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Effect of cytochalasin B during oocyte maturation for parthenogenetic embryos generation in goat

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ABSTRACT

The present experiment was conducted to study the effect of cytochalasin B (CCB) in maturation medium for parthenogenetic embryos generation in caprine (*Capra hircus*). The cumulus oocyte complexes (COC) were isolated, evaluated, graded and matured in maturation medium having different concentration of CCB (0 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml). Matured oocytes were activated by 7% ethanol for 5 min followed by incubation with 2mM 6-dimethyl amino purine (DMAP) for 4 h and cultured in modified synthetic oviductal fluid (mSOF) in CO_2 incubator at 37°C, 5% CO_2, and 95% relative humidity. The cleavage rate and further embryo development was recorded after 48 h and then 96 h of *in vitro* culture. The overall oocyte recovery rate was 1.13±0.02/ovary. The overall cleavage rate was 77.22±1.04 and percentage of 2–4 cell, 8–16 and morula stage embryos production were 22.90±1.38, 34.46±0.89 and 42.64±1.04, respectively irrespective of different concentration of CCB used in maturation medium. There was nonsignificant difference of cleavage rate among different concentration of CCB used. The embryos development beyond 16 cell stage was higher in 15 µg/ml CCB compared to other concentration of CCB but nonsignificant compared to control (0 µg/ml CCB). The presence of CCB all throughout maturation (27 h) did not inhibit cleavage rate as well as embryo development, rather increased the embryo development beyond 16 cell stage.

Key words: Caprine, Cytochalasin B, Embryos, Ethanol, Maturation media, Parthenogenesis

In many invertebrates parthenogenesis occur as a natural process in the life history. In many sexually reproducing animals, the activation of egg by artificial methods to start the development without fertilization is called artificial parthenogenesis. Physical stimuli, temperature, chemical stimuli or stimulus provided by sperm entry are the stimulus for artificial parthenogenesis. Parthenogenetic embryos were generated in pig (Yi and Park 2004), sheep (Loi *et al.* 1998), bovine (Fukui *et al.* 1992), goat (Pankaj *et al.* 2012) and buffalo (Venkatesh 2009, Sathisha 2009).

Parthenogenesis does not occur in goats naturally, but artificially parthenogenetic embryo can be produced by various methods. Parthenogenetically produced goat embryos are important tools to study the origin of goat, phylogeny and genes regulating the growth and development of early goat embryos by comparing IVF and *in vivo* produced embryo. Stem cells production from parthenogenetic goat embryo is cost effective and these are important in therapeutic and research fields in future. It is postulated that in mammal, parthenogenetic embryos fail to produce live birth because of haploidy and lack of paternal genomes (Young *et al.* 2003). Cytochalasin B (CCB) inhibits first polar body extrusion creating diploid heterozygous parthenogenetic embryos in mice (Jacek *et al.* 1991) and this chemical can also be used for diploid parthenogenetic embryos in caprine also (Ranjan *et al.* 2013). Most of the researchers used CCB in embryo development media after oocyte maturation to prevent second polar body for the production of parthenogenetic embryos. In this study we used CCB in maturation medium to prevent first polar body extrusion for the generation of heterozygous diploid parthenogenetic embryos.

So, the present experiments were conducted to study the effect of cytochalasin B in maturation media for parthenogenetic embryos generation in caprine (*Capra hircus*).

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MATERIALS AND METHODS

Ethics: All the experiments were approved by ethics committee of Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India.

Oocytes collection and processing: Goat ovaries were collected from the local slaughter house and transported to

the laboratory in normal saline solution fortified with gentamycin (10µg/ml) in a thermo flask at 37°C within 2 h of slaughter. Oocytes were aspirated from all visible non-atretic follicles by aspiration method using oocyte collection media (OCM) (pH 7.3–7.4, 280–290 mOSM/kg; NaCl-136.89mM; KCl-2.68mM; KH_2PO_4 -1.46mM; Na_2HPO_4–8.09mM; $CaCl_2H_2O$-0.90 mM;$MgCl_2$ (anhydrous)-1.00 mM; D- glucose-5.54 mM; sodium pyruvate- 0.32 mM; gentamicin (50µg/ml); phenol red-10µl/ml; working OCM 3mg/ml BSA (V), 10% EGS). The COCs were searched out, evaluated and graded**.** Only excellent (i.e., more than 5 layers of cumulus cell and evenly granulated cytoplasm) and good quality (more than 3 layers of cumulus cell and evenly granulated cytoplasm) COCs were collected and drop washed 6 to 7 times with OCM and finally with maturation media (MM-TCM-199 with HEPES media, 10% FBS, 10% goat follicular fluid, 0.5µg/ml FSH, 10µg/ml LH, 0.5µg/ml estradiol)) having different concentration of CCB (0 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml) (Ranjan *et al*. 2013).

In vitro *maturation, activation and culture of oocytes:* Oocytes were matured in MM having different concentration of CCB. The droplets were covered with warm non toxic paraffin oil and cultured at 37°C, 5% CO_2 for 27 h. After 27 h of incubation, the extent of oocytes maturation was evaluated based on the visual assessment of degree of cumulus expansion under inverted microscope (Kobayashi *et al*. 1994). Matured oocytes were activated by 7% ethanol for 5 min followed by incubation with 2mM DMAP for 4 h. The activated oocytes were taken out of 6-DMAP drop, washed several times in modified synthetic oviductal fluid (mSOF- NaCl-107.70 M; KCl-7.16mM; KH_2PO_4– 0.35mM $CaCl_2H_2O$-1.71mM; $MgCl_2$ (anhydrous)-0.49mM; sodium lactate-3.30mM; $NaHCO_3$–25.07 mM; sodium pyruvate- 0.30mM; L-glutamine-0.20mM; gentamicin (50µg/ml); phenol red-10µg/ml); working solution: mSOF stock solution up to 10 ml**-** BSA fraction V (0.8%) NEAA-50 µl; EAA-100 µl; ß-merceptaethanol (0.1mM); IGF1 (100ng/ml) and cultured in 100µl of same media in CO_2 incubator at 37°C, 5% CO_2, and 95% relative humidity. The cleaved oocytes were subsequently transferred in fresh drop of mSOF for further embryo development. The cleavage rate and further embryo development were recorded after 48 h and then 96 h of *in vitro* culture.

Statistical analysis: The embryo development was recorded as percentage of cleaved oocytes. The data were analyzed by applying one-way ANOVA using the SPSS 16 computer program as different variables and results were presented as mean±SE.

RESULTS AND DISCUSSION

Culturable cumulus oocyte complexes (COCs; 4,059) were harvested from 3,592 goat ovaries by aspirating the surface follicles. The overall oocyte recovery rate was 1.13±0.02 / ovary, which was higher (0.98) than the earlier reports of Pankaj *et al.* (2012). The lower oocyte recovery rates might be due to the different lots of ovaries used, different season as well as the status of the animals. Goats show an evident reproductive seasonality and seasonal variations in ovulation (Duarte *et al*. 2008) and might have attributed to more COC recovery rate.

The overall cleavage rate was 77.22±1.04 and percentage of 2–4 cell, 8–16 and morula stage embryos production were 22.90±1.38, 34.46±0.89 and 42.64±1.04, respectively, irrespective of different concentration of CCB used in maturation media. There was nonsignificant difference ($P<0.05$) of cleavage rate among different concentration of CCB used. The overall percentage of 2–4 cell stage embryo production was 18.08±2.76, 35.22±3.70, 29.65±1.71, 17.35±1.42 and 29.18±3.51, respectively, in different concentration of CCB (0 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml) used in maturation media followed by activation with ethanol (7%) and 6-DMAP. The overall percentage of 8–16 cell stage embryo production was 38.38±2.56, 29.72±3.57, 29.56±1.25, 36.97±0.97 and 30.85±1.40, respectively, in different concentration of CCB, viz. 0 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml. The overall percentage of morula stage embryo production were 43.54±2.97, 34.96±3.45, 40.79±1.63, 45.68±1.33 and 39.96±2.41, respectively, in different concentration of CCB viz. 0 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml. The further embryo developments were higher ($P<0.05$) in 15µg/ml CCB compared to other concentration of CCB (Fig. 1) but nonsignificant ($P<0.05$) with control condition of 0 µg/ml CCB.

The cleavage rate was significantly higher following ethanol + 6-DMAP, ethanol + cyclohexamide and ethanol + cyclohexamide + 6-DMAP activation (52.5%, 52.5% and 44.4%, respectively) (Mishra *et al*. 2008). Oocytes activated by treatment with ethanol (7%) followed by 4 h incubation in 2mM 6-DMAP had greater cleavage rate (58%) as compared to *in vitro* fertilized oocyte (47.2%) in goat (Ongeri *et al*. 2001). The earlier studies in our laboratory also showed that ethanol activation induces high development rates of parthenogenetic embryos in caprine. By using ethanol and DMAP as activating media for goat oocyte, about 72% cleavage rates was achieved (Pankaj *et al*. 2012). This chemical was found most effective for inducing parthenogenesis, presumably because it mimics fertilization very closely (Jones *et al*. 1995, Kim *et al*. 1996). Ethanol induces both extracellular and intracellular calcium (Loi *et al*. 1998) and promotes single intracellular calcium increase of greater and longer amplitude than initial increase observed at fertilization (Grupen *et al*. 2002). It was also reported that ethanol-induced repetitive transient intracellular Ca^{2+} concentration increases in activated mouse oocytes (Deng and Fan 1994). An extended incubation in 6-DMAP impaired the development of goat parthenotes, quickened both the release from metaphase arrest and the pronuclear formation, and inhibited the chromosome movement at anaphase II (A-II) and telophase II (T-ll), leading to the formation of one pronucleus without extrusion of second polar body (Lan *et al*. 2005).

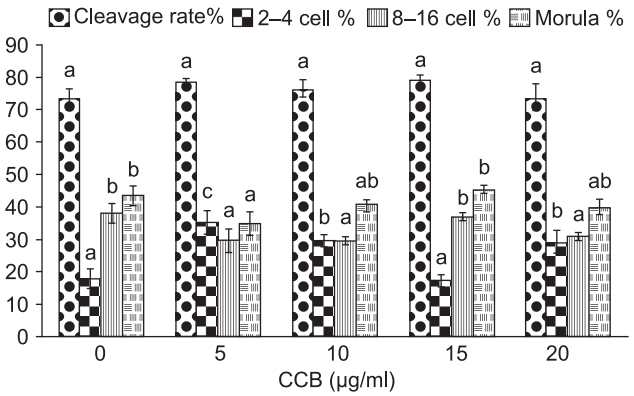

Fig. 1. Effect of different concentration of CCB during oocyte maturation on parthenogenetically embryos generation in caprine (mean±SE).

The presence of CCB all throughout maturation (27 h) did not inhibit cleavage rate as well as embryo development, rather increased the embryo development beyond 16 cell stage (Fig. 1). In earlier studies it was observed that short term treatment of bovine eggs after maturation with this drug does not impair further embryonic development (Fukui *et al*. 1992). The present results could not be explained as comparable studies are not available in any species. Diploid parthenogenetic mouse embryos produced by heart shock and CCB developed beyond implantation (Hanna and Tarkowski 1976). In porcine, activation of oocytes with CCB alone or in combination with cyclohexamide and DMAP, produced high percentage of parthenogenetic embryos (Yi and Park 2004). It was also reported that CCB in combination with cyclohexamide and DMAP after initial treatment with ethanol, strontium or calcium ionophores increase the cleavage rate and blastocysts development in porcine (Yi and Park 2004). When porcine oocytes matured for 22 h were activated with 8% ethanol in NCSU-23 medium followed by 3 h incubation supplemented with 7.5 µg/ml CCB, the cleavage rate was 48% compared to 24.9% when ethanol alone was used for activation. Chemicals like CCB is an actin polymerization inhibitor, shortens actin filaments by blocking monomer addition at the fast-growing end of polymers and in the presence of CCB, segregation of the chromosomes occurred, but cytokinesis does not take place. Therefore, this chemical was used to produce diploid zygote with 2 pronuclei (Liu *et al*. 1998). Cytochalasin B may also help to prevent fragmentation of embryos (Yang *et al*. 1992).

The presence of CCB all throughout maturation (27 h) did not inhibit cleavage rate as well as embryo development, rather increased the embryo development beyond 16-cell stage. So, this chemical may be used for parthenogenetic embryos production.

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