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Comparison of parthenogenetic chemical activation of sheep prepubertal oocytes in Telangana

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Abstract
Parthenogenetic embryos were produced by using different concentrations of calcium ionophore + 6-dimethyl aminopurine (6-DMAP) in the concentrations of 1 µg/ml + 1 mM, 5 µg/ml + 2 mM and 10 µg/ml + 5 mM in protocol I, II and III. respectively and also a control was maintained using TCM 199 B + synthetic oviduct fluid (SOF). The results of embryonic development showed that there was significant (P<0.05) difference in cleavage rate between the activation treatments. Treatment II resulted in significantly (P<0.05) higher cleavage rate (66 ± 0.37%) followed by *in vitro* fertilization (IVF) (57.5 ± 0.38%), Treatment III (46.75 ± 0.62%) and Treatment I (38.5 ± 0.41%). No significant difference was observed between Treatment II and IVF pertaining to attainment of 2-cell stage, but differed significantly (P<0.05) when compared to Treatments I (21.75 ± 0.24%) and III (27.5 ± 0.32%). Pertaining to attainment of 4-cell stage, Treatment II resulted in significantly higher rate (30.25 ± 0.32%) followed by *in-vitro* fertilization (27.25 ±0.28%), Treatment III (23 ± 0.36%) and Treatment I (16.5 ± 0.18%). Pertaining to attainment of 8-cell stage, Treatment II resulted in significantly higher rate (26.75 ± 0.33%) followed by *in-vitro* fertilization (24.5± 0.23%), Treatment III (13.25± 3.20%) and Treatment I (15 ± 0.19%). Pertaining to attainment of Morula stage, Treatment II resulted in significantly higher rate (16.5 ± 0.23%) followed by *in-vitro* fertilization (14 ± 0.16%), Treatments III (12 ± 0.20%) and I (9.5± 0.16%).
Based on the results of the present study, it can be concluded that the exposure of *in vitro* mature oocytes to calcium ionophore at a concentration of 5 µg/ml for 5 min followed by 3 hrs incubation with 2 mM 6-DMAP (Protocol-II) was found to be the best for parthenogenetic embryo production in prepubertal ovine ovaries. Comparison of 2-cell stage between treatment II and IVF was similar. Other stages (4-cell, 8-cell and Morula stage) were significantly (P<0.05) higher in parthenogenetic activation of oocytes.

Keywords: Calcium ionophore, 6-DMAP

Introduction
Parthenogenesis is a reproductive strategy in which a female gives birth to offspring without a paternal contribution. It is found in some invertebrate species such as flies, ants and honey bees, and vertebrates such as lizards, snakes, fish and amphibians. Activation of metaphase II oocytes in mammals can be induced by a wide variety of chemical and physical stimuli whose effectiveness increases with increasing post-ovulatory aging of the oocyte (Kaufman, 1981). More recent studies have focused on the improvement of activation protocols in non-aged oocytes by administrating multiple electrical stimuli (Ozil, 1990) [1] or by coupling an activation stimulus with the administration of chemical factors known to suppress MPF kinase activity (Szollosi *et al.*, 1993; Presicce and Yang, 1994) [2]. Loi *et al.* (1998) [4] have determined the effectiveness of combining standard activation protocols with techniques that inhibit protein kinase activity in the oocyte. Ledda *et al.* (1996) [3] have described the effects on nuclear progression and DNA synthesis in sheep oocytes of combining ethanol activation with kinase inhibition by 6-dimethylaminopurine (6-DMAP).

Materials and Methods
Parthenogenetic activation of prepubertal sheep oocytes After maturation, the oocytes were treated with 0.1% hyaluronidase in TCM 199 B followed by pipetting to remove the cumulus cells. Cumulus free oocytes were again washed 3-4 times with handling medium; finally the oocytes were activated using one of the protocols given below.
Protocol 1: 1 µg/ml Ca ionophore in TCM 199B for 2 min followed by 2 h incubation with 1 mM 6-DMAP in mSOF.
Protocol 2: 5 µg/ml Ca ionophore in TCM 199B for 2 min followed by 2 h incubation with 2 mM 6-DMAP in mSOF.

Protocol 3**:** 10 µg/ml Ca ionophore in TCM 199B for 2 min followed by 2 h incubation With 5 mM 6-DMAP in mSOF.
Protocol 4**:** No chemical was added and treated as control

***In vitro* culture of parthenogenetically activated or *in vitro* fertilized oocytes**

After activation, treatment oocytes were taken out of 6-DMAP drop, washed several times in modified synthetic oviductal fluid (mSOF) and cultured in 100 µl of same media in CO_2, incubated at 38 ^{0}C, 5% CO_2, 20% O_2 level and 95% relative humidity. The cleavage rate and embryo development were observed after 48 and 96 h post-insemination and further observations were made to monitor the development of embryos.

Statistical Analysis: Snedecor and Cochran, 199

Results and Discussion

The present study was designed with the objectives to study the effect of different chemical activation protocols on cleavage rate of *in vitro* matured prepubertal sheep oocytes

Total four treatments were used for parthenogenetic embryo development using calcium ionophore and 6-DMAP at a concentration of 1, 5 and 10 µg/ml, and 1, 2 and 5 mM, respectively.

In protocol I, out of total 400 culturable oocytes that were incubated with Calcium ionophore at a concentration of 1 µg/ml and 1 mM 6 DMAP, the percentage of cleaved oocytes was 38.5 ± 0.41%, and 21.75 ± 0.24% attained 2-cell stage of development, 16.5 ± 0.18% attained 4-cell stage of development, 15± 0.19% attained 8 cell stage of development, and finally 9.5 ±0.1% attained morula stage.

In protocol II, total 400 culturable oocytes were incubated with Calcium ionophore at a concentration of 5 µg/ml and 2 mM 6 DMAP. The percentage of cleaved oocytes was 66 ± 0.37, out of which 38.5 ± 0.40% attained 2-cell stage of development, 30.25 ± 0.32% attained 4-cell stage of development, 26.75 ± 0.33% attained 8 cell stage of development, and finally 16.5 ± 0.23% attained morula stage.

In protocol III, total 400 culturable oocytes were incubated with Calcium ionophore at a concentration of 10 µg/ml and 5 mM 6 DMAP, The percentage of cleaved oocytes was 46.75 ± 0.62 and about 27.5 ± 0.32% attained 2-cell stage of development. 23 ± 0.36% attained 4-cell stage of development. 13.25 ± 3.20% attained 8 cell stage of development and finally12 ±0.20% of total oocytes attained morula stage.

In protocol IV, total 100 culturable oocytes were incubated with no chemical added (ie control medium). The percentage of cleaved oocytes was 2± 0.21 and about 1±0.16% attained 2 –cell stage of development, while no further cleavages were observed in this group.

The results on embryonic development showed that there was significant (P<0.05) difference in cleavage rate between the activation protocols. Protocol II resulted in significantly (P<0.05) higher cleavage rate (66 ± 0.37%) followed by *in-vitro* fertilization (57.5 ± 0.38%), protocol III (46.75 ± 0.62%) and protocol I (38.5 ± 0.41%). No significant difference was observed between protocol II and IVF (38.5 ± 0.40%) pertaining to attainment of 2 cell stage, but differed significantly (P<0.05) compared to protocol I (21.75 ± 0.24%) and III (27.5 ± 0.32%). Pertaining to attainment of 4 cell stage, protocol II resulted in significantly higher rate (30.25 ± 0.32%) followed by *in-vitro* fertilization (27.25 ±0.28%), protocols III (23 ± 0.36%) and I (16.5 ± 0.18%). Pertaining to attainment of 8 cell stage Protocol II resulted in significantly higher rate (26.75 ± 0.33%) followed by *in-vitro* fertilization (24.5± 0.23%), protocols III (13.25± 3.20%) and I (15 ± 0.19%). Pertaining to attainment of morula stage, protocol II resulted in significantly higher rate (16.5 ± 0.23%) followed by *in vitro* fertilization (14 ± 0.16%), protocols III (12 ± 0.20%) and I (9.5± 0.16%)The results of embryonic development showed that there was significant (P<0.05) difference in cleavage rate between the activation treatments. Treatment II resulted in significantly (P<0.05) higher cleavage rate (66 ± 0.37%) followed by *in vitro* fertilization Combined treatments with different chemicals for Parthenogenetic activation have been widely used for reconstructed oocytes (sheep: Schnieke *et al*., 1997; cattle: Cibelli *et al*., 1998; goat: Keefer *et al*., 2001 & 2002) [15, 11, 9] to increase intracellular Ca^{2+} concentration (such as calcium ionophore with 6-DMAP) and to inhibit protein synthesis or MPF activity (e.g., 6-DMAP). In this study, a cleavage rate of parthenogenetic activation in treatment-II was achieved at a higher rate (66.0 ± 0.37%) followed by IVF (57.05 ± 0.38%). This is in accordance with the reports of Kouamo and Kharche (2014) [8] and Raja *et al.* (2016) [13]. The latter hypothesized that because all oocytes used for parthenogenetic activation were denuded prior to activation, which allowed for selection based on the presence of a polar body and evenly granulated cytoplasm, a higher proportion of developmentally competent oocytes might have been selected for these procedures. The combination of calcium ionophores with 6-DMAP induces high rates of activation, pronucleus formation and development to blastocyst stage (Loi *et al*., 1998) [4].

From the present study, it can be concluded that calcium ionophore at a concentration of 5 µg/ml for 5 min followed by 3 h incubation with 2 mM 6 DMAP (Protocol-II) was found to be the best for parthenogenetic embryo production in prepubertal ovines and comparison of parthenogenetic embryo revealed similarity in 2-cell stage between treatment II and IVF. Other stages (4-cell, 8-cell and Morula stage) were significantly (P<0.05) higher in parthenogenetic activation of embryos.

Conclusion

a) Calcium ionophore at a concentration of 5 µg/ml for 5 min followed by 3 hrs incubation with 2 mM 6 DMAP was found to be the best for parthenogenetic embryo production in prepubertal ovines.
b) Comparison of parthenogenetic embryo revealed similarity in 2-cell stage between calcium ionophore 5 µg/ml + 2 mM 6-DMAP and IVF. Other stages (4-cell, 8-cell and Morula stage) were significantly (P<0.05) higher in parthenogenetic activation of embryos.

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