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Ramsingh L
P.V. Narsimha Rao Telangana Veterinary University
College of Veterinary Science
Korutla, Telangana, India

KRC Reddy
P.V. Narsimha Rao Telangana Veterinary University
College of veterinary science
Korutla, Telangana, India

Gopala Reddy A
P.V. Narsimha Rao Telangana Veterinary University
College of Veterinary Science
Korutla, Telangana, India

Corresponding Author:
Ramsingh L
P.V. Narsimha Rao Telangana Veterinary University
College of Veterinary Science
Korutla, Telangana, India

Efficacy of Ca ionophore and 6-dimethylaminopurine on activation of prepubertal ovine oocytes

Ramsingh L, KRC Reddy and Gopala Reddy A

Abstract
The present study was carried out to compare the different activation protocols for production of parthenogenetic embryos from prepubertal sheep ovarian oocytes and to compare the *in vitro* fertilization derived embryos. *In vitro* fertilization (IVF) embryos were produced by adopting IVFMC of oocytes collected from prepubertal sheep ovaries from local slaughter house, Ziaguda (Hyderabad). The overall cleavage rate was 57.5 ± 0.38, 53 ± 0.87 and 8 ± 0.24%, respectively in treatments I (10% ESS + 10 µg/ml FSH + 10 µg/ml LH + µg/ml estradiol + 50 µg/ml gentamicin + TCM 199 B), II (10 µg/ml FSH + 10 µg/ml LH + µg/ml estradiol + 50 µg/ml gentamicin + TCM 199 B) and III (50 µg/ml gentamicin + TCM 199 B), respectively. The cleaved cells attained 2-cell, 4-cell, 8-cell and Morula stage were 38.5 ± 0.28, 27.25 ± 0.28, 24.5 ± 0.23 and 14 ± 0.16%, 31 ± 0.71, 20 ± 0.60, 11 ± 0.51 and 2 ± 0.21%,, 3 ± 0.24, 1 ± 0.20, 0 and 0%, respectively in treatments I, II and III. Among the three treatments, Treatment I achieved significantly (P<0.05) higher percentage of cleaved cells.
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, *in vitro* fertilization

Introduction
Sheep is an important species of livestock around the world and especially in India contributing greatly to mutton and wool in rural development. India possesses total sheep population of 65.06 millions, which contribute wool 47.9 million kg and 65.06 million tonnes of meat (FAO 2015-2016). Improvement of reproductive efficiency of sheep through technologies such as artificial insemination, embryo transfer and cloning could enhance their contribution to the Indian economy. The incorporation of juvenile *in vitro* embryo technology into breeding programmes is advantageous because it can reduce the generation interval and increase the genetic gain. 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) [6] 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

Ovaries from sheep were recovered from local slaughter house at Ziaguda, Hyderabad, immediately after slaughter. TCM 199 H was supplemented with 10% Estrus Sheep Serum, 25 IU/ml of heparin (H3393, Sigma, USA) and 50µg/ml gentamicin sulphate. The medium was sterilized by filtration through a 0.22 µm filter and incubated at 39 ºC under humidified atmosphere in 5% CO_2 in air for 1 h prior to use. *In vitro* fertilization medium and culture medium as per standard procedure. Collection of estrus sheep serum (ESS).Blood was collected aseptically from the jugular vein of ewes on the day of standing heat into sterile glass test tubes. The blood was allowed to clot for 1 to 2 h. After the clot formation, the test tubes were transferred to a refrigerator (4-5 ºC) and serum was allowed to ooze. Then the serum was separated and centrifuged at 1000 rpm for 10 min. The separated serum was filtered through a 0.22 µm filter. 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

3.1.12 *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 and to compare the development of parthenogenetic embryos with the standard IVF protocol for embryo development using different concentrations of chemical (calcium ionophore and 6-DMAP) activation protocols.

Oocyte recovery rate from prepubertal sheep ovaries colletred from slaughter house

A total of 1800 prepubertal sheep ovaries were collected from the slaughter house and from them 3150 culturable cumulus oocyte complexes (COCs) were harvested. The overall recovery rate of COCs was 1.7 per ovary. However, Pathak and Kharche (2013) [9] and Sharma *et al.* (2015) reported oocyte recovery rate as 2.24 and 2.27, respectively in goats. Oocyte recovery depends on several factors viz., species, age of the donar, effect of follicle size, stage of estrous cycle etc.

In vitro fertlization of *in vitro* matured oocytes

In treatment I, a total of 400 M-II stage oocytes were co-incubated with spermatozoa and the percentage of cleaved oocytes was 57.5 ± 0.38 and about 38.5 ± 0.28% of total oocytes attained 2-cell stage of development, 27.25 ± 0.28% of total oocytes attained 4-cell stage of development, 24.5± 0.23% of total oocytes attained 8 cell stage of development, and finally 14 ± 0.16% of total oocytes attained morula stage.

In treatment II totally 400 M-II oocytes were co-incubated with sperm and the percentage of cleaved oocytes was 53 ± 0.87 and about 31 ± 0.71% of total oocytes attained 2-cell stage of development, 20 ± 0.60% of total oocytes attained 4-cell stage of development, 11 ± 0.51% of total oocytes attained 8 cell stage of development, and finally 2 ± 0.21% of total oocytes attained morula stage.

In Treatment III (control), totally 400 M-II oocytes were co-incubated without addition of spermatozoa and the percentage of cleaved oocytes was 8 ± 0.24 and about 3 ± 0.24% attained 2-cell stage of development, 1 ± 0.20% attained 4-cell stage of development and none of the oocytes attained 8 cell stage of development and morula stage. The results revealed significant (P<0.05) difference among the treatment groups. Among the three treatments, Treatment I achieved significantly (P<0.05) higher percentage of cleaved oocytes The observations of germinal vesicle in Treatment I was 51±0.73%, which was significantly higher when compared to other treatments. These results are in accordance with the reports of Ledda *et al.* (1999) [6] and Shirirazi *et al.* (2006). On the contrary, Shankariah *et al.* (2013) and Divya (2014) reported lesser values The overall embryo cleavage rate in IVF was 57.5 ± 0.38, 53.0 ± 0.87 and 8.0 ± 0.24% in treatments I, II and III, respectively. The cleavage rate was found highest in Treatment I that contained ESS. Li *et al.* (2006) [5]

Comparison of activation treatments for parthenogenetic embryo production by using *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) [14, 10, 8] 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) [7] and Raja *et al.* (2016) [12]. 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].

However, in the present study, IVF and parthenogenetic groups of treatment –II had almost similar cleavage and further developmental rates. Hence, in terms of developmental competence, it could be inferred that PA embryos can be used as an alternative source to IVF embryos for the production of embryonic stem cells, although further investigation on molecular study related to gene expression is warranted.

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

In vitro maturation rate of prepubertal sheep oocytes by using different IVM media revealed that the treatment I (TCM 199B + 10% ESS + FSH 10 µg/ml + LH 10 µg/ml + estradiol 17 β 1 µg/ml) was found superior to the remaining treatments.

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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