Indian Journal of Animal Sciences **85** (2): 139–142, February 2015/Article

Effect of protein phosphorylation inhibitor on production of parthenogenetic caprine embryos

S D KHARCHE[1], A K GOEL[2], S K JINDAL[3], PUJA GOEL[4], JUSTIN KOUAMO[5] and SONIA SARSWAT[6]

Central Institute for Research on Goats, Makhdoom, Uttar Pradesh 281 122 India

Received: 4 May 2013; Accepted: 8 October 2014

ABSTRACT

The objective of the present study was to compare the effect of different concentrations of 6-Dimethylaminopurine (6-DMAP) treatment in chemical activated oocytes to compare the developmental potency of parthenogenetic caprine embryos. Morphologicaly matured oocytes were denuded and activated with 5 µm calcium ionophore (ionomycin) for 5 min. Activated oocytes were randomly divided into 5 groups: Group 1 oocytes were cultured in modified Charles Rosenkrans medium (mCR2aa), without DMAP treatment, group 2 cultured in mCR2aa with 2.5 mM DMAP, group 3 cultured in mCR2aa with 5 mM DMAP, group 4 cultured in mCR2aa with 10 mM DMAP, group 5 cultured in mCR2aa with 20 mM DMAP for 4 h. The presumptive zygotes of 5 groups were washed and cultured in the embryo development medium. Development of parthenotes was observed at every 48 h till day 12 post insemination. The percentage of cleavage, morula, blastocyst and hatched blastocyst production in groups 1, 2, 3, 4 and 5 were 61.76, 6.67, 0.00 and 0.00; 59.79, 27.43, 10.62 and 0.00; 72.43, 41.62, 10.66 and 1.52; 64.61, 50.44, 7.83 and 0.00; 63.19, 19.42, 3.88 and 0.00% respectively. Results indicated that 5 mM DMAP treatment for chemically activated oocyte is the best treatment for development of parthenogenetic caprine embryos. In conclusion, it can be stated that for the production of caprine parthenogenetic embryos in vitro, concentration of protein phosphorylation inhibitor plays an important role in the activation treatment. Optimized activation protocols could enhance the production of viable parthenogenetic embryos.

Key words: Caprine embryo, Cleavage, 6-Dimethylaminopurine, *In vitro* maturation, Parthenogenesis

Activation of the oocyte is a complex process during which the oocyte passes from meiotic control of the cell cycle to mitotic control. Artificial stimuli through elevating the cytoplasmic levels of calcium ions of oocytes aim to mimic the action of sperm cells during fertilization (Nakada and Mizuno 1998). Some artificial activation treatments promote an increase in intracellular free calcium concentrations by the release of calcium from cytoplasmic stores, such as ethanol (Kharche *et al.* 2012) and calcium ionophore (Shirazi *et al.* 2009) while there are elements that promote influx of calcium from the extra-cellular medium, such as electrical stimulus. These treatments are commonly followed by the application of an inhibitor of protein phosphorylation (6-DMAP).

The release from M II arrest is characterized by a slow and progressive inactivation of some protein kinases and activation of protein phosphatases (Szollosi *et al.* 1993). 6-DMAP is the non-specific inhibitor of protein phosphorylation (Néant and Guerrier 1988). During parthenogenetic activation and subsequent culture of oocytes, DMAP interfere with the normal course of protein phosphorylation during entry into the first interphase. 6-DMAP is able to induce maturation promoting factor (MPF) inactivation in M II-arrested oocytes, nor it modify the kinetics of MPF inactivation after oocyte activation. However, following oocyte activation, 6-DMAP inhibits the phosphorylation of the 35 kDa complex, thus dephosphorylation of the 30 kDa protein and 35 kDa complex was accelerated (Szollosi *et al.* 1993).

Literature on activation protocols for production and development of parthenogenetic goat embryos is limited (Ongeri *et al*. 2001), creating the need for effective activation protocols that can be used to attain maximum number of developmentaly competent parthenogenetic embryos. One common, universal method or activation agents has not been developed for all species because the process is highly species specific. Similarly concentration of protein phosphorylation inhibitor for parthenogentic treatment is also species specific and need to be optimized. There is no relevant study on dose dependent effect of protein phosphorylation inhibitor on *in vitro*

Present address: [1,2,3]Principal Scientist (kharche62 @gmail.com, akgcirg2012@gmail.com, jindalmathura @gmail.com), [4]Senior Research Fellow (pujabiotech2k @gmail.com), [6]Research Associate (sonia.saraswat@gmail.com), Physiology Reproduction and Shelter Management Division, Central Institute for Research on Goats, Makhdoom, P.O. Farah-281122, Mathura. [5]Senior lecturer (kouamojustin14@yahoo.fr), School of Veterinary Medicine and Sciences, The University of Ngaoundere, PO BOX: 454. Ngaoundere-Cameroon.

parthenogenetic embryo development potential in caprine species. Therefore objective of the present study was to compare the effect of protein phosphorylation inhibitor and standardize the effective parthenogenetic activation protocol for caprine oocyte.

MATERIALS AND METHODS

Collection of ovaries: Goat ovaries were collected from the abattoir at Agra. Ovaries were collected just after the slaughter in normal saline containing antibiotics. Ovaries were maintained at 35–37°C and brought to the laboratory within 3 h of slaughter to avoid any detrimental effect. Upon reaching the laboratory, surplus tissue on the ovaries was removed, ovaries were washed (8–10 times) in sterile normal saline containing antibiotics.

Recovery of oocytes and in vitro *maturation:* The oocytes were retrieved from each ovary in a petri dish containing oocyte collection media (OCM) (Dulbecco's phosphate-buffered saline with 1 mg/ml BSA, 50 µg/ml streptomycin and 60 µg/ml penicillin) by follicle slicing method using sterile blade. Only grade A and B oocytes (Kharche *et al.* 2008) were chosen as they have evenly granulated cytoplasm which represents their active physiological state with having bunch of compact cumulus cell mass around them. Selected oocytes (1015) were washed 2 to 3 times in oocyte holding medium (OHM) containing (TCM-199 with Hepes modification, FBS 10%, sodium pyruvate 0.25 mM, gentamicin 50 µg/ml, glutamine 100 µg/ml, BSA 3 mg/ml) and subsequently 2 to 3 times in oocyte maturation media (TCM-199 with 10% FBS, sodium pyruvate 0.25 mM, glutamine 100 µg/ml, LH 10µg/ml, FSH 5 µg/ml, BSA 3 mg/ml and gentamicin 50 µg/ml) and allowed for maturation in 50 µl drop of IVM medium in 35 mm × 10 mm petri dish for 27 h in a CO_2 incubator maintained at 38.5°C, 5% CO_2 and 90% humidity.

Activation of oocytes: After 27 h of maturation, morphologicaly matured oocytes (972) were denuded with 0.1% hyaluronidase by gentle pipetting. Oocytes were washed in embryo culture medium (mCR2aa medium; modified Charles Rosenkrans medium) and activated with 5 µm calcium ionophore (A23187) (Ongeri *et al*. 2001) for 5 min. Activated oocytes were randomly divided into following five groups and cultured for 4 h.

Group 1: Activated oocytes (170) were cultured in embryo development medium without DMAP treatment (control).

Group 2: Activated oocytes (189) were cultured in embryo development medium containing 2.5 mM DMAP.

Group 3: Activated oocytes (272) were cultured in embryo development medium containing 5 mM DMAP.

Group 4: Activated oocytes (178) were cultured in embryo development medium containing 10 mM DMAP.

Group 5: Activated oocytes (163) were cultured in embryo development medium containing 20 mM DMAP for 4 h.

After 4 h of treatments, the presumptive zygotes of each group were washed and cultured in the embryo culture medium (mCR2aa medium).

Embryo development and evaluation: Development of embryos was observed at every 48 h till day 12 post activation under inverted phase contrast microscopy. In this evaluation, the cleavage rate, morula formation, blastocyst formation rates and the capacity of the blastocyst to hatch from the zona pellucida were analyzed.

Statistical analysis: Cleavage rates, morula, blastocyst and hatched blastocyst formation rate among the different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

In the present study, the effect of different concentration of DMAP on *in vitro* parthenogenetic embryo development was observed. DMAP concentration varied from 0 mM, 2.5 mM, 5 mM, 10 mM and 20 mM. The percentage of cleavage, morula, blastocyst and hatched blastocyst production in groups 1, 2, 3, 4 and 5 were 61.76, 6.67, 0.00 and 0.00; 59.79, 27.43, 10.62 and 0.00; 72.43, 41.62, 10.66 and 1.52; 64.61, 50.44, 7.83 and 0.00; 63.19, 19.42, 3.88 and 0.00% respectively. Significant differences ($P < 0.05$) were recorded among treatments for these variables. The dose dependent effect of DMAP on cleavage rate, morula formation, blastocyst and hatched blastocyst production from in vitro activated goat oocytes are summarized in Table 1. In this study, cleavage rate in 5 mM concentration DMAP was significantly higher than all other treated groups. Significantly higher blastocyst formation rate was also found in 5 mM DMAP treated group than 20 mM DMAP treated and control groups. However morula production rate was significantly higher in 10 mm DMAP treated group than all other treated groups. No significant difference was found in the blastocyst formation rate at 2.5 and 5 mM of

Table 1. Developmental potential of chemically activated *in vitro* matured caprine oocytes cultured in different concentration of 6-DMAP

Treatment	Cleavage (%)	2cell (%)	4 cell (%)	8 cell (%)	Morula (%)	Blastocyst (%)
Control	61.76^{a}	22.86^{a}	29.52^{a}	40.95^{a}	6.67^{a}	0.0^{a}
2.5 mM DMAP	59.79^{ab}	8.85^{b}	23.89^{ab}	29.20^{ab}	27.43^{b}	10.62^{b}
5 mM DMAP	72.43^{c}	8.63^{bc}	10.66^{c}	26.90^{c}	41.62^{c}	10.66^{bc}
10 mM DMAP	64.61^{abcd}	7.83^{bcd}	4.35^{d}	29.57^{ad}	50.44^{d}	7.83^{bcd}
20 mM DMAP	63.19^{abde}	23.30^{abe}	30.10^{be}	23.30^{e}	19.42^{e}	3.88^{bde}

^{a,b,c,d,e} Values in a column with different superscripts are significantly different ($P < 0.05$).

DMAP concentration.

Optimized activation protocols could enhance the production of viable parthenogenetic embryos, which may increase success rates in parthenogenetic cloning. There are no relevant studies on the effect of different and higher concentration of protein phosphorylation inhibitor on parthenogenetic embryo development in caprine species. Therefore, the present study assessed the effect of different concentrations of 6-DMAP, a protein phosphorylation inhibitor with Ca- ionophore, one of the intracellular calcium elevating agents to optimize the effective protocol for parthenogenetic embryo development. Calcium ionophore (A23187) treatment can induce a single intracellular calcium rise in MII oocytes and its consequence is the activation of several calcium dependent proteolytic pathways, leading to the destruction of cyclin B, reduction of MPF activity, and resumption of meiosis (Vincent *et al.* 1992).

In the present study, significantly higher morula and blastocyst formation rate were obtained in calcium ionophore+DMAP treated groups as compared to control (without DMAP treatment). In general, if activated oocytes are allowed to extrude a second polar body, an aneuploid, specifically haploid, parthenote is induced i.e exposure of oocyte to activation agent calcium ionophore only; the probability of extrusion of the second polar body is increased leading to the formation of a haploid parthenote. This method is rarely used since, in this case, the developmental competence is reduced compared to normal embryos and to diploid parthenotes (Henery and Kaufman 1992). In order to overcome this problem, additional agents inhibiting protein synthesis or protein phosphorylation can be added and higher activation and *in vitro* development rates are observed in several species including primates (Mitalipov *et al*. 2001). Diploid parthenotes are easily obtained and these can be derived from M II oocytes whose sister chromatids of the chromosome segregate without being extruded into the second polar body. Thus the oocyte retains its diploid status, either homozygous or including cross-over associated heterozygosity in a single pronucleus (Paffoni *et al*. 2008).

This result showed the arrested growth of embryo development in control group as compared to protein phosphorylation inhibitor treated group. Using only activating agent, such as ethanol, Ca ionophores and electroporation, oocyte is released from metaphase arrest but levels of MPF are not properly reduced and further development is impaired (Rinaudo *et al*. 1997). Reason for the arrested embryo development in control group may be the higher incidence of haploidy as compared to the DMAP treated groups. Indeed, the instant destruction of the spindle in 6-DMAP-treated oocytes could lead to the inhibition of polar body extrusion. There is a team of researchers who demonstrated the developmental potential of parthenotes, especially in young oocytes, was improved by the addition of 6-DMAP to the activation regimen (Gasparrini *et al.* 2004, Hou *et al.* 2009, Shirazi *et al*. 2009).

Our data demonstrated that 5 mM DMAP treatment was more effective for parthenogenetic embryo production in terms of cleavage rate and hatching of blastocyst as compared to other treatments. However, no other research team obtained results at the concentration of 5 mM 6-DMAP and above. Development of parthenogenetic embryos with 2–2.5 mM concentration of DMAP treatment was found effective among different species as reported by others (Gasparrini *et al*. 2004, Mishra *et al*. 2007, Shirazi *et al*. 2009). Mishra *et al*. (2007) observed 51.9 and 7.5% cleavage rate and blastocyst production at 2.5 mM concentration. Gasparrini *et al*. (2004) also demonstrated that both parthenogenetic treatments i.e. ionomycin and ethanol were able to sustain embryo development up to the blastocyst stage. Although Hou *et al*. (2009) observed 46.1% and 21.8% of cleavage rate and blastocyst production with 5 µM concentration of 6-DMAP.

Our results showed that at 20 mM concentration of DMAP, blastocyst formation significantly decreased as compared with low concentration of DMAP treated groups. At 10 mM concentration of 6-DMAP, morula formation was found significantly higher as compared with other groups. No significant difference was found in blastocyst production rate between 2.5 mM, 5mM and 10 mM concentration DMAP treated groups. However, the production of hatched blastocyst were observed only at 5mM concentration of 6-DMAP. Our results demonstrated that the 5 mM DMAP is effective treatment for *in vitro* parthenogenetic embryo development as compared to other groups.

In conclusion, concentration of protein phosphorylation inhibitor plays an important role in the activation treatment. Optimized activation protocols could enhance the production of viable parthenogenetic embryos.

ACKNOWLEDGEMENT

The authors wish to thank the Director, NAIP, New Delhi for providing the funding and Director, CIRG, Makhdoom, India for providing the facilities needed.

REFERENCES

Gasparrini B, Boccia L, Rosa A D, Palo R D, Campanile G and Zicarelli L. 2004. Chemical activation of buffalo (*Bubalus bubalis*) oocytes by different methods: effects of aging on post-parthenogenetic development. *Theriogenology* **62**: 1627–37.

Henery C C and Kaufman M H. 1992. Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. *Molecular Reproduction and Development* **31**: 258–63.

Hou Y, Liu Y, Dai Y, Li R, Shi W, Wang H, Wang L, Li N and Zhu S. 2009. Improved parthenogenetic development of vitrified-warmed bovine oocytes activated with 9% ethanol plus 6-DMAP. *Theriogenology* **72**: 643–49.

Kharche S D, Goel A K, Jindal S K, Jha B K and Goel P. 2012. Assessment of parthenogenetic embryo production by activation of in vitro matured caprine oocytes with different concentrations of ethanol. *Small Ruminant Research* (http://dx.doi.org/10.1016/j.smallrumres.2012.08.008.).

Kharche S D, Goel A K, Jindal S K and Sinha N K. 2008. Birth of a female kid from in vitro matured and fertilized caprine oocytes. *Indian Journal of Animal Sciences* **78**: 680–85.

Mishra V, Misra A K and Sharma R. 2007. Effect of ambient temperature on in vitro fertilization of Bubaline oocyte: *Animal Reproduction Science* **100**: 379–84

Mitalipov S M, Nusser K D and Wolf D P. 2001. Parthenogenetic activation of rhesus monkey oocytes and reconstructed embryos. *Biological Reproduction* **65**: 253–59.

Nakada K and Mizuno J. 1998. Intracellular calcium responses in bovine oocytes induced by spermatozoa and by reagents. *Theriogenology* **50**: 269–82.

Neant I and Guerrier P. 1988. Meiosis reinitiation in the mollusk Patella vulgata.Regulation of MPF, CSF and chromosome condensation activity by intracellular pH, protein synthesis and phosphorylation. *Development* **102**: 505–16.

Ongeri E M, Bormann C L, Butler R E, Melican D, Gavin W G, Echelard Y, Krisher R L and Behboodi E. 2001. Development of goat embryos after in vitro fertilization and Parthenogenetic activation by different methods. *Theriogenology* **55**: 1933–45.

Paffoni A, Brevini T A L, Gandolfi F and Ragni G. 2008. Parthenogenetic Activation: Biology and Applications in the ART Laboratory. *Placenta* **29**: S121–25

Rinaudo P, Pepperell J R, Buradgunta S, Massobrio M and Keefe D L. 1997. Dissociation between intracellular calcium elevation and development of human oocytes treated with calcium ionophore. *Fertility and Sterility* **68**: 1086–92.

Shirazi A, Bahiraee A., Ahmadi E, Nazari H, Heidari B and Borjian S. 2009. The Effect of the duration of *in vitro* maturation (IVM) on parthenogenetic development of ovine oocytes. *Avicenna Journal of Medical Biotechnology* **1**: 181–91.

Snedecor G W and Cochran W G. 1989. *Statistical Methods*. 6th edn. The lowa State University Press, USA.

Szollosi M S, Kubiak J Z, Debey P, Pennart H D, Szollosi D and Maro B. 1993. Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. *Journal* of *Cell Science* **104**: 861–72.

Vincent C, Cheek T R and Johnson M H. 1992. Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. *Journal* of *Cell Science* **103**: 389–96.