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Article

Supplementation of 9-cis retinoic acid in the in vitro maturation medium increase blastocyst development rate and quality

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Abstract: The 9-cis retinoic acid (9-cisRA) enhances early embryonic development in both in vitro and in vivo conditions. This experiment was conducted to evaluate the effect of supplementation of 9-cisRA in the in vitro maturation (IVM) medium on embryo development efficiency and embryo quality. For this purpose, immature cumulus oocyte complexes (COC) collected from slaughterhouse derived bovine ovaries were matured in three different IVM media (control group, DMSO group and DMSO+RA group). In the control group, base IVM medium were used without supplementation of 9-cisRA and DMSO. In the DMSO group, base IVM medium was supplemented with 0.5 µl DMSO per ml IVM medium without 9-cisRA. In DMSO+RA group, base medium was supplemented with 5 nm 9-cisRA dissolved in 0.5 µl DMSO. Data were analyzed using one way ANOVA method and means were compared using Duncan's multiple range test. Results showed that, supplementation of 9-cisRA in the maturation medium has no effect on embryonic development uptocleavage stage. However, blastocyst development rates (P>0.01), total blastomere number (P> 0.01), number of apoptotic blastomere per blastocyst (P>0.05) and percent of apoptotic blastomere per blastocyst (P>0.05) were significantly influences by 9-cisRA. In conclusion, 9-cisRA may be supplemented into the maturation medium for increasing bovinein vitro blastocyst development efficiency and blastocyst quality.

Keywords: bovine; in vitro embryo production; 9-cis retinoic acid; blastocyst; apoptosis

1. Introduction

The in vitro embryo production (IVP) and embryo transfer techniques have been adopted with traditional cattle breeding programme for multiplying high genetic merit cattle in many countries. The IVP enhancesgenetic gain in the traditional cattle breeding programme through increasing selection intensity and reducing generation interval (Camargo *et al.*, 2005; Marquez and Garrick, 2007; Manjunatha *et al.*, 2008). Considering these facts, Bangladesh Livestock Research Institute (BLRI) has adopted IVP technique (Deb *et al.*, 2016) with the aim of multiplication of high yielding dairy cows in Bangladesh. However, application of the technology required consistent embryo development efficiency. Consistent embryo development in *in vitro* condition depends on the efficiency of laboratory technician, culture condition, media used and many other factors. Retinoic acid (all-transRA and 9-cisRA) has been used in many *in vivo* and *in vitro* study for increasing reproduction efficiency of different laboratory and farm animal species. *In vivo* administration of retinoic acid increases birth numbers in rabbits (Besenfelder *et al.*, 1993) and litter size in swine (Coffey *et al.*, 1993). Moreover, supplementation of retinoic acid in the *in vitro* maturation medium affects blastocyst development rate, trophectoderm differentiation, total cell number and inner cell mass-trophoblast cell ratios (Hidalgo *et al.*, 2003; Gomez *et al.*,

2003). Efficiency of *in vitro* embryo development technology are generally defined by embryo development rate and blastocyst quality. Embryo development rates are defined by percent of blastocyst developed from number of immature oocytes set in the maturation medium or number of presumptive zygote set the in vitro culture medium. Blastocyst quality is generally predicted from their blastomere number including total blastomere numbers, proportion of inner cell mass and percent apoptotic blastomerein the blastocyst (van Soom *et al.*, 1997; Wrenzycki *et al.*, 2003; Jeong *et al.*, 2009). Number of blastomeres (total, inner cell mass, percent apoptotic cell) in a blastocyst determined their post-transfer development potential (van Soom *et al.*, 1997; Chang *et al.*, 2010). Considering the above facts, this study evaluated the effect of addition of 9-cis retinoic acid in the *in vitro* maturation medium on subsequent bovine *in vitro* blastocyst development rate and quality in Bangladesh.

2. Materials and Methods

This experiment was conducted in reproductive biotechnology laboratory of Biotechnology Division, Bangladesh Livestock Research Institute, Savar, Dhaka.

2.1. In vitro embryo development

During this study, blastocyst were developed in the laboratory as described in Deb et al., 2016.A short description of the IVP procedure was given below.

2.2. Ovary collection, oocyte aspiration and grading

Ovaries of slaughtered cows were collected from slaughterhouse located at Mohammodpur, Dhanmondi, Dhaka in physiological saline (0.9% sodium chloride supplemented with 100 IU/mL penicillin, 0.1 g/mL streptomycin sulfate) at ambient temperature. The ovary were transported to the laboratory within 4 to 5 h of slaughter. The cumulus-oocyte-complexes (COC) were aspirated using a 10-mL disposable syringe attached with a 21G needle. The aspirated material was poured onto a 100-mm Petri dish containing TL-HEPES (114-mM sodium chloride, 3.2-mM potassium chloride, 2-mM sodium bicarbonate, 0.34-mM sodium biphosphate, 10-mM sodium lactate, 0.5-mM magnesium chloride, 2.0-mM calcium chloride, 10-mM hepes, 1 µL/mL phenol red, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin) solution and the COC were searched under a steriomicroscope at low magnification (10x). The COC possessing an even cytoplasm and covered with minimum 3 layers of compact cumulus cells were selected for in vitro maturation (Stojkovic *et al.*, 2001). The selected COC (50 to 70 per well) were washed 2-3 times in TL-HEPES and 2-3 times in IVM medium (TCM199 + 10% FBS, 1 µg/mL β-estradiol, 10 µg/mL FSH, 0.6-mM cystein, and 0.2-mM sodium pyruvate). Then the selected COC were placed into a well of 4-well dish containing 500 to 700 µL IVM medium as per experimental design for 22 to 24 hr. The matured COC were fertilized in vitro by fresh semen collected from BLRI cattle Breed-1 (BCB-1) using artificial vagina method. Five micro litters (5 µL) semen were placed in a 15-mL conical tube containing 10 mL D-PBS and pelleted by centrifugation at 750 × g for 5 min. The supernatant was removed carefully and 10 mL D-PBS was added in the tube. The sperm was washed for 2-3 times accordingly. Then sperm were capacitated through incubation with 500 µL IVF medium (Tyrode's lactate solution supplemented with 6 mg/mL BSA, 22 µg/mL sodium pyruvate, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin) containing heparin sodium salt (20 µg/mL) for 15 min. After capacitation, the spermatozoa were diluted at approximately 1×10^6 spermatozoa/mL with IVF medium. The matured COC were co-cultured with capacitated spermatozoa for 18 to 20 h through placing them into IVF medium (500 to 700 µL per well of 4-well dish). After IVF, the cumulus cells were removed by gentle pipetting into TL-HEPES and the denuded presumed zygotes were placed into a 100 µL droplet of CR1-aa medium (Rosenkrans *et al.*, 1993), supplemented with 44 µg/mL Na-pyruvate, 14.6 µg/mL glutamine, 10 µL/mL penicillin/streptomycin, 3 mg/mL BSA and 310 µg/mL glutathione for 3 days (IVC-I). These were then cultured until day 8 of embryonic development in a medium of the same composition, except that the BSA was replaced with 10% (v/v) FBS (IVC II). The incubation conditions during IVM, IVF and IVC were 5% CO2 in air at 38.5°C with maximum humidity. The media (IVM, IVF and IVC) were pre-incubated for minimum two hours under the culture condition. Cleavage development rates were evaluated at day 3 (day 0: day of IVF) as a proportion of the presumed zygote transferred into IVC-I medium. Blastocyst development rates were calculated at day 8 as a proportion of the presumed zygote transferred into IVC-I medium. Blastocyst developed during each culture session were fixed in 4% (v/v) paraformaldehyde in PBS and kept at 4 °C until detection of blastomere numbers by terminal deoxynucleotidyl transferased UTP nick and Labeling (TUNEL) staining.

2.3. TUNEL staining

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Fluorescein; Roche Diagnostics Corp.; Indianapolis, IN; Cat. 1684795) as described by Deb *et al*. (2011). In brief, the fixed embryos were washed twice with 0.3% (w/v) polyvenylpyruvic acid (PVP) in 1-M PBS (PVP-PBS). Then embryos were incubated in a permeabilization solution (0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate) for 30 min at room temperature. The embryos were washed twice in PVP-PBS after permeabilization and incubated with fluorescence-conjugated dUTP and terminal deoxynucleotide transferase for 1 h at room temperature in the dark condition. The embryos were then washed in PVP-PBS and incubated in PVP-PBS containing 10 µg/mL Hoechst 33342 for 10 min. The embryos were washed twice in the PVP-PBS to remove excess Hoechst 33342. After washing in PVP-PBS, the blastocysts were mounted on glass slides under cover slips. Total number of blastomere were counted under an epifluorescence microscope equipped with a mercury lamp. Apoptotic blastomere were identified by their bright red fluorescence and total blastomere were identified by their green/blue fluorescence.

2.4. Data analysis

Data were analyzed using one way ANOVA procedure and mean differences were tested by Duncan's multiple range test.

2.5. Experimental design

To evaluate the effect of 9-cisRA on IVP efficiency, the cumulus-oocyte-complexes (COC) were matured in three different combinations of IVM medium. In the control group, neither 9-cisRA nor DMSO was used. In the DMSO group, 0.5 µl DMSO was added per ml IVM medium. In DMSO+RA group, the IVM medium was supplemented with 5 nm 9-cisRA. DMSO was used to dilute the 9cisRA.

3. Results and Discussion

Results showed no significant differences in cleavage rates among the three treatment groups. The genome of gamete is activated at around 8 cell stage in bovine. Until this stage the growth and development of the gamete is regulated by maternal factors borne by the oocytes. Therefore, development rate up to cleavage is generally not affected by culture condition and supplementation of medium (Deb *et al.*, 2011). The influence of culture condition, media and other factors has less influence on cleavage during IVP.During this study, no variations ($P>0.05$) were observed in blastocyst development rates between control and DMSO groups (Table 1). However, blastocyst development rates were increased ($P<0.01$) in DMSO+9-cisRA group over control and DMSO groups. The stimulatory effects of retinoic acid on blastocyst development that are found in this study are consistent with previous reports (Deb *et al.*, 2011; First and Parrish, 1987; Hidalgo *et al.*, 2003). DMSO, used as carrier of 9-cisRA have no effect on cleavage, blastocyst development rates and blastocyst quality. This data indicating that increased blastocyst development rate and blastomere numbers of blastocyst in the DMSO+RA group are associated with 9cisRA. Generally DMSO or absolute ethanols are used for dissolving 9-cisRA and they have no effect on in vitro embryo developments (Deb *et al.*, 2011).

Total blastomere numbers were increased in DMSO+RA group compared to contro and DMSO groups (Table 2). However, number of apoptotic blastomere ($P>0.05$) and percent of apoptotic blastomere per blastocyst ($P>0.05$) were decreased in DMSO+RA group compared to control and DMSO groups (Figure 1). These results are consistent with previous reports (Deb *et al.*, 2011; Hidalgo *et al.*, 2003; Gómez *et al.*, 2003).

The beneficial effects of RA during IVM are mediated by enhancing oocyte maturation through its effect on FSH or LH receptor expression (Hattori *et al.*, 2000), increasing mRNA quality and processing (Gomez *et al.*, 2004), growth factors signaling (Gómez *et al.*, 2003) by endogenous oxidative-stress protection mechanism (Guerin, 2001) and /or prevention of apoptosis (Deb *et al*., 2011).

Table 1. Effect of supplementation of 9-cis retinoic acid in to in vitro maturation medium on cleavage and blastocyst development rates.

Treatment	Presumed zygote	% Cleaved (Mean±SD)	% Blastocyst (Mean±SD)
Control	115	76.73±10.03	21.10b±1.02
DMSO	105	74.30±2.59	20.20b±1.27
DMSO+RA	300	78.23±3.07	31.98a±3.82
Significance		Non-significant	P> 0.01

Table 2. Effect of supplementation of 9-cis retinoic acid in to in vitro maturation medium on blastomere number and apoptosis in blastocyst.

Treatment	N	Total blastomere numbers per blastocyst (Mean±SD)	Number of apoptotic blastomere per blastocyst (Mean±SD)	% Apoptotic blastomere per blastocyst (Mean±SD)
Control	15	125.73[b]±21.33	6.93b±2.66	5.62b±2.22
DMSO	15	127.73[b]±20.38	6.60b±2.41	5.22b±1.96
DMSO + RA	15	141.87[a]±15.92	4.20[a]±2.21	3.07[a]±1.77
Significance		P> 0.01	P>0.05	P>0.05

[a,b] Figure with different superscript in the same column differ significantly.

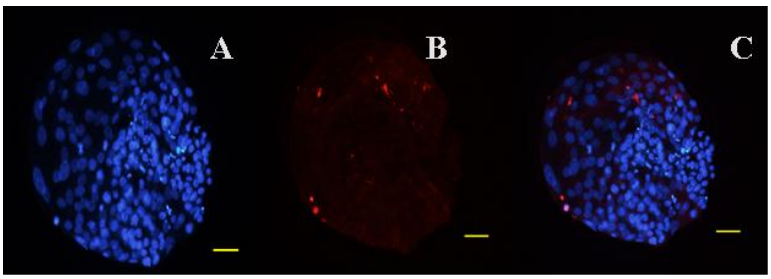

Figure 1. Representative photograph showing total blastomeres (A), apoptotic blastomeres (B) and merged of picture A and B. The blastocyst was stained using TUNEL procedure. Arrowhead showing apoptotic blastomere.

4. Conclusions

Supplementation of 9-cisRA in the in vitro maturation medium increased blastocyst development rates. Moreover, 9-cisRA also increased blastocyst quality by increasing total blastomere number and by decreasing apoptosis in the blastocyst.

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