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Development of water buffalo *(Bubalus bubalis*) embryos after parthenogenetic activation of oocytes with calcium ionophore, ethanol and 6-dimethylaminopurine

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ABSTRACT

The present study was carried out to investigate the effects of various activation treatments on the parthenogenetic development of buffalo oocytes and their comparison with the IVF embryos and to investigate the mean total cell number per blastocysts produced. The rates of cleavage and blastocyst development were significantly higher when the oocytes were activated by combined treatment of calcium ionophore (A23187) and ethanol, followed by 6-dimethylaminopurine (6-DMAP) than those activated by individual treatments with these chemicals, as well as blastocysts produced through IVF. Our results showed significant difference in the number of blastomeres between parthenotes and IVF buffalo embryos. Our results can be used to establish effective protocols for activation of buffalo oocytes and development of embryos which can be used to study mechanisms involved in the initiation of the embryonic development, and derivation of embryonic stem (ES) cells for various applications.

Key words: Blastocyst, Ionophore, Parthenote

Buffalo embryos produced by *in vitro* maturation (IVM) and fertilization (IVF) of oocytes collected from the abattoir-derived ovaries to develop blastocysts can be used to establish pregnancies and produce live offspring (Madan *et al.* 1994, Chauhan *et al.* 1998). One of the major problems associated with IVM/IVF in buffalo is the very poor recovery of immature oocytes. The average recovery of acceptable quality oocytes in buffaloes is 40 per ovary (Totey *et al.* 1992) compared to 80 per ovary in cattle (Sato *et al.* 1990). The normal rate of blastocysts produced through IVF ranges from 8–10%. Researchers are attempting to improve buffalo reproduction through innovative assisted reproduction approaches like cryopreservation of oocytes (Gautam *et al.* 2008), establishing stem cells (Verma *et al.* 2007), through trangenesis (Verma *et al.* 2008) and cloning. Parthenogenetic activation of the oocytes could be exploited as an alternative strategy to produce embryos in buffaloes to derive ES cells or to study basic developmental dynamics in the species.

The methods used to activate the animal oocytes include physical stimuli, such as an electrical pulse (Ozil 1990), chemical stimuli such as ethanol (Liu *et al*. 1998), ionophores, and ionomycin (Susko-Parrish *et al.* 1994) etc. The chemical stimuli could be used alone or combined with a protein phosphorylation inhibitor, 6-dimethylaminopurine (6-DMAP). For instance, bovine oocytes activated with ethanol in combination with calcium ionophore, followed by the 6-DMAP treatment resulted in blastocyst development at a rate comparable to that of IVF embryos (Liu *et al.* 1998). However, calcium ionophore and ethanol followed by treatment with 6-DMAP, a suitable combination of chemical treatments for activation of oocytes ungulates has not been studied in water buffaloes. The present study was taken to investigate the effects of chemical agents on parthenogenetic activation of bubaline oocytes, and development of embryos in the species.

MATERIALS AND METHODS

Oocyte collection and in vitro *maturation:* The *in vitro* maturation of buffalo oocytes was carried out as per Madan *et al.* (1994).

Parthenogenetic activation: After maturation *in vitro* for 24 h, the oocytes were denuded manually. The oocytes with an extruded first polar body (MII oocytes) were assigned to

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the following treatments: exposure to 7% ethanol for 5 min or 5 µM calcium ionophore (A23187) for 5 min, or in a combination of 7% ethanol and 5 µM calcium ionophore for 5 min with subsequent treatment with 2 mM 6-DMAP (4 h) under a humidified atmosphere of 5% CO2 at 385°C. Parthenogenetically activated oocytes were cultured in a co-culture system comprising monolayer of cumulus cells and freely floating oviductal epithelial cell clumps, and synthetic oviductal fluid (SOF) supplemented with 10% FBS. The percentages of cleavage, morulae and blastocyst development were evaluated on days 2, 5, 7 and 10 of initiating the culture, respectively.

Sperm preparartion and in vitro *fertilization:* The spermatozoa used for IVF throughout the study were from the same batch and the same donor, and had been tested for IVF earlier. The spermatozoa were prepared for fertilization as per Chauhan *et al.* (1998). The cleavage rate was observed after 42–44 h of culture.

In vitro *culturing of fertilized and parthenogenetically activated oocytes:* The fertilized and parthenogenetically activated oocytes were transferred to 200 µl droplets of SOF with 10% FBS and cultured in CO_2 incubator at 385°C. After 24 h, the cells were washed 3 times in SOF with 10% FBS and transferred to each of the culture droplets. The medium in the droplets was changed on alternate days by replacing 50µl of the old medium with fresh medium. The development of the embryos was monitored for 7–10 days.

Cell count: Embryos at different developmental stages i.e. 4–8, 8–16, morulae and blastocysts were stained on glass slides in a PBS solution and 10% glycerol containing 1 mg/ml of Hoechst 33342. A drop (20 µl) of staining solution containing 1±3 embryos was placed in the center of a slide and a cover slip was placed over the drop and the edges were sealed. Nuclei were visualized and counted using an inverted microscope.

Experimental design: A total number of 1056 oocytes, over an average of 6 replicates, were matured *in vitro* and randomly assigned to 4 experimental groups. In first experimental group, the oocytes (N= 300) were fertilized *in vitro* which served as a positive control group. In second, third and fourth groups, the remaining oocytes were activated by ethanol+6-DMAP (N= 252), A23187+6-DMAP (N=246) and ethanol+ A23187+6-DMAP (N=258), followed by immediate exposure to 6-DMAP (4 h) respectively.

Statistical analysis: Data of parthenogenetic activation experiments and *in vitro* fertilized oocytes were collected from 6 replicates. The rate of cleavage and blastocyst development in parthenotes and IVF were analyzed by ANOVA. The data was considered for significant differences at $P < 0.05$. The rate of blastocysts development of oocytes treated with ethanol +calcium ionophore+6-DMAP was significantly higher ($P<0.05$) than ethanol+6-DMAP and those activated with calcium ionophore +6-DMAP or IVF groups. The mean blastocyst cell number was statistically different ($P< 0.05$) between parthenotes and IVF buffalo embryos. But no significant difference was found among parthenotes generated by various activation treatments with respect to cell number.

RESULTS AND DISCUSSION

Our results (Tables 1, 2) showed that high efficiency of oocyte activation can be achieved by a combination of ethanol+calcium ionophore, followed by incubation in 6-DMAP.The cleavage rate and percentage of blastocyst development obtained by ethanol + calcium ionophore + 6-DMAP was significantly higher than all other groups (Table 1), while the percentage of cleavage and blastocyst development in both ethanol+6-DMAP and calcium ionophore+6-DMAP was not significantly different ($P<0.05$). The mean blastocyst cell number was statistically different ($P< 0.05$) between parthenotes and IVF buffalo embryos. But no significant difference was found among parthenotes generated by various activation treatments with respect to cell number (Table 2). Our results demonstrated the possibility of chemically activating the bubaline *in vitro* matured oocytes. All the treatments were able to sustain embryo development to blastocyst stage (Fig. 1 A- B).

Parthenogenetic activation has been used as a tool to study mechanisms involved in the initiation of embryonic development. The present study compared embryo development by parthenogenetic activation using two

Table 1. Parthenogenetic development of buffalo oocytes after various activation

Treatment	Treatments		
	No of oocytes (N)	Cleavage rate N (%)	Blastocyst N (%)
Ethanol+6-DMAP	252	168 (67%) [a]	80 (32%) [a]
A23187+6-DMAP	246	164 (67%) [a]	88 (36%) [a]
Ethanol+A23187+6DMAP	258	216 (84%) [b]	144 (56%) [b]
IVF (control)	300	122 (41%) [b]	50 (17%) [b]

Different superscripts within columns are significantly different ($P < 0.05$). Values are mean±SEM.

Table 2. Total cell number of parthenotes and IVF buffalo embryos

Treatment	4–8 cell	8–6 cell	Morulae	Blastocyst N (%)
Ethanol+6-DMAP	40±13[a]	110±22 [a]	32±12 [a]	950±20 [a]
A23187+6-DMAP	40±10 [a]	100±22 [a]	30±12 [a]	960±10 [a]
Ethanol+A23187+ 6DMAP	30±10 [a]	110±205 [a]	32±12 [a]	950±20 [a]
IVF (control)	68±20[b]	140±269 [b]	490±16 [b]	1480±20 [b]

Different superscripts within columns are significantly different ($P < 0.05$). Values are mean±SEM.

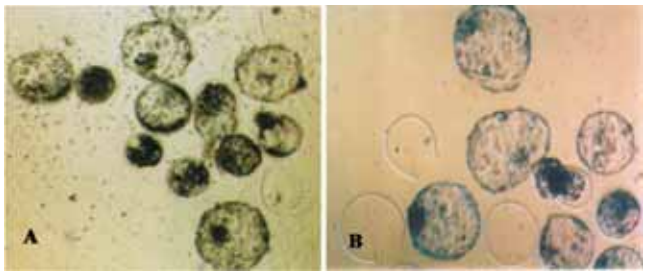

Fig. 1. (A-B). A, parthenote buffalo blastocysts, (10×); B, blastocysts produced through IVF (10×).

chemical agents i.e. ethanol and calcium ionophore individually or in combination followed by immediate exposure to 6-DMAP (4 h) with IVF. The results show that a high efficiency of oocyte activation could be achieved by treatment with a combination of ethanol+calcium ionophore+6-DMAP (4 h) (Table 1). Previous findings show that the treatment of calcium ionophore+6-DMAP or ethanol+6-DMAP (which increase intracellular calcium), are effective in inducing activation and the subsequent development of buffalo oocytes (Gasparrini *et al.* 2004). In our study, when the combination treatment of calcium ionophore + ethanol + 6-DMAP was used, the cleavage rate and the number of blastocysts produced were significantly higher (P< 005) than the individual treatments with ethanol+6-DMAP or calcium ionophore+6-DMAP or IVF produced embryos. However, no significant difference was found among the parthenotes produced through individual treatments with ethanol+6-DMAP or calcium ionophore+6-DMAP (Table 2). It could be because they both successfully mimic the fertilization events.

It is well known that an increase in intracellular Ca^{2+} concentration is a global response elicited by the sperm during fertilization. It was also observed that Ca^{2+} is released in the form of a long series of oscillations (Miyazaki *et al.* 1986). Given the importance of the Ca^{2+} release during fertilization, most of the currently used oocyte activation procedures rely on methods to induce an intracellular Ca^{2+} increase Calcium ionophores determine a prolonged Ca^{2+} rise and the source is both external and from the intracellular stores. However when ethanol+calcium ionophore+6-DMAP were used in combination, it resulted in prolonged and consistent Ca^{2+}rise. Previous studies shows decrease in histone kinase activity, which is a prerequisite for oocyte activation, was rapid in oocytes treated with calcium ionophore followed by 6-DMAP, while it increased slowly in oocytes treated with a calcium ionophore only (Liu *et al.* 1998). It was demonstrated that a single Ca^{2+}rise which is evoked by most of the currently used activation procedure causes only a transient decline of MPF and CSF activity, which is not adequate for oocytes activation (Collas *et al.* 1993). Based on this concept, alternative method of activation were developed that combine a transient inactivation of MPF obtainable with a Ca^{2+} rise with a persistent inhibition of MPF, induced by addition of either protein synthesis inhibitors or non-specific kinase inhibitors (Liu *et al.* 1998).

Differences in the efficiency of blastocyst development after oocytes activation with various methods were observed between species. As sperm is the natural activating agent of the oocytes, IVF could be expected to be more efficient method of producing embryos, as also observed in the present study. On the contrary, we found that activation of MII oocytes, induced by ethanol + calcium ionophore+6-DMAP, had significantly improved cleavage rates and blastocyst production when compared with ethanol+6-DMAP or calcium ionophore+6-DMAP. In this context, the blastocyst production rate in this report (by using these three combinations) is higher (36%) than that of Liang *et al.* (2010) where the blastocyst development rate was 29%.

Our last objective was to investigate the mean total cell number per blastocyst between embryos produced through parthenotes and IVF (Fig 1). Using ethanol alone or in combination with cytochalasin B, Boediono *et al.* (1995) reported a lower total cell number per blastocyst in parthenogenetic embryos than in IVF produced embryos. Our results showed a significant (P<0.05) difference in the number of blastocyst cells between parthenotes and IVF buffalo embryos, but no significant difference in number of blastomeres were found among the parthenotes. When compared with the results of Liang *et al*. (2010), the no of cells in blastocyst was higher in all the combinations.

In conclusion, buffalo oocytes treated with ethanol+ calcium ionophore, followed by incubation in 6-DMAP resulted in higher blastocyst development than IVF controls, suggesting that these treatments may be optimal for the activation of oocyte. The fact that oocytes activated with the treatments developed more efficiently than fertilized oocytes probably reflects deficiencies in the buffalo culture systems used for IVM, IVF and culture (IVMFC), which emphasizes the need for continued research in this area.

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