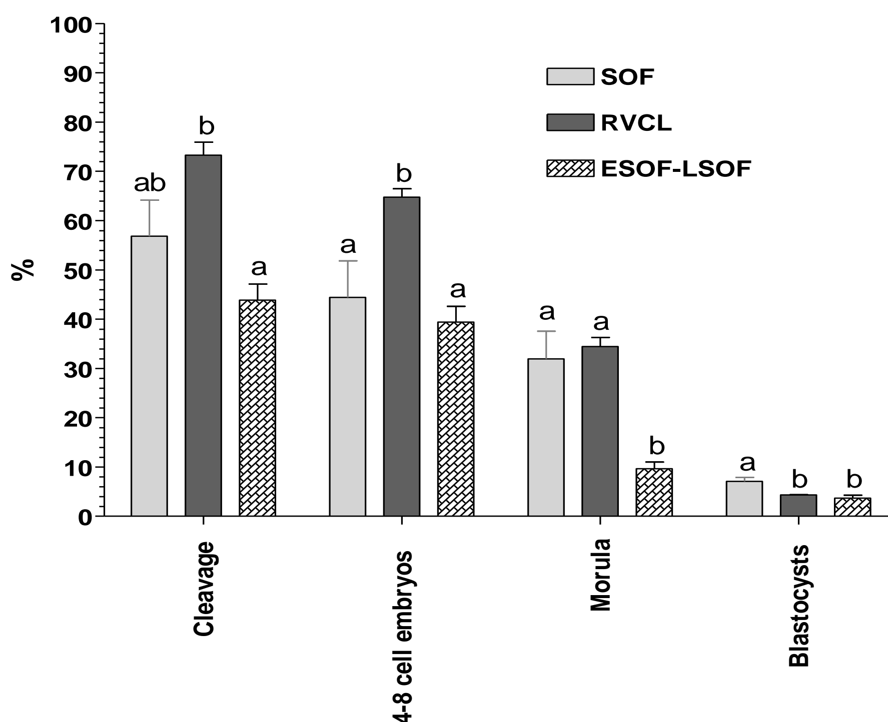

Figure 1: Effect of different *in vitro* culture media on the rate (%, mean±SE) of cleavage and formation of 4-8 cell embryos, morula and blastocysts. Ovine cumulus-oocyte complexes were matured and fertilized *in vitro* and presumptive zygotes were cultured *in vitro* in synthetic oviductal fluid (SOF), research vitro cleave (RVCL) or early SOF (ESOF)-late SOF (LSOF) media until 192 h. a, b above error bars indicate a significant difference (p<0.05) among the groups.

Hence, in the experiment 2, the effect of DNP supplementation around the time of morula formation on the development of ovine embryos was assessed (Figure 2). DNP was supplemented in SOF as the medium was found to be most superior in supporting the formation of ovine blastocysts. DNP was supplemented in the culture medium at 96 (5 and 10 µM) or 120 h (5µM) post-culture onwards. No significant differences in the rate of cleavage or formation of 4-8 cell embryos and morula were observed among the experimental groups.

In contrast, significantly ($p<0.05$) greater blastocyst rate (%) was observed with the supplementation of 5µM DNP at 120 h (12.1±1.11) as compared to that of the 10 (7.99±0.84) and 5µM (5.73±0.58) DNP supplementation at 96 h or no supplementation (6.24±1.26) groups.

It is important to reduce oxidative stress during *in vitro* culturing as it causes defective embryo development and numerous types of embryo damage[13]. Embryo metabolism produces ROS via several enzymatic mechanisms. *In vitro* culture conditions and exogenous factors increase the production of ROS by embryos and surroundings materials[13]. The relative influence of each ROS source appears different depending on the species, the stage of development and the culture conditions. ROS can alter several intracellular molecules and can impose development block and delayed development of embryos[13].
Previous studies report the favorable effects of DNP at low concentration on the *in vitro* development of bovine embryos[26,31]. Nevertheless, at high concentration, DNP decreases the mitochondrial ATP production and exerts negative effects on blastocyst development[12, 31]. The current study revealed that the supplementation of 5 µM of DNP in the culture medium during the period of 120 to 196 h of *in vitro* culturing was beneficial for the development of ovine embryos to blastocyst stage.

There is an increasing interest among the researchers to perform embryo culture in sequential media to augment the success of IVEP[9,11]. The sequential media are formulated based on the concept that embryo's metabolic capability and requirements for growth are different during the different development stages[3,19,32]. In contrast, the monoculture medium system uses a specific formulation to provide the necessary nutrients to support full embryo development[11].

In the current study, the experiments 1 and 2 revealed that RVCL medium was superior in supporting early stages of embryo development, but DNP supplemented SOF medium supported better blastocyst development. Therefore, in the experiment 3, the effect of combination of RVCL and DNP supplemented SOF media on the development of ovine embryos was assessed (Figure 3).

The embryos were cultured sequentially in RVCL until 120 h and then in DNP supplemented (5 µM) SOF until 192 h. In the control group, the embryos were cultured in SOF until 120 h and then in DNP supplemented (5 µM) SOF until 192 h.

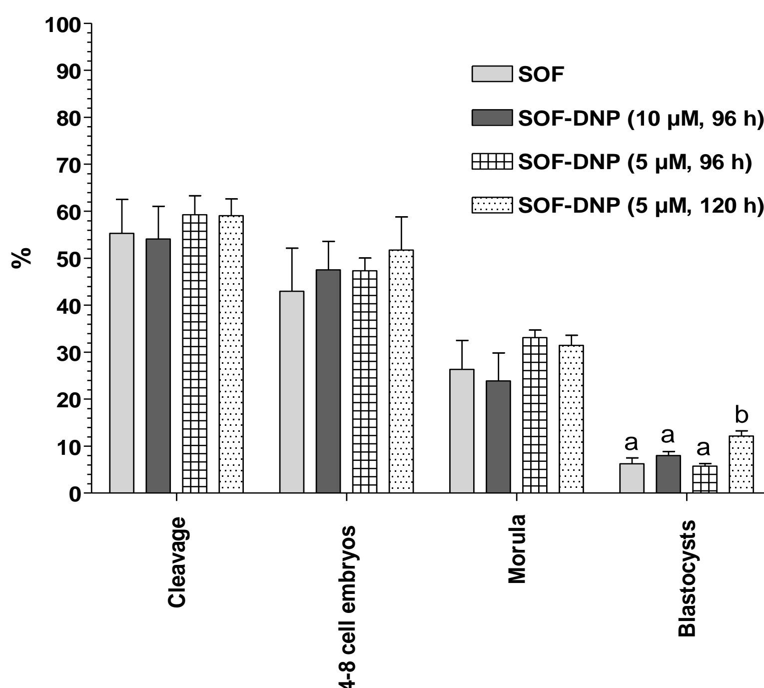

Figure 2: Effect of supplementation of 2, 4-dinitrophenol (DNP) in synthetic oviductal fluid (SOF) culture medium on the rate (%, mean±SE) of cleavage and formation of 4-8 cell embryos, morula and blastocysts. Ovine cumulus-oocyte complexes were matured and fertilized *in vitro* and presumptive zygotes were cultured *in vitro* in SOF until 192 h. DNP was supplemented in SOF at the 96 h (10 and 5 µM) or 120 h (5 µM) post-culture onwards. a, b above error bars indicate a significant difference ($p<0.05$) among the groups

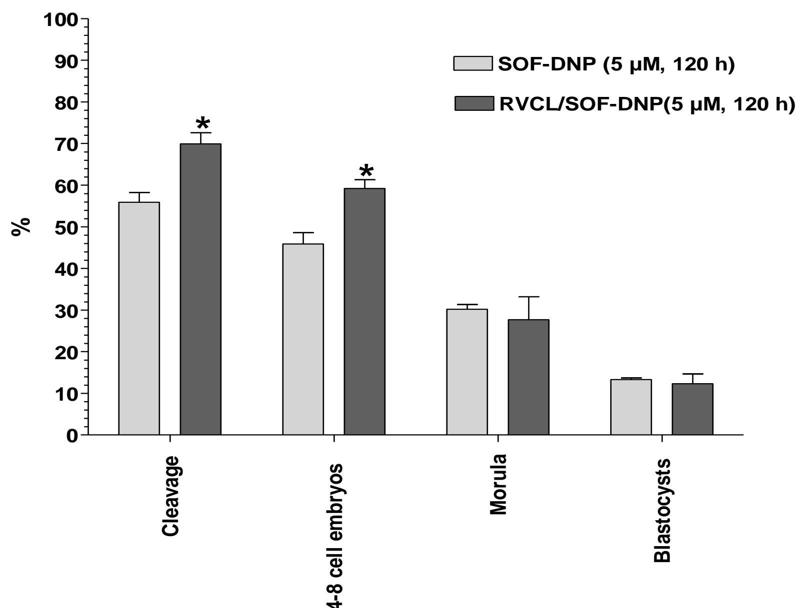

Figure 3: Effect of combination of research vitro cleave (RVCL) and synthetic oviductal fluid (SOF) culture media and supplementation of 2,4-dinitrophenol (DNP) on the rate (%, mean±SE) of cleavage and formation of 4-8 cell embryos, morula and blastocysts. Ovine cumulus-oocyte complexes were matured and fertilized *in vitro* and presumptive zygotes were cultured *in vitro* in RVCL or SOF medium until 120 h and then in DNP supplemented (5 µM) SOF medium until 192 h. * above error bar indicates a significant difference (p<0.05) between the groups

As expected, the rate (%) of cleavage and formation of 4-8 cell embryos were found significantly ($p<0.05$) greater in the RVCL-SOF group (69.9±2.64 and 59.2±2.08 respectively) as compared to that of the control group (55.9±2.35 and 45.9±2.74 respectively). In contrast, the formation of morula and blastocysts did not differ significantly between the RVCL-SOF and control groups. The results indicated that sequential culturing of the ovine embryos in the RVCL and SOF media supported better embryo development at the early stages. Nevertheless, the strategy did not have any additional benefits towards the embryo development at the late stages.

Conclusion

In conclusion, the effect of different IVC media and supplementation of DNP in IVC medium on the *in vitro* development of ovine embryos was assessed. It was evident that RVCL resulted in better cleavage and formation of 4-8 cell embryos as compared to SOF and the ESOF-LSOF media.

In contrast, SOF resulted better formation of blastocysts. The supplementation of 5 µM of DNP in SOF medium at the 120 h post-culture onwards further improved the formation of blastocysts. However, sequential culturing of embryos in RVCL and DNP supplemented SOF medium did not have any additional benefits towards the formation of blastocysts.

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