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Effects of different activation protocols on cleavage rate and blastocyst production of caprine oocytes

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Summary

The present study was undertaken to assess the effect of different chemical activators along with 6-DMAP on *in vitro* matured caprine oocytes. From 4332 ovaries, 14235 cumulus oocyte complexes (COCs) were collected which were matured in TCM-199 medium containing follicle stimulating hormone (FSH) (5 µg/ml), Leutinizing hormone (LH) (10 µg/ml), oestradiol-17β (1 µg/ml) supplemented with 10% fetal bovine serum, 10% follicular fluid and 3 mg/ml bovine serum albumin (BSA) at 38.5°C and 5% CO_2 in an incubator under humidified air for 27 h. In group 1 (control), 3117 *in vitro* matured oocytes were co incubated with sperms for 18 h in ferttalp medium. In group 2, 3563 *in vitro* matured oocytes were activated with 7% ethanol for 5-7 min followed by treatment with 2.0 mM DMAP for 4 h in mCR_2aa medium. In group 3, 3109 *in vitro* matured oocytes were activated with 5 µM ionomycin for 5-7 min followed by treatment with 2.0 mM DMAP for 4 h in mCR_2aa medium. In group 4, 3455 *in vitro* matured oocytes were activated with 5 µM calcium ionophore for 5-7 min followed by treatment with 2.0 mM DMAP for 4 h in mCR_2aa medium. Oocytes were cultured in 50 µL drops of research vitro cleave (RVCL) medium for embryo development. The cleavage rate, morula and blastocyst production in group 1, 2, 3 and 4 were 26.07 ± 2.37%, 14.91 ± 2.91 & 1.45 ± 0.71%, 49.57 ± 3.79%, 20.07 ± 2.38% & 5.29 ± 1.42%, 50.18 ± 3.59%, 15.26 ± 2.87% & 1.85 ± 0.72% and 80.26 ± 2.30%, 35.33 ± 2.67 & 7.10 ± 0.89%, respectively. These results indicated that the activation of *in vitro* matured oocytes by 5 µM calcium ionophore for 5-7 min followed by treatment with 2.0 mM DMAP for 4 h is most favorable for parthenogenetic caprine embryos production.

Key words: Blastocyst, Calcium ionophore, Caprine, Cleavage, Parthenogenesis

Introduction

Parthenogenetic activation of oocytes is used for studying the comparative roles of paternal and maternal genomes in controlling early embryo development and as an alternative tool for optimizing culture conditions for *in vitro* embryo production, especially in domestic animals (Abdoon *et al*., 2012). Furthermore, parthenogenetic activation is relevant to cloning research, because artificial activation of oocytes is an essential component of nuclear transfer protocols (Kim *et al*., 1996). An optimized activation protocol may enhance better or complete reprogramming of the reconstructed embryo, which might in turn increase the chance of success in cloning (Wang *et al*., 2008).

There have been several attempts to generate parthenogenetic embryos in caprine using different activation protocol (Kharche and Birade, 2013; Kharche *et al*., 2013; Pathak *et al*., 2013; Kharche *et al*., 2014; Sharma *et al*., 2015; Kharche *et al*., 2016). The *in vitro* developmental competencies of the embryos have also been studied, and it has been observed that the embryos up to blastocyst stage can be developed using different activation protocols (Kharche *et al*., 2014; Sharma *et al*., 2015; Kharche *et al*., 2016). Furthermore, it has also been observed that the embryo developmental rate was found to be quite higher in parthenogenetic activation than IVF (Kharche and Birade, 2013; Kouamo and Kharche, 2015).

There are several methods for induction of parthenogenetic activation which include ethanol (Kharche *et al*., 2013; Pathak *et al*., 2013), calcium ionophore (Kharche *et al*., 2014; Sharma *et al*., 2015; Kharche *et al*., 2016), calcium ionophore combined with cycloheximide (Nussbaum *et al*., 1995) or combined with protein phosphorylation inhibitor (Liu and Yang, 1999), $CaCl_2$ (Machaty *et al*., 1996), protein kinase inhibitors (Mayes *et al*., 1995), G protein stimulation (Machaty *et al*., 1996), ionomycin (Loi *et al*., 1998), ultrasound (Sato *et al*., 2005), strontium (Meo *et al*., 2004), Ca-EDTA (Lee *et al*., 2007), electrical shock (Kono *et al*., 1989; Kim *et al*., 1996), and magnetic field (Max *et al*., 2007).

Despite extensive research in this area, there is no absolute agreement toward using a single common protocol. Therefore, this study was conducted to evaluate the effect of ethanol, ionomycin and calcium ionophore compounds along with 6-DMAP on developmental competence of caprine oocytes matured *in vitro*.

Materials and Methods

All organic and inorganic chemicals were purchased from Sigma Chemicals Co. except research vitro cleave (RVCL) medium from Cook Medical, Australia.

Collection of ovaries

4332 goat ovaries (group1 (942), group 2 (1204), group 3 (958) and group 4 (1228)) were collected in a Thermos flask containing sterile warm (35-37°C) physiological normal saline solution (NSS) supplemented with antibiotics (100 IU/ml penicillin G and 100 µg/ml streptomycin sulphate) within 4 h of slaughter from a local abattoir located in Agra. In the laboratory, the working area was cleaned with 70% alcohol and ovaries were handled aseptically at room temperature. All ovaries were cleared of the attached tissue and mesovarium (trimming). The trimmed ovaries were then subject to washings (5-6 times) with warm saline fortified with antibiotics and then transferred into laminar flow. Subsequently, all experimental procedures were conducted in laminar flow.

Recovery of oocytes and *in vitro* maturation (IVM)

A total of 14235 oocytes were recovered (group 1 (3259), group 2 (3916), group 3 (3344) and group 4 (3716)) from ovaries by using slicing technique; the ovaries were sliced separately in a sterile disposable petri-dish containing OCM (oocyte collection medium) using a sterile surgical blade. The oocytes were isolated and placed in another petri-dish containing oocyte holding medium (OHM). The collected oocytes were finally graded under the inverted phase contrast as per the method of Kharche *et al*. (2008) and only grade A, B, C quality oocytes (Fig. 1A) were chosen for further work. The cumulus oocyte complexes (COCs) were selected and placed in a sterile disposable culture dish containing OHM (TCM-199) containing L-glutamine (100 µg/ml), sodium pyruvate (0.25 mmol) and gentamycin (50 µg/ml). The selected oocytes were washed in 5 to 6 drops (50 µL each) of OHM by serial drop washing method. Further washings were given 3 to 4 times in drops (50 µL each) of maturation media (TCM-199) containing L-glutamine (100 µg/ml), sodium pyruvate (0.25 mmol), gentamycin (50 µg/ml), follicle stimulating hormone (FSH) (5 µg/ml), Leutinizing hormone (LH) (10 µg/ml) and oestradiol-17β (1 µg/ml) supplemented with 10% FBS, 10% follicular fluid and 3 mg/ml bovine serum albumin (BSA). Selected COCs were matured in 50 µL droplets of maturation media covered with sterile mineral oil for 27 h in humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator.

Assessment of *in vitro* maturation

After 27 h of IVM, matured oocytes (Fig. 1B) were assessed for maturation morphologically on the basis of cumulus cell expansion under inverted phase contrast microscope. The oocytes with degenerative changes in

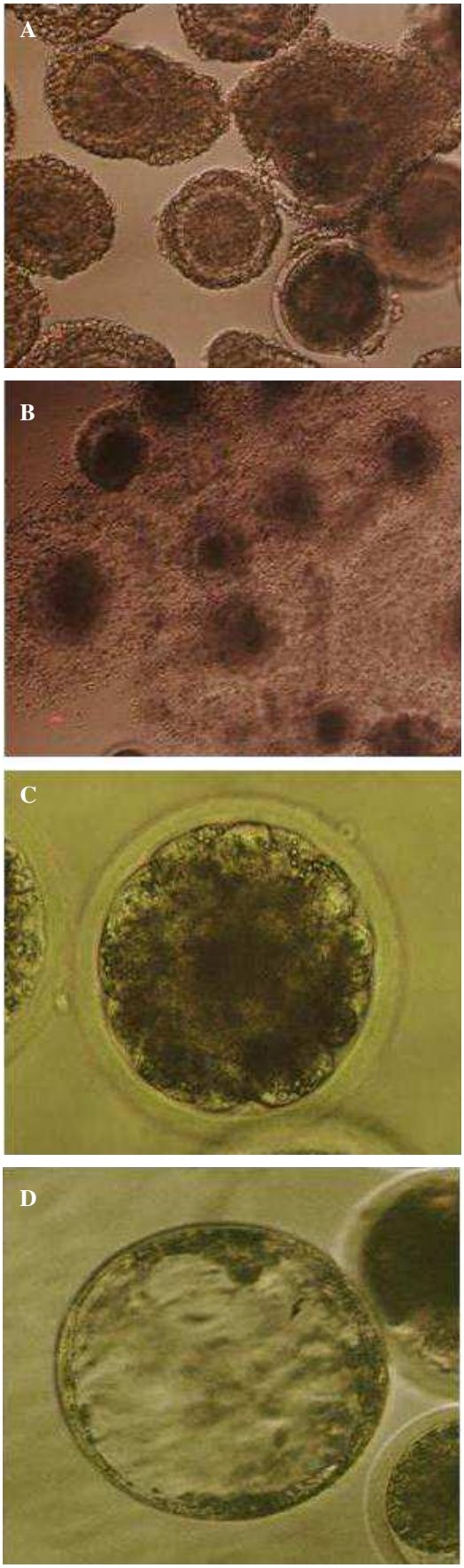

Fig. 1: Developmental stages of oocytes and embryos. **A**: Immature oocytes (×15), **B**: Mature oocytes (×10), **C**: Morula (×20), and **D**: Blastocyst (×20)

the cytoplasm and without cumulus cell expansion were discarded. The degree of expansion was determined by visual assessment and the oocytes with moderate and fully expanded cumulus cell mass were used for further work. The morphological cumulus expansion rate of selected COCs in groups 1, 2, 3 and 4 was 95.30 ± 0.32, 93.76 ± 0.57%, 95.74 ± 0.55% and 94.21 ± 0.46%, respectively.

Assessment of *in vitro* embryo production

After 27 h of maturation, oocytes were stripped off their cumulus cells by gentle pipetting for 10 seconds in 0.1% hyalurunidase enzyme. After removal of cumulus cells, denuded oocytes with polar body and evenly distributed cytoplasm were selected for embryo production. A total of 13244 *in vitro* matured oocytes were collected for *in vitro* fertilization and different activation treatments.

Group 1 (control) embraced 3117 *in vitro* matured oocytes for *in vitro* fertilization which served as control group to activation treatments. *In vitro* fertilization was carried out as per the method described by Kharche *et al*. (2011) with slight modifications. Fifty µL drops of Fert-TALP medium containing oocytes were inseminated with 15-20 µL of the final diluted semen so as to obtain a sperm concentration of 1-2 × 10^6 sperm/ml. After 18 h of sperm-oocytes co-incubation, extra sperm cells adhered to zonapellucida were removed.

Group 2 (7% ethanol) embraced 3563 *in vitro* matured oocytes after 27 h of IVM, activated with 7% ethanol for 5-7 min followed by treatment with 2.0 mM DMAP for 4 h in mCR_2aa medium.

Group 3 (5 µM ionomycin) embraced 3109 *in vitro* matured oocytes after 27 h of IVM, activated with 5 µM ionomycin for 5-7 min followed by treatment with 2.0 mM DMAP for 4 h in mCR_2aa medium.

Group 4 (5 µM calcium ionophore) embraced 3455 *in vitro* matured oocytes after 27 h of IVM, activated with 5 µM calcium ionophore for 5-7 min followed by treatment with 2.0 mM DMAP for 4 h in mCR_2aa medium.

In vitro culture of oocytes

The oocytes were washed and cultured in embryo development medium (RVCL) in a CO_2 incubator at 38.5°C and 5% CO_2 in a humidified atmosphere. The cleavage rate was evaluated after 48 h under inverted phase contrast microscope. The embryo development was observed at 48-72 h interval under inverted phase contrast microscope.

Statistical analysis

The morphological cumulus expansion rate of oocytes was calculated as a percentage. Cleavage rates between the different treatment groups were compared using ANOVA method after Arcsine transformation with SPSS software (16.1 version). Post-hoc multiple comparisons were carried out using the Tukey test. The level of significance was recorded at 5%.

Results

Parthenogenetic activation of *in vitro* matured oocytes

The cleavage rate of *in vitro* matured goat oocytes in group 1 (control), group 2 (7% ethanol + DMAP), group 3 (5 µM ionomycin + DMAP) and group 4 (5 µM calcium ionophore + DMAP) was 26.07 ± 2.37%, 49.57 ± 3.79%, 50.18 ± 3.59% and 80.26 ± 2.30%, respectively.

Developmental potential of parthenogenetic embryos

The embryo development among group 1 (control), group 2 (7% ethanol + DMAP), group 3 (5 µM ionomycin + DMAP) and group 4 (5 µM calcium ionophore + DMAP) was compared. In group 1 (control), the percentage of morula and blastocysts was 14.91 ± 2.91% and 1.45 ± 0.71, respectively. In group 2 (7% ethanol + DMAP), the percentage of morula and blastocyst was 20.07 ± 2.38% and 5.29 ± 1.42%, respectively. In group 3 (5 µM ionomycin), the percentage of morula and blastocyst was 15.26 ± 2.87% and 1.85 ± 0.72%. In group 4 (5 µM calcium ionophore), the percentage of morula (Fig. 1C) and blastocyst (Fig. 1D) was 35.33 ± 2.67% and 7.10 ± 0.89%, respectively. The relevant data for embryo development in RVCL medium are shown in Table 1.

Discussion

The cleavage rate was significantly higher ($P<0.05$) in group 4 (5 µM calcium ionophore + DMAP) than that of group 1 (control), group 2 (7% ethanol + DMAP) and group 3 (5 µM ionomycin + DMAP) (Table 1).

A significant improvement in cleavage rate was observed in group 4 (5 µM calcium ionophore + DMAP), suggesting that the *in vitro* matured goat oocytes had acquired more cleavage potential after 5 µM calcium ionophore + DMAP activation treatment. This indicates a critical role of calcium ionophore activation treatment in the signaling pathways of embryo development and also it could be due to the synergistic effect produced by the use of both calcium ionophore + DMAP.

Table 1: Effects of different activation protocols on cleavage rate and blastocyst production (mean±SE) of caprine oocytes

S. No.	Groups	Total cleaved (%)	Morula (%)	Blastocyst (%)
1	Group 1 (control)	752^a (26.07 ± 2.37)	14.91 ± 2.91^a	1.45 ± 0.71^a
2	Group 2 (7% ethanol + DMAP)	1645^b (49.57 ± 3.79)	20.07 ± 2.38^a	5.29 ± 1.42^b
3	Group 3 (5 µM ionomycin + DMAP)	1464^b (50.18 ± 3.59)	15.26 ± 2.87^a	1.85 ± 0.72^a
4	Group 4 (5 µM CAI + DMAP)	3026^c (80.26 ± 2.30)	35.33 ± 2.67^b	7.10 ± 0.89^c

Values in a column with different superscripts are significantly different ($P<0.05$)

The result observed following activation with 7% ethanol + DMAP (group 2) are higher than that reported by Wang *et al*. (2008) (28.1%) in bovine and are comparable with (Ongeri *et al*., 2001) (55%) in caprine oocytes and Ongeri *et al*. (2001) and Hosseini *et al*. (2008) (40.6% and 68.9%) in bovine oocytes. Furthermore, the results observed following activation with ionomycin + DMAP (group 3) are higher than that reported by Wang *et al*. (2008) (30.7%) in bovine and are lower than (Ongeri *et al*., 2001) (61.1%) in caprine oocytes. However, our results of cleavage rate following calcium ionophore + DMAP activation (group 4) are higher than De *et al*. (2012) (70.44%) and Jena *et al*. (2012) (75.7%) in caprine oocytes and Hosseini *et al*. (2008) (73.7%) and Wang *et al*. (2008) (19.7%) in bovine oocytes.

It is suggested that the difference in the cleavage rate might be due to difference in the species, culture media and activation treatments used for parthenogenetic activation. The phenomenon of parthenogenesis would be understood by the following explanation. The type of parthenote formed depends on the activating stimulus, its intensity, the postovulatory age of an oocyte, and the conditions of activation i.e. osmolality, pH and temperature of culture media (Dyban *et al*., 1989; King *et al*., 1998). Matured mammalian oocytes spontaneously block their meiotic maturation at the stage of metaphase II. Further progress in meiosis beyond this spontaneous block depends on the activating stimulus. Without this stimulus, the mammalian oocytes underwent spontaneous death. Under natural conditions, this stimulus is brought into the oocyte by the sperm. When a spermatozoon penetrates the zonapellucida and attaches to the vitelline membrane of the MII oocyte, oscillations of intracellular calcium (Ca^{2+}) occur and the elevation of Ca^{2+} triggered by sperm penetration inhibits dephosphorylation of P34cdc2 kinase, and in turn reduces maturation-promoting factor (MPF) activity and activates the oocytes (Whitaker and Patel, 1990). So, for activation of oocytes, mitogen promoter factor (MPF) and mitogen activated protein kinase (MAPK) must be suppressed. Multiple and periodic oscillations in intracellular calcium concentrations are responsible for this suppression (White and Yue, 1996). Namely, MAPK and cytostatic factor (CSF), important factors for MII maintenance, are inactivated and cyclin B, a subunit of MPF, is destroyed and low MPF activity can be maintained by either inhibition of continuous synthesis of cyclin B by cycloheximide (Soloy *et al*., 1997) or by phosphorylation of cdc2/dephosphorylation of ERK2 by 6-DMAP (Liu and Yang, 1999). In contrast to cycloheximide or IVF-induced activation, 6-DMAP induces earlier dephosphorylation of MAPK and therefore, is responsible for earlier pronuclear development (Liu and Yang, 1999).

Methods used for parthenogenetic activation have different activation mechanisms. Ionophores such as calcium ionophore and ionomycin induce a great single intracellular calcium rise in MII oocytes which originates exclusively from the internal deposits (Meo *et al*., 2004) while ethanol induces a single Ca^{2+} rise, whose main source is external although a minor intracellular release is also involved (Shiina *et al*., 1993). So, cleavage rate in all treatments (ethanol + DMAP, ionomycin + DMAP and calcium ionophore + DMAP) suggests that ethanol, ionomycin and calcium ionophore plays a significant role in oocytes activation, by increasing intracellular Ca^{2+} (Presicce and Yang, 1994; Pathak *et al*., 2013) and probably lead to the development of embryos. Thereby, the sequence of events occurring during parthenogenetic activation mimics the cascade of intracellular activities produced by the penetrating spermatozoa.

Early embryonic development is a complex process that consists of oocyte maturation, fertilization, and embryo growth (2-cell, 4-cell, 8-cell, morula, and blastocyst), and both maternal and paternal genomes are required for normal embryo development of mammals (Singh *et al*., 2014). Additionally, low blastocyst rate has also been reported by Abdullah *et al*. (2011). The low development may be caused improper activation of method, media or condition of *in vitro* embryo culture. The activation process relies on chemicals that can stimulate calcium transients in the oocyte and prevent the second polar body extrusion after activation (Srirattana *et al*., 2013).

Our data demonstrated that the morula (35.33 ± 2.67%) and blastocyst (7.10 ± 0.89%) production was significantly higher in group 4 (Ca ionophore + DMAP) than group 1 (control), group 2 (7% ethanol + DMAP) and group 3 (5 µM ionomycin + DMAP). Furthermore, blastocyst production in group 2 (7% ethanol + DMAP) was also significantly higher than group 1 (control) and group 3 (5 µM ionomycin + DMAP). Group 4 (Ca ionophore + DMAP) yielded significantly higher number of morula and blastocysts suggesting that more embryos were able to cross the barrier from 2-cell, 4-cell and 8-16 cell to morula and blastocyst.

As per the results of blastocyst production, in 7% ethanol + DMAP activation (group 2), our results are higher than Ongeri *et al*. (2001) (3.6%), Hosseini *et al*. (2008) (1.6%) and Wang *et al*. (2008) (3.9%) in bovine oocytes but are lower (17%) than that reported by Ongeri *et al*. (2001) in caprine oocytes. Further, in 5 µM ionomycin + DMAP activation (group 3), our results are higher than Wang *et al*. (2008) (4.2%) in bovine oocytes but are lower than Ongeri *et al*. (2001) (17.6%) in caprine oocytes. However in Ca ionophore + DMAP activation (group 4), our results are higher than Jena *et al*. (2012) (6.8%) and De *et al*. (2012) (21.09%) in caprine oocytes and Hosseini *et al*. (2008) and Wang *et al*. (2008) (5.4% and 4.6%, respectively) in bovine oocytes.

Our results also verified that of the activation methods the highest efficiency in terms of morula and blastocyst rates was observed with calcium ionophore + DMAP activation (group 4) than the other experiments (group 1 (control), group 2 (ethanol + DMAP) and group 3 (ionomycin + DMAP)). This clearly indicates that Ca ionophore + DMAP activation treatment yielded significantly higher number of morula and blastocysts

suggesting that more embryos were able to cross the barrier from 2-cell, 4-cell and 8-16 cell to morula and blastocyst.

The results indicated that parthenogenetic activation of caprine oocytes in RVCL media can be effectively done by ethanol + DMAP, ionomycin + DMAP and calcium ionophore + DMAP combinations, which may enhance better development of parthenogenetic embryos. Thus, considering the fact that cleavage, morula and blastocyst production following chemical activation (calcium ionophore + DMAP) was significantly higher; our study indicates that caprine oocytes had better inherent developmental competence following calcium ionophore + DMAP chemical activation.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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