



Effect of different activation protocol on generation of parthenogenetic embryos in caprine

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Received: 24 January 2012; Accepted: 21 February 2012

ABSTRACT

The present study was carried out to compare the different activation protocol for generation of parthenogenetic embryos in caprine. For parthenogenetic embryo production, 6 activation protocols, viz. 7% ethanol in modified synthetic oviductal fluid (mSOF) for 5 min (P-I), 5µM ionomycin in mSOF for 5 min (P-II), 27 h maturation in maturation media containing 10 and 20 µg/ml cytochalasin B (CCB) followed by activation with (a) 7% ethanol in mSOF for 5 min (P-III, P-IV), or (b) 5µM ionomycin in mSOF for 5 min (P-V and P-VI). All these treatments were followed by 4 h incubation with 2mM 6-dimethylaminopurine (6-DMAP) in mSOF. The activated oocytes were further cultured in mSOF for embryo development. The cleavage rate was higher when oocytes were activated by ionomycin than ethanol but further embryo development was better in ethanol activated oocytes. When oocytes were matured in presence of CCB followed by ionomycin or ethanol activation, the cleavage rate and embryo development did not change significantly and was almost similar to when oocytes were activated simply by ethanol or ionomycin. The study indicated that ethanol and ionomycin alone or even in combination with cytochalasin B induced activation and parthenogenetic development of *in vitro* matured caprine oocytes.

Key words: Caprine, Cytochalasin B, Ionomycin, Ethanol, Parthenogenetic embryo

Parthenogenesis is a reproductive strategy in some species where a female gives birth to offspring without a paternal contribution. Thus far, parthenotes obtained *in vitro* have been studied and transferred in the uterus of recipient female in a variety of mammals including mice, sheep, cow, pigs and monkeys (Kono *et al.* 1996, Loi *et al.* 1998, Boediono and Suzuki 1994, Kawarasaki *et al.* 2009, Cibelli *et al.* 2002). Some studies showed that mouse parthenotes can develop beyond implantation until the forelimb bud stage Kono *et al.* (1996); rabbit parthenotes until day 11th post activation (Kure-bayashi *et al.* 2000) while parthenotes from primates have only been shown to reach the implantation stage (Cibelli *et al.* 2002). Chemical activating stimuli such as ethanol (Nagai 1987, Yang *et al.* 1992), calcium ionophore, ionomycin (Jones *et al.* 1995) strontium chloride etc were used to induce the artificial activation of mammalian oocytes. The histone kinase inhibitor, 6-dimethylaminopurine (6-DMAP), accelerated and enhanced the formation of pronuclei in non-age metaphase II oocytes from mice and cattle (Liu *et al.* 1998). Chemicals like cytochalasin B (CCB) is actin polymerization inhibitor, shortens actin filaments by blocking

monomer addition at the fast-growing end of polymers and in the presence of cytochalasin segregation of the chromosomes occurred, but cytokinesis does not take place (Presicce and Yang 1994a, b, Liu *et al.* 1998). Therefore, this chemical can be used to produce parthenogenetic embryos with diploid. It is observed that when matured bovine oocytes were activated in presence of CCB, diploid zygotes with 2 pronuclei (Presicce and Yang 1994a, b, Liu *et al.* 1998) were formed. However, it was not found whether oocytes matured in presence of CCB would go for cleavage and further embryo development with diploidy in livestock. Further, there is very scanty information available on parthenogenetic embryo development in caprine. Therefore, the present study was carried out to investigate the (i) effect of different activation protocol, (ii) effect of cytochalasin B during oocyte maturation on generation and developmental competence of parthenogenetic embryos in caprine.

MATERIALS AND METHODS

All the experiments were approved by the Indian Veterinary Research Institute, Izatnagar, Bareilly ethics committee.

Materials: Chemicals and media were purchased commercially.

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Recovery and in vitro maturation of oocytes: Goat ovaries collected from goats of unknown reproductive status slaughtered at local abattoir were carried to the laboratory in normal saline solution (NSS) (0.85%) fortified with antibiotic in a thermos flask at 35–37°C within 2 h of slaughter. In laboratory, the extra ovarian tissues were trimmed off and ovaries were washed several times with NSS (35–37°C). Cumulus oocyte complex (COCs) were aspirated from all visible non atretic follicles by an 18 gauge needle attached to 5 ml syringe containing oocyte collection media (OCM). The COCs along with follicular fluid were pooled into 50 ml sterile plastic tube and were allowed to settle for 10 min. Sediments were taken in large petridish (90 mm) and the COCs were searched under zoom stereomicroscope. Only excellent (i.e., more than 5 layers of cumulus cells and evenly granulated cytoplasm) and good (more than 3 layers of cumulus cells and evenly granulated cytoplasm) COC were selected and drop washed several times in OCM. The COCs were further drop-washed 5–6 times with *in vitro* maturation (IVM) medium. Groups of 15–20 selected oocytes were placed in 60 µl droplets of IVM medium. IVM medium was TCM 199, supplemented with 10% fetal bovine serum (FBS), bovine serum albumin (BSA) (3mg/ml), FSH (0.5 µg/ml); LH (10 IU/ml); goat follicular fluid (5%); L- glutamine (0.1mg/ml) and gentamycin (50 µg/ml). The droplets were covered with warm non-toxic mineral oil and cultured at 37°C, in an atmosphere of 5% CO₂ and 20% oxygen under humidified air (95%) for 27 h in CO₂ incubator. The evaluation of maturation were based on the visual assessment of degree of cumulus expansion under inverted microscope (Kobayashi *et al.* 1994).

Oocyte activation

After maturation, the oocytes were treated with 0.1% hyaluronidase in TCM-199 followed by pipetting to remove the cumulus cells. Finally cumulus free oocytes were activated using one of the protocols given below:

Experiment 1: Two protocols given below were without cytochalacin B

Protocol I: 7% ethanol in mSOF for 5 min followed by 4h incubation with 2mM 6-DMAP in mSOF.

Protocol II: 5µM Ionomycin in mSOF for 5 min followed by 4 h incubation with 2mM 6-DMAP in mSOF.

Experiment 2: Oocytes were matured for 27 h in

maturation media supplemented with different concentrations of cytochalacin B and then activated:

Protocol III: 27 h maturation in maturation media containing 10 µg/ml cytochalacin B followed by activation with 7% ethanol in mSOF for 5 min then 4 h incubation with 2mM 6-DMAP in mSOF.

Protocol IV: 27 h maturation in maturation media containing 20 µg/ml cytochalacin B followed by activation with 7% ethanol in mSOF for 5 min then 4 h incubation with 2mM 6-DMAP in mSOF.

Protocol V: 27 h maturation in maturation media containing 10 µg/ml cytochalacin B followed by activation with 5µM ionomycin in mSOF for 5 min then 4 h incubation with 2mM 6-DMAP in mSOF.

Protocol VI: 27 h maturation in maturation media containing 20 µg/ml cytochalacin B followed by activation with 5µM ionomycin in mSOF for 5 min then 4 h incubation with 2mM 6-DMAP in mSOF.

In vitro embryo development: After activation treatment, oocytes were taken out of 6-DMAP drop, washed several times in modified synthetic oviductal fluid (mSOF) supplemented with 0.8% BSA and essential and non-essential amino acids and cultured in 100µl of same media in CO₂ incubator at 38°C, 5% CO₂, 20% O₂ and 95% relative humidity until assessment to determine cleavage rates. For embryo development, cleaved oocytes were subsequently transferred in fresh drop of mSOF and were cultured up to 5 days. Observations were made to see the cleavage rate and embryo development at different time interval (48 and 96 h) after activation for each activation protocol.

Statistical analysis: Data were collected for each activation treatment represented. The cleavage rate was recorded as a percentage of total treated oocytes. Further, embryo development was recorded as percentage of cleaved oocytes. The data were analyzed by use of one-way ANOVA using the SPSS 16 computer program as different activation protocols as variables and results have been presented as mean±SE.

RESULTS AND DISCUSSION

The cleavage rate was (P<0.05) higher when oocytes were activated by ionomycin than ethanol (Table 1). When oocytes were matured in presence of CCB (10 or 20 µg/ml) followed by ionomycin or ethanol activation (P-II to P-VI), the

Table 1. Effect of different activation protocol on generation of parthenogenetic embryos in goat

Protocols	Treatment	No of oocytes	% Cleavage	% 4–8 cell	% 8–16cell	% Morula
P-I	Ethanol+6-DMAP	305	218(72.68±4.17) ^a	165(77.01±5.00) ^b	70(32.33±1.96) ^b	121(53.69±4.01) ^{bc}
P-II	Ionomycin+6-DMAP	220	185(86.19±3.77) ^b	140(78.00±6.19) ^b	51(29.94±2.07) ^b	83(37.45±7.09) ^a
P-III	10µg CCB+ethanol+6-DMAP	111	81(74.99±4.78) ^{ab}	57(69.34±3.88) ^a	15(19.40±4.51) ^a	34(41.09±3.25) ^{ab}
P-IV	20µg CCB+ethanol+6-DMAP	106	83(77.95±1.99) ^{ab}	61(73.94±1.94) ^{ab}	18(21.65±1.48) ^a	33(39.86±3.93) ^{ab}
P-V	10µg CCB+ionomycin+6-DMAP	105	85(81.20±4.97) ^{ab}	68(82.20±4.50) ^c	22(26.88±3.54) ^{ab}	41(46.46±5.42) ^{abc}
P-VI	20µg CCB+ionomycin+6-DMAP	116	94(83.08±4.30) ^{ab}	70(74.27±8.25) ^{ab}	21(20.26±4.28) ^a	55(57.05±5.42) ^c

cleavage rate did not change significantly ($P>0.05$) and was almost similar to when oocytes were activated simply by ethanol or ionomycin (P-I, P-II). But the cleavage rate were comparatively higher when oocytes were cultured in presence of CCB (10 or 20 $\mu\text{g/ml}$) followed by ionomycin treatment (P-V, P-VI) as compared to ethanol activation (P-III and IV) preceded by oocyte culture with similar concentration of CCB (Table 1).

The number of 4–8 cell stage cell stage embryo after 48 h of activation differ ($P>0.05$) among the different activation protocols although it was comparatively higher when oocytes were matured in presence of 10 $\mu\text{g/ml}$ CCB followed by ionomycin activation (P-V) than any other activation protocol (Table 1).

The percentage of 16 cell stage embryos did not differ significantly when oocytes were activated by ethanol or ionomycin (P-I, P-II) alone (32.33 ± 1.96 vs 29.94 ± 2.07). But CCB treatment with 10 or 20 $\mu\text{g/ml}$ (except P-V) during maturation significantly reduced the percentage of 16 cell stage embryos in P-III, P-IV, P-VI ($P<0.05$) (Table 1).

The percentage of embryos with >16 cell stage was significantly higher ($P<0.05$) when oocytes were activated with ethanol (53.69 ± 4.01) as compared to ionomycin treatment (37.45 ± 7.09). CCB treatment did not significantly alter the development of activated oocytes beyond 16 cell stage when compared with activation with either ethanol or ionomycin. Further, CCB+ ionomycin treatment produced comparatively higher percentage (P-V and P-VI) of >16 cell stage parthenogenetic embryos as compared to CCB+ethanol (in P-III and P-IV), however the difference was nonsignificant among P-III, P-IV, P-V as well as P-V and P-VI. The results also indicated that 20 μg CCB + ionomycin (P-VI) as well as only ethanol activation (P-I) resulted comparatively higher number of >16 cell stage embryos as compared to other protocols.

In the present study, we compared parthenogenetic activation treatments by ethanol which causes single intracellular calcium increase of greater and longer amplitude than initial increase observed at fertilization (Nakada and Mizuno 1998). It was also reported that ethanol induced repetitive transient intracellular Ca^{2+} concentration increases in activated mouse oocytes with ionomycin which causes single intracellular Ca^{2+} rise in caprine oocyte (Jellerette *et al.* 2006).

Our results suggested that ethanol and ionomycin

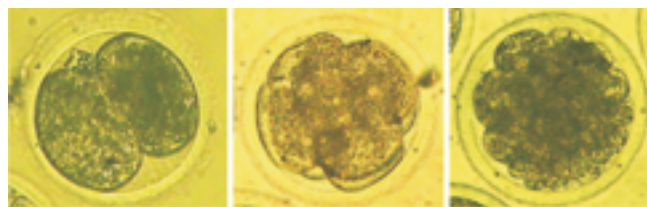


Fig. 1. Parthenogenetic embryos of different stages in caprine (10X).

activation induce high development rates of parthenogenetic embryos in caprine. These 2 chemicals were found the most effective for inducing parthenogenesis in bovine oocytes, presumably because they mimic fertilization very closely (Eusebi and Siracusa 1983, Jones *et al.* 1995, Kim *et al.* 1997). In this study, it was found that the cleavage rate was significantly higher ($P<0.10$) in when oocytes were activated with ionomycin ($86.19\pm3.77\%$, P-II) than with ethanol ($72.68\pm4.17\%$, P-I). In caprine, similar cleavage rate, viz. 58 and 57.8%, respectively, for ethanol (7% for 5 min) and ionomycin (5 μM for 5 min) treatment both followed by incubation in 6-DMAP were reported (Ongeri *et al.* 2001). In buffalo, activation with 7% ethanol for 7 min and 5 μl ionomycin for 5 min both followed by 4 h incubation in 6-DMAP resulted in 71.4 and 59.4% cleavage rate, respectively (Bianca *et al.* 2004).

The numbers of embryos at 16-cell stage were $32.33\pm1.96\%$ in protocol-I, compared to $29.94\pm2.07\%$ in protocol-II. Number of embryos >16-cell stage were significantly higher ($P<0.50$) in protocol-I compared to protocol-II. The present results showed that though ionomycin gave higher cleavage rate after 48 h of activation than ethanol, later embryo development was better in ethanol activated oocytes.

In the present study, CCB was tested with the primary objective to test whether it will allow the formation of parthenogenetic embryos when used throughout oocytes maturation in ruminant species. The results indicated that CCB treatment did not inhibit cleavage rate as well as embryo development, rather increased the cleavage rate as well as embryo development beyond 16 cell stage. There is no reports available regarding use of cytochalacin B during maturation of caprine oocyte before activating with different agents.

From this study it can be concluded that both ethanol and ionomycin activation can result in producing high percentage of parthenogenetic embryos with well developmental potency in caprine. CCB treatment during maturation did not have adverse effect on activation of oocytes with either ethanol or ionomycin rather enhanced cleavage rate as well as there was no constraint on further embryo development.

ACKNOWLEDGEMENTS

Authors are thankful to Director, IVRI, Izatnagar for providing all necessary facilities to conduct this experiment.

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