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A combined treatment with ethanol and 6-dimethylaminopurine is effective for the activation and further embryonic development of oocytes from Sprague-Dawley and Wistar rats

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Summary

In nuclear-transferred or round spermatid-injected oocytes, artificial activation is required for further development in mammals. Although strontium chloride is widely used as the reagent for inducing oocyte activation in mice, the optimal method for oocyte activation remains controversial in rats because ovulated rat oocytes are spontaneously activated *in vitro* before artificial activation is applied. In our previous study, we found that cytostatic factor activity, which is indispensable for arrest at the MII stage, is potentially low in rats and that this activity differs greatly between two outbred rats (Slc: Sprague-Dawley (SD) and Crj: Wistar). Therefore, it is necessary to establish an optimal protocol for oocyte activation independent of strains. Given that comparative studies of the *in vitro* development of oocytes activated by different activation protocols are very limited, we compared four different protocols for oocyte activation (ethanol, ionomycin, strontium and electrical pulses) in two different SD and Wistar rats. Our results show that oocytes derived from SD rats have significantly higher cleavage and blastocyst formation than those from Wistar rats independent of activation regimes. In both types of rat, ethanol treatment provided significantly higher developmental ability at cleavage and blastocyst formation compared to the other activation protocols. However, the initial culture in a fertilization medium (high osmolarity mR1ECM) for 24 h showed a detrimental effect on the further *in vitro* development of parthenogenetic rat oocytes. Taken together, our results show that ethanol treatment is the optimal protocol for the activation of rat oocytes in SD and Wistar outbred rats. Our data also suggest that high-osmolarity media are inadequate for the *in vitro* development of parthenogenetically activated oocytes compared with fertilized oocytes.

Keywords: Development, Ethanol, Oocytes, Parthenogenetic activation, Rat

Introduction

In almost all mammalian species, ovulated oocytes are arrested at the MII stage during meiosis and this arrest continues until fertilization. Following sperm penetration, Ca^{2+} oscillations, cortical granule exocytosis, inactivation of cytostatic factor (CSF), extraction of the second polar body and pronuclear formation occur (Schultz & Kopf, 1995; Ducibella *et al.*, 1998; 2002; Kurokawa *et al.*, 2005, 2007; Miyazaki *et al.*, 2006); these phenomena are collectively known as 'oocyte activation'. Many researchers have demonstrated that artificial stimulation using chemicals or instruments mimics this activation induced by sperm and that such stimulation is sufficient for development to term. Indeed, generation from oocytes injected with round spermatids or oocytes to which somatic cell nuclei have been transferred requires artificial activation because

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these recipient oocytes cannot be activated by sperm (Ogura *et al.*, 1994; Wakayama *et al.*, 1998). For example, strontium chloride is commonly used as an activation protocol for somatic cell nuclear transfer in mice (Inoue *et al.*, 2002; Kishigami *et al.*, 2006). Ultrasound (Miyoshi *et al.*, 2007), thimerosal (Machaty *et al.*, 1997) and Ca^{2+} ionophore (Ito *et al.*, 2003) have also been reported as agents for inducing oocyte activation in other species. Moreover, ethanol treatment is also well known as a popular regent that induces oocyte activation in mice (Cuthbertson *et al.*, 1983), cattle (Nagai *et al.*, 1987) and pigs (Yamauchi *et al.*, 1996). It is also known that, after the use of these stimuli, additional treatment with 6-dimethylaminopurine (6-DMAP) (Méo *et al.*, 2005), cycloheximide (Yamanaka *et al.*, 2007) or puromycin (Roh *et al.*, 2003) improves the development of parthenogenetic or nuclear-transferred embryos *in vitro*.

Nevertheless, the optimal activation method in rats remains controversial. It has been reported that the rate of development of rat embryos from the 2-cell stage to morulae/blastocysts and live pups was significantly lower in a group treated with strontium than in a group treated with electrical pulses plus 6-DMAP when the oocytes were activated prior to the injection of round spermatids (Kato *et al.*, 2004). Furthermore, Jiang *et al.* (2002) report that ethanol treatment showed lower developmental ability. They conclude that ethanol treatment is unsuitable for the activation of rat oocytes. Moreover, the percentage of activated oocytes has been found to be dependent on the strain of rat (Kato *et al.*, 2001). In our previous study (Ito *et al.*, 2007), oocytes from two different types of rat (Sprague-Dawley, SD and Wistar rats) showed different CSF activity. Given that the inactivation of CSF is indispensable for oocyte activation in vertebrate oocytes (Tunquist & Maller, 2003), it is essential to establish an optimal protocol for oocyte activation in rats. However, there have been very few comparative experiments to date.

In the present study, we compared four activation protocols (ethanol, ionomycin, electrical pulse and strontium chloride) for the activation of oocytes collected from two different types of outbred rat, Wistar and SD, that are commonly used in research on reproductive biology. We also examined the effects of initial culturing in a fertilization medium on the further *in vitro* development of parthenogenetic embryos.

Materials and methods

All chemicals and reagents were purchased from Sigma–Aldrich Corporation unless otherwise stated. All procedures for the handling and treatment of the animals were conducted according to the guidelines established by the Animal Research Committee of Azabu University.

Oocyte preparation

Specific-pathogen-free Crj: Wistar and Slc: SD female rats (4 to 5 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and Japan SLC, Inc. (Shizuoka, Japan), respectively. The rats were housed in an environmentally controlled room with a 12 h dark/12 h light cycle at a temperature of 23 ± 2 °C and humidity of 55 ± 5% with free access to laboratory diet and filtered water. The females were superovulated by intraperitoneal injections of 300 IU/kg equine chorionic gonadotropin (eCG; Nippon Zenyaku Kogyo Co.) and 300 IU/kg human chorionic gonadotropin (hCG; Asuka Pharmaceutical Co.) at 48 h intervals, as previously reported by Nakai *et al.* (2005). Eighteen hours after the hCG injection, cumulus–oocyte complexes were collected from the oviductal ampullae of the donor females with calcium-free modified Dulbecco's phosphate-buffered saline PB1(Ca^-) (Whittingham, 1971) supplemented with 0.1% hyaluronidase. After the cumulus cells were removed, the denuded oocytes were washed three times with PB1(Ca^-) and kept in the same medium at 37 °C until being subjected to the treatments.

Parthenogenetic activation and *in vitro* culture

Denuded oocytes were divided into the following five groups: non-treatment, electrical pulse treatment, ionomycin treatment, strontium treatment and ethanol. Following the procedure described by Mizutani *et al.* (2004) for ionomycin treatment, oocytes were placed in mR1ECM (Miyoshi *et al.*, 1995) supplemented with 5 μM ionomycin for 5 min. The ethanol treatment was carried out following the protocol given by Jiang *et al.* (2002), under which oocytes were put in a fertilization medium (mR1ECM containing 110 mM NaCl and 4 mg/ml bovine serum albumin, BSA, and omitting polyvinyl alcohol, as per Oh *et al.*, 1998) supplemented with 7% (v/v) ethanol (Kanto Chemical Co.) for 3 min. The electrical pulse treatment was carried out as previously reported (Ito *et al.*, 2005): oocytes were artificially stimulated by two direct current pulses (100 V/mm, 99 μs). The strontium treatment was carried out according to the protocol described by Tomashov-Matar *et al.* (2005), under which oocytes were placed in Ca^{2+}-free fertilization medium supplemented with 2 mM $SrCl_2$ for 30 min. Each treatment group included more than 60 oocytes.

Half of the activated oocytes and control (non-treated) oocytes were washed three times in mR1ECM, after which they were cultured in mR1ECM supplemented with 2 mM 6-DMAP for 4 h to

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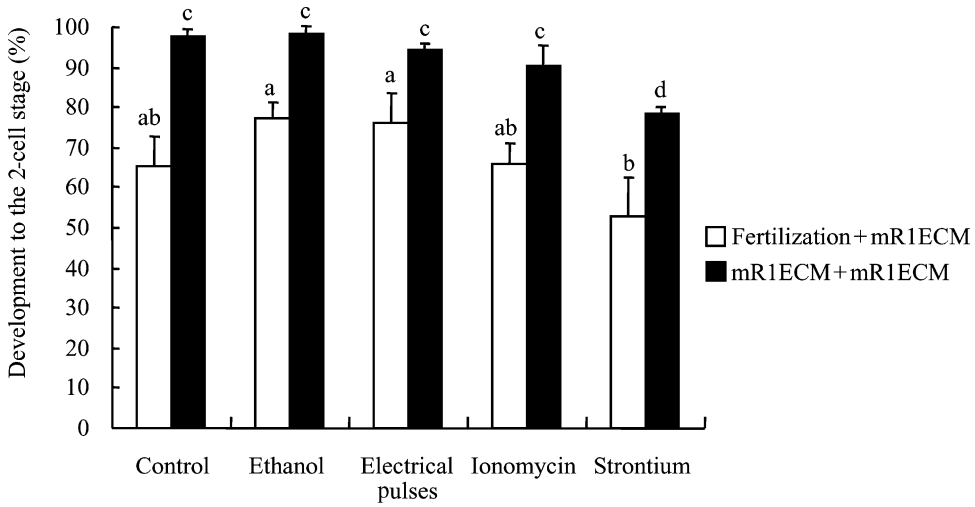

Figure 1 Development to the 2-cell stage in Wistar oocytes activated by different treatments. After activation, the oocytes were cultured for 24 h in a fertilization medium (open bar) or in mR1ECM (black bar). The different superscripts on the SEM bars denote significant differences among the treatment groups ($p < 0.05$).

form diploids. The oocytes were then transferred to mR1ECM and cultured for up to 120 h in the same medium. The proportions of cleavage and blastocyst formation were assessed at 24 h and 120 h, respectively. In order to examine the effect of osmolarity in the initial culture medium on *in vitro* embryonic development, the remaining activated and control oocytes were washed three times in fertilization medium and cultured for 24 h. They were then washed three times in mR1ECM and cultured for up to 120 h. Cleavage and blastocyst formation of the embryos were assessed as described above.

Statistical analysis

Each experiment included at least three replicates. Statistical analysis was carried out by Fisher's protected least significant difference (PLSD) using the STATVIEW program (Abacus Concepts, Inc.). All percentage data were subjected to arcsine transformation before statistical analysis. Data are shown as means ± SEM.

Results

Ethanol treatment efficiently induces blastocyst formation in both Wistar and SD rats

Oocytes collected from Wistar rats were activated by different protocols and cultured for up to 120 h. The results are shown in Figs. 1 and 2. At 24 h after stimulation, the cleavage rate of ethanol-treated oocytes was 98.0 ± 1.9% (black bar), and those of the electrical pulse, ionomycin and strontium treatments were 95.4 ± 1.0%, 90.6 ± 4.7% and 78.2 ± 1.7%, respectively. The cleavage rate of control (non-treated) oocytes was 97.8 ± 1.9%. There were significant differences between the strontium group and the other treatment groups. With respect to blastocyst formation, the ethanol treatment showed a significantly higher percentage (74.5 ± 5.1%, black bar) than the other treatments (electrical pulses, 52.3 ± 4.4%; ionomycin, 60.4 ± 4.9%; strontium, 60.0 ± 2.8%; control, 59.2 ± 2.4%). There were no significant differences in the cell numbers of the blastocysts among the treatment and control groups (data not shown).

In oocytes collected from SD rats, the rates of cleavage and blastocyst formation of oocytes activated by the different protocols are shown in Figs. 3 and 4, respectively. The cleavage rates of these oocytes were 95.1 ± 1.7% (ethanol, black bar), 95.1 ± 2.8% (electrical pulses), 94.7 ± 3.2% (ionomycin) and 79.7 ± 2.2% (strontium) after culturing for 24 h. The cleavage rate of the control (non-treated) oocytes was 81.7 ± 3.0%. There was a significant difference only between the ethanol and strontium treatment groups. The rates of blastocyst formation in the activated oocytes were 78.7 ± 1.7% (ethanol), 67.2 ± 20.0% (electrical pulses), 66.7 ± 2.3% (ionomycin) and 60.9 ± 9.0% (strontium). The blastocyst formation rate of control group was 65.0 ± 6.7%. There were significant differences between the ethanol treatment group and the ionomycin, strontium and control groups; however, there were no significant differences among these groups in the cell numbers of the blastocysts (data not shown). These results suggest that ethanol treatment can effectively induce activation and further embryonic development in rat oocytes derived from Wistar and SD rats. Additionally, blastocyst formation in SD rat oocytes was significantly higher than that in Wistar rat oocytes independent of the activation treatment.

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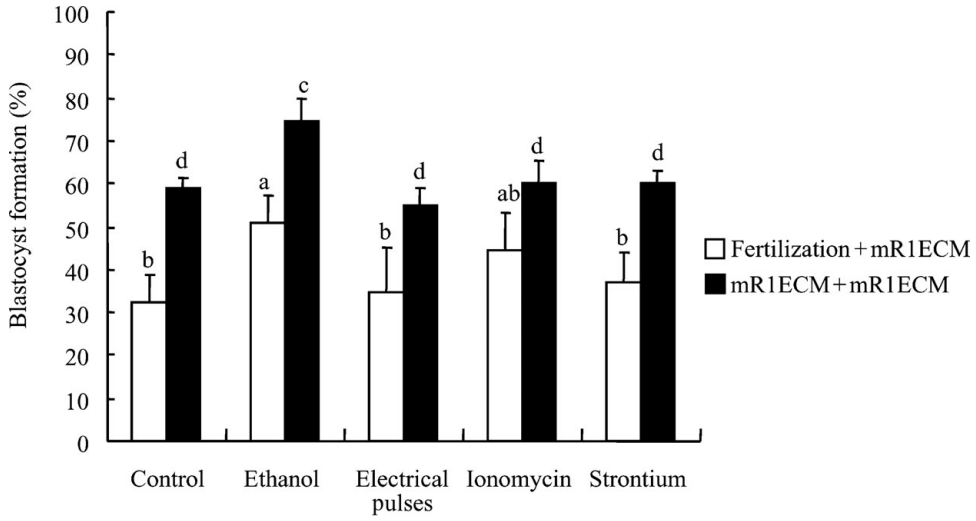

Figure 2 Blastocyst formation in Wistar oocytes activated by different treatments. After activation, the oocytes were cultured in a fertilization medium for 24 h and then further cultured in mR1ECM for up to 120 h (open bar). After activation, the oocytes were cultured in mR1ECM for 24 h and then further cultured in the same medium for up to 120 h (black bar). The different superscripts on the SEM bars denote significant differences among the treatment groups ($p < 0.05$).

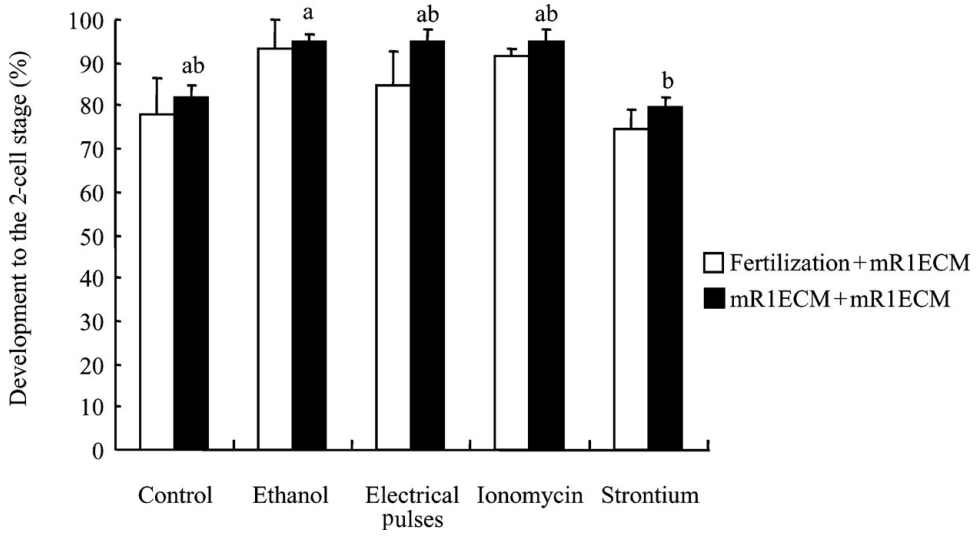

Figure 3 Development to the 2-cell stage in SD oocytes activated by different treatments. After activation, the oocytes were cultured for 24 h in a fertilization medium (open bar) or in mR1ECM (black bar). The different superscripts on the SEM bars denote significant differences among the treatment groups ($p < 0.05$).

Initial culture in a fertilization medium detrimentally affects further development of parthenogenetic embryos

For *in vitro* fertilization of rats, it has been reported that culturing in a fertilization medium (310 mOsm) produces higher developmental ability than culturing in mR1ECM (246 mOsm) (Oh *et al.*, 1998). In contrast, Mizutani *et al.* (2004) demonstrated that oocytes activated by ionomycin in a fertilization media and/or treated with 6-DMAP in a fertilization media after ionomycin treatment showed a significantly lower rate of embryonic development than that of oocytes treated with ionomycin and further 6-DMAP in mR1ECM. To clarify the effect of initial culture in a fertilization medium on further embryonic development, oocytes activated by different protocols were cultured in a fertilization medium for 24 h and then further cultured for up to 120 h in mR1ECM.

When Wistar oocytes were cultured in a fertilization medium for 24 h, the cleavage rate was lower than that of oocytes cultured in mR1ECM (ethanol, 77.4 ± 3.9%; electrical pulses, 76.4 ± 7.3%; ionomycin, 66.0 ± 4.8%; strontium, 53.1 ± 9.4%; control, 65.3 ± 7.2%; Fig. 1, open bar). There were significant differences between the ethanol and strontium groups, and between the

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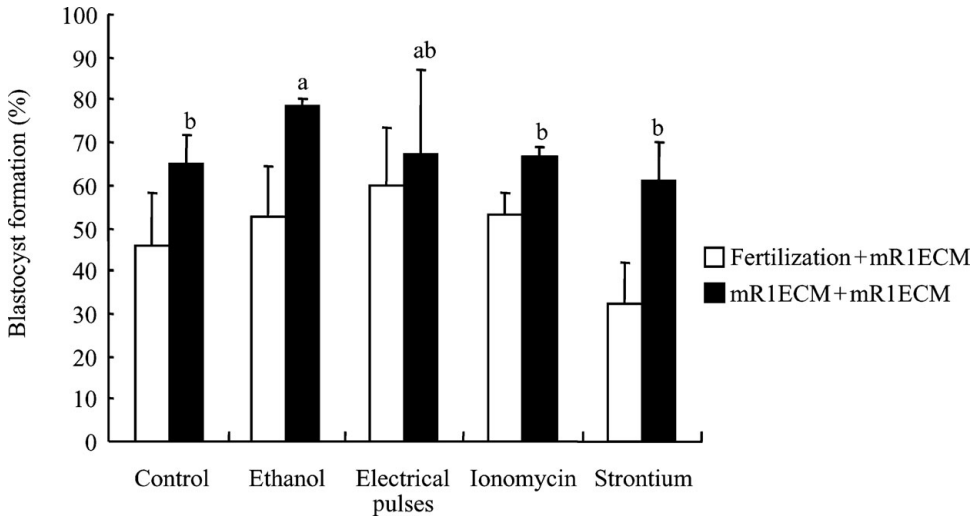

Figure 4 Blastocyst formation in SD oocytes activated by different treatments. After activation, the oocytes were cultured in a fertilization medium for 24 h and then further cultured in mR1ECM for up to 120 h (open bar). After activation, the oocytes were cultured in the mR1ECM for 24 h and then further cultured in the same medium for up to 120 h (black bar). The different superscripts on the SEM bars denote significant differences among the treatment groups ($p < 0.05$).

electrical pulse and strontium groups. When oocytes were cultured in a fertilization medium for 24 h and then cultured in mR1ECM for 120 h, the rates of blastocyst formation were also lower (ethanol, 50.9 ± 6.4%; electrical pulses, 34.5 ± 10.3%; ionomycin, 44.2 ± 8.8%; strontium, 36.7 ± 7.1%; control, 32.7 ± 6.0%). There were significant differences between the ethanol treatment group and the control, electrical pulse and strontium groups.

The cleavage rates of SD oocytes cultured in fertilization media for 24 h were lower than those cultured in mR1ECM (ethanol, 93.2 ± 6.7%; electrical pulses, 85.0 ± 7.5%; ionomycin, 91.7 ± 1.7%; strontium, 74.6 ± 4.3%; control, 78.1 ± 8.2%; Fig. 3, open bar). The rates of blastocyst formation were also lower (ethanol, 52.5 ± 4.8%; electrical pulses, 60.0 ± 13.4%; ionomycin, 53.3 ± 5.0%; strontium, 32.2 ± 9.8%; control, 45.9 ± 12.1%). These results suggest that initial culturing in a fertilization medium for 24 h after activation is not conducive to the further development of parthenogenetic oocytes in rats.

Discussion

In the present study, we compared the developmental abilities of rat oocytes activated by four different treatments (ethanol, electrical pulses, strontium and ionomycin), which have been reported as popular agents for oocyte activation in other species (Cuthbertson *et al.*, 1983; Ito *et al.*, 2004; Méo *et al.*, 2005; Jellerette *et al.*, 2006). We also examined the developmental abilities of oocytes collected from two different outbred types of rat, SD and Wistar, that have been widely used in studies on reproductive technologies (Roh *et al.*, 2003; Shinozawa *et al.*, 2004; Hirabayashi *et al.*, 2005; Ross *et al.*, 2006). The oocytes collected from SD rats showed a significantly higher developmental ability than those collected from Wistar rats independent of activation treatment. In addition, our previous study (Ito *et al.*, 2007) demonstrated that SD oocytes have higher $p34^{cdc2}$ kinase activity, which is involved in arrest at the MII stage. It has been reported that low $p34^{cdc2}$ kinase activity in oocytes contributes to abnormal oocyte activation (Borsuk, 1991; Naito *et al.*, 1992).

The present study demonstrated that ethanol treatment effectively induces activation and further embryonic development of rat oocytes in both SD and Wistar rats when oocytes are cultured in mR1ECM for 120 h after activation. These results differ in some respects from those reported by Jiang *et al.* (2002), possibly due to the use of different media for the initial culture after activation. Jiang *et al.* (2002) used the fertilization medium reported by Oh *et al.* (1998) for both the ethanol treatment and the initial 24 h culture, specifically, mR1ECM containing 110 mM NaCl and 4 mg/ml BSA but omitting polyvinyl alcohol, and showing high osmolarity (310 mOsM). The osmolarity of the mR1ECM usually used as an *in vitro* culture medium for rat embryos is 246 mOsM (Miyoshi *et al.*, 1994, 1995). The present results suggest that a high-osmolarity medium may have a detrimental effect on the embryonic development of parthenogenetically activated oocytes even though it has been reported that high osmolarity

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improves *in vitro* fertilization and the subsequent development of rat embryos (Oh *et al.*, 1998). Indeed, the present study clearly demonstrated that oocytes cultured in a high-osmolarity medium after activation showed lower rates of blastocyst formation compared with those cultured in a low-osmolarity medium, independent of activation protocol. In contrast, in our preliminary study, oocytes treated with ethanol in a low-osmolarity medium (mR1ECM) did not develop (data not shown), though Mizutani *et al.* (2004) report that high-osmolarity media together with ionomycin treatment caused lower developmental ability of parthenogenetic rat embryos. These results suggest that high-osmolarity fertilization media are required for ethanol treatment, and low osmolarity media are needed for further culture after ethanol treatment.

Interestingly, more than 80% of oocytes cultured in both high- and low-osmolarity media cleaved, suggesting that the high osmolarity may affect not the cleavage but the later embryonic development of the parthenotes. One possible explanation for the detrimental effect of high osmolarity may have to do with the regulation of sodium hydrogen exchangers (NHEs). NHEs play an essential role in the regulation of both cell volume and Na^+ in epithelia and intracellular pH, and in the transduction of signals that promote cell proliferation (Tse *et al.*, 1994; Yun *et al.*, 1995). In rodents, it has been reported that NHEs are involved in embryonic development since NHE inhibitors dramatically decrease development from the 2-cell stage to the blastocyst stage (Lane *et al.*, 1998; Kawagishi *et al.*, 2004). Barr *et al.* (1998) also report that two isoforms of NHE (NHE1 and NHE3) are expressed in embryos at the blastocyst stage. Moreover, ethanol treatment plays a crucial role in the up-regulation of NHEs during oocyte activation in pigs, since a NHE inhibitor, amiloride, was found to abolish the effect induced by ethanol (Ruddock *et al.*, 2000). Since oocytes cultured in a high-osmolarity medium for the first 24 h after ethanol treatment showed low developmental ability, high osmolarity not of the activation medium but of the initial culture medium may affect the down-regulation of NHEs, resulting in interference with the embryonic development of the rat. Based on these reports as well as our present results, ethanol treatment is more suitable for parthenogenetic activation and embryonic development in the rat. However, in other rodents, for example in mice, strontium treatment is widely used as the activation regime for somatic cell nuclear transfer (Wakayama *et al.*, 1998; Ogura *et al.*, 2000), because it can mimic the Ca^{2+} oscillations that are usually observed during fertilization (Brind *et al.*, 2000; Zhang *et al.*, 2005). It may be that rat oocytes are more resistant to strontium treatment; further studies are required to clarify the effect of ethanol treatment on the embryonic development of rat parthenotes.

In conclusion, we have shown here for the first time that a combined treatment with 7% ethanol for 3 min in a high-osmolarity medium and 2 mM 6-DMAP for 4 h in a low-osmolarity medium is an effective protocol for the activation and further embryonic development of rat oocytes from both SD and Wistar rats. Moreover, high osmolarity of the initial culture medium had a detrimental effect on development from the 2-cell stage to the blastocyst stage. This activation protocol can also applied to somatic cell nuclear transfer and round spermatid injection in rats.

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