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Fluoxetine Treatment during *In Vitro* Fertilization and Culture Increases Bovine Embryonic Development

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ABSTRACT

K^+ channels are involved in the regulation of a variety of physiological functions, including proliferation, apoptosis and differentiation, in mammalian cells. Our previous study demonstrated that the blockage of K^+ channels inhibits mouse early embryonic development. This study was designed to identify the effect of K^+ channels during bovine embryonic development. K^+ channel blockers (tetraethylammonium (TEA), $BaCl_2$, quinine, ruthenium red and fluoxetine) were added to the culture medium during *in vitro* fertilization (IVF) for 6 h to first identify the short-term effect of these chemicals. Among K^+ channel blockers, fluoxetine, which is used as a selective serotonin reuptake inhibitor, significantly increased the blastocyst formation rate by approximately 6% when compared to control. During the *in vitro* maturation (IVM) of immature oocytes and the *in vitro* culture (IVC) of embryos, the oocytes and embryos were exposed to fluoxetine for either a short-term (6 h) or a long-term (24 h) to compare the embryonic development in response to exposure time. The 6 h exposure to fluoxetine during IVM did not affect the blastocyst formation rate, but the rate of blastocyst formation was reduced after the 24 h exposure. On the other hand, embryonic development increased approximately 10% in both groups of embryos exposed to fluoxetine for 6 and 24 h during IVC. Taken together, fluoxetine treatment during IVF and IVC, but not IVM, enhances bovine embryonic development. These results suggest that fluoxetine-modulated signals in oocytes and embryos could be an important factor towards enhancing bovine embryonic development.

(Key words : cattle, embryonic development, potassium channel, selective serotonin reuptake inhibitor)

INTRODUCTION

The well-known functions of potassium (K^+) channels are to control resting membrane potential, the neuronal firing rate, muscle contraction and hormone secretion. K^+ channels also control cell proliferation, death and differentiation (Lang *et al.*, 2005; Enkvetchakul, 2010). The level of K^+ efflux and/or K^+ channel expression is an important factor that can determine cell fate (Heitzmann and Warth, 2008; Manikkam *et al.*, 2002; Jehle *et al.*, 2011). The K^+ influx via a Na pump keeps the K^+ concentration in cells constant. Opening and closing K^+ channels could affect cell viability. By opening K^+ channels, a slight increase in K^+ efflux may be helpful for cell survival by causing hyperpolarization, but a large K^+ efflux may be detrimental to the cell by decreasing the K^+ concentration and volume in the cell. Closure of K^+ channels could induce depolarization and cell death. Contradictorily, their closure could keep the cell volume constant and induce protection against cell death. Blocking K^+ efflux greatly reduces cell death in cultured cerebellar granule neurons (Lauritzen *et al.*, 2003), and over-expression of the K^+ channel in cultured hippocampal slices reduces cell-death when compared to control (Liu *et al.*, 2005).

Mammalian gametes and embryos express many types of K^+ channels that regulate maturation, fertilization and the cell cycle (Darszon *et al.*, 2007; Tosti and Boni, 2004; Winston *et al.*, 2004; Day *et al.*, 1993; Day *et al.*, 1998). Two-pore domain K^+ channels, a type of K^+ channel, are responsible for the K^+ efflux in apoptotic mouse zygotes, resulting in a decrease in cell volume (Trimarchi *et al.*, 2002). Our recent studies have demonstrated that a blockage of K_{2P} channels inhibits mouse early embryonic development (Hur *et al.*, 2012; Kim *et al.*, 2012). The K_{2P} channels expressed in bovine and mouse early

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embryos (Hur *et al.*, 2009) are modulated by a variety of factors, including temperature, ROS, pH and volume and these factors can be manipulated during *in vitro* culture (IVC). Compared to mammalian cells, only a few studies have demonstrated the role of K^+ channels in germ cells. Moreover, little is known about the role of K^+ channels in bovine germ cells.

Our previous study reported that fluoxetine, one particular K^+ channel blocker, has a unique effect on mouse and bovine embryonic development (Kim *et al.*, 2012). Low concentration and short-term exposure of fluoxetine enhanced mouse embryonic development, whereas high concentration and long-term exposure of fluoxetine decreased blastocyst formation in mouse embryos. However, bovine embryonic development were increased regardless of the incubation time for fluoxetine treatment during IVC (Kim *et al.*, 2012). These results suggest that we should investigate the effect of K^+ channel blockers on bovine embryonic development. In this study, K^+ channel blockers including fluoxetine were treated to bovine oocytes and embryos during different *in vitro* processes, such as *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and IVC.

MATERIALS AND METHODS

1. Chemicals

All components of the medium and other chemicals were tissue culture grade and were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise specified.

2. Oocyte Isolation and IVM

All experiments were performed with the approval of the Animal Ethics Committee of the National Institute of Animal Science and Gyeongsang National University. Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 3 h in sterile 0.9% NaCl solution at 38℃. The procedures of oocyte isolation and IVM were performed as described previously (Kim *et al.,* 2012). For IVM, sets of 30～90 cumulus-oocyte complexes were cultured in TCM-199 containing 10% (v/v) FBS (Invitrogen, Grand Island, NY), 5 mM HEPES, 2.3 mM sodium pyruvate, 5.5 mM calcium lactate, 36 mM $NaHCO_3$, 50 μg/ml gentamycin, and hormones (35 μg/ml FSH and 10 μg/ml LH) in four-well dishes at 39℃ in a humidified atmosphere with 5% CO_2 in air for 22 h.

3. IVF and IVC

Frozen-thawed Korean proven bull number semen was used for sperm isolation. The procedures of IVF and IVC were performed as described previously (Kim *et al.*, 2012). Briefly, sperm were washed with Brackett and Oliphant's (BO) medium supplemented with 5 mM/l caffeine (Caff-BO) by centrifugation at 1,500 g for 5 min. The sperm pellet was resuspended in Caff-BO supplemented with 1% (w/v) BSA and 20 μg/ml heparin to a sperm concentration of 2×10^5/ml. The sperm were incubated at a 45° angle and at 39℃ in a humidified atmosphere of 5% CO_2 in air for 1 h to allow the motile sperm to swim upwards. There suspended sperm were used for the IVF procedure. The sperm were transferred to oocytes matured *in vitro*. After incubation for 6 h, cumulus cells were removed by vortexing and transferred into 500 μl of CR1aa medium supplemented with 0.3% (v/v) BSA for 3 days and additionally cultured in CR1aa medium containing 10% (v/v) FBS for 4 days at 39℃ in a humidified atmosphere with 5% (v/v) CO_2 in air. Development of the embryos was observed every 24 h under an inverted microscope at 100 × magnification. The embryos were cultured for 7 days to determine cell number and blastocyst formation.

4. Statistical Analysis

Data are represented as the mean±S.D. Significant differences among the groups were analyzed using one-way ANOVA test and post hoc comparisons using Tukey's test (SPSS18 software; SPSS, Inc., Chicago, IL, USA). Differences were considered significant at $p<0.05$.

RESULTS

To assess the effect of K^+ channel blockers on bovine embryonic development, K^+ channel blockers (1 mM tetraethylammonium (TEA), 1 mM $BaCl_2$, 10 μM quinine, 10 μM ruthenium red and 10 μM fluoxetine) were added to the culture medium during IVF (6 h). The concentrations used in this study were chosen to block each specific K^+ channel by approximately 30% (Hur *et al.*, 2012; Kim *et al.*, 2012). Compared to IVM and IVC, IVF requires a short incubation time; therefore, we first tried to identify the effect of K^+ channel blockers on IVF. Cleavage and blastocyst formation rates were analyzed for both 2-cell and blastocyst development, respectively. During cleavage, there was no significant difference in the groups treated with K^+ channel blockers when compared to the control group (Table

1). During development to the blastocyst stage, fluoxetine treatment showed a significant increase when compared to control group (28.2±2.9% in the fluoxetine-treatment group versus 22.5 ±1.6% in the control group, $p<0.05$). TEA, $BaCl_2$, quinine and ruthenium red had no significant effect on blastocyst formation ($p>0.05$). Following these results, we decided to focus on the effect of fluoxetine on embryonic development.

To identify the time-dependent effect of fluoxetine (short-term and long-term) on embryonic development, fluoxetine was added to the culture medium for 6 h or 24 h for IVM. As shown in Table 2 and Table 3, the cleavage rates were not affected by fluoxetine treatment regardless of exposure time. Fluoxetine treatment for 6 h had no significant effect on the formation of blastocysts between the two groups ($p>0.05$, 25.6 ±8.5% in the fluoxetine-treated group versus 26.0±0.1% in the control group), whereas treatment for 24 h with fluoxetine decreased the rate of blastocyst formation (13.7±8.8% in the fluoxetine-treated group versus 23.5±5.0% in the control group).

The effect of fluoxetine treatment during IVC was also investigated. Fluoxetine was added to the culture medium for 6 h or 24 h. As shown in Table 4 and Table 5, fluoxetine treatment for 6 hand 24 h showed no significant difference in the cleavage rate when compared to control. However, fluoxetine-treatment increased blastocyst formation in both groups when compared to control group (by 8% and 12% in fluoxetine-treated groups for 6 h and 24 h, respectively). Total cell number of blastocyst was not significantly different between control and fluoxetine treatment, as judged by propidium iodide staining ($p>0.05$).

Table 1. Effect of K^+ channel blockers treated during IVF on bovine embryonic development

Treatment	No. of oocytes	No. of embryos developed to	
		2-cell(%)	Blastocyst(%)
Control	255	191(74.9± 2.1)[a]	43(22.5±1.6)[a]
Tetraethylammonium	250	180(72.0± 2.8)[a]	40(22.2±2.1)[a]
$BaCl_2$	245	160(65.3± 6.0)[a]	36(22.3±4.0)[a]
Quinine	223	146(65.6±12.1)[a]	36(24.6±4.1)[ab]
Ruthenium red	251	154(61.3± 9.4)[a]	30(19.1±2.9)[a]
Fluoxetine	252	176(69.8± 6.0)[a]	50(28.2±2.9)[b]

[a,b] Values with different superscripts within each column are significantly different ($p<0.05$). Percentages are the means ±S.D. of five independent experiments.

Table 2. Effect of fluoxetine treated during IVM (6 h) on bovine embryonic development

Treatment	No. of oocytes	No. of embryos developed to	
		2-cell (%)	Blastocyst (%)
Control	263	169(64.3±4.0)[a]	44(26.0±0.1)[a]
Fluoxetine	115	86(74.8±8.3)[a]	22(25.6±8.5)[a]

[a] Values with same superscripts within each column are not significantly different ($p<0.05$). Percentages are the means± S.D. of three independent experiments.

Table 3. Effect of fluoxetine treated during IVM (24 h) on bovine embryonic development

Treatment	No. of oocytes	No. of embryos developed to	
		2-cell (%)	Blastocyst (%)
Control	388	298(76.8±5.7)[a]	70(23.5±5.0)[a]
Fluoxetine	376	284(75.5±4.5)[a]	39(13.7±8.8)[b]

[a,b] Values with different superscripts within each column are significantly different ($p<0.05$). Percentages are the means ± S.D. of five independent experiments.

Table 4. Effect of fluoxetine treated during IVC (6 h) on bovine embryonic development

Treatment	No. of oocytes	No. of embryos developed to	
		2-cell (%)	Blastocyst (%)
Control	259	184(70.9±7.1)[a]	42(23.0±2.4)[a]
Fluoxetine	250	183(73.2±3.0)[a]	57(31.0±4.7)[b]

[a,b] Values with different superscripts within each column are significantly different ($p<0.05$). Percentages are the means ±S.D. of five independent experiments.

Table 5. Effect of fluoxetine treated during IVC (24 h) on bovine embryonic development

Treatment	No. of oocytes	No. of embryos developed to	
		Cleavage (%)	Blastocyst (%)
Control	150	117(78.0±6.9)[a]	26(22.2±2.9)[a]
Fluoxetine	150	117(78.0±7.2)[a]	40(34.6±6.9)[b]

[a,b] Values with different superscripts within each column are significantly different ($p<0.05$). Percentages are the means ±S.D. of three independent experiments.

DISCUSSION

In this study, we reported, for the first time, the effect of fluoxetine treatment during three different *in vitro* procedures (IVM, IVF and IVC) on bovine embryonic development. Fluoxetine treatment during IVF showed an increase in blastocyst formation, but fluoxetine treatment during IVM demonstrated a different effect on blastocyst formation, with no effect following short-term exposure of fluoxetine and a decrease following long-term exposure of fluoxetine. Fluoxetine treatment during IVC increased blastocyst formation regardless of exposure time. However, it was difficult to determine what caused this different effect following fluoxetine treatment. Immature oocytes, mature oocytes and zygotes subjected to IVM, IVF and IVC procedures, respectively, could be modulated by many different factors elicited from their microenvironments in eggs at different stages. Different environments could also likely induce a different effect from any chemicals added to the culture medium.

Among the K^+ channel blockers tested during IVF in this study, fluoxetine treatment had an effect on bovine embryonic development. Fluoxetine modulates K^+ channels, such as the voltage-activated K^+ channel (Kv1.3) (Choi *et al.*, 1999), G protein-activated inwardly rectifying K^+ channels (GIRK) (Kobayashi *et al.*, 2003) and two-pore domain K^+ (K_{2P}) channels (Kennard *et al.*, 2005; Heurteaux *et al.*, 2006; Lee *et al.*, 2011; Thummler *et al.*, 2007; Kim *et al.*, 2012). Kv1.3 is inhibited by application of TEA and Ba^{2+}(Choi *et al.,* 1991; Somodi *et al.,* 2004), and GIRK is inhibited by Ba^{2+}(Walsh, 2011). In this study, TEA and Ba^{2+} did not affect bovine embryonic development. These results suggest that fluoxetine-mediated changes in bovine embryonic development could be affected by channel types other than Kv1.3 and GIRK channels. In our previous study, fluoxetine affected TREK channels, a family of K_{2P} channels; therefore, the blockage of TREK channels inhibited mouse embryonic development (Kim *et al.*, 2012). TREK channels expressed in bovine oocytes and embryos could be a target for fluoxetine (Hur *et al.*, 2009). In addition to K^+ channels, fluoxetine affects a variety of signaling molecules, such as protein kinase C (PKC), mitogen-activated protein kinase (MAPK), calcium/calmodulin-dependent protein kinase II (CaMKII), and the extracellular signal-regulated kinase (ERK)-cyclic AMP-responsive-element-binding protein (CREB) signaling pathway (Edgar *et al.*, 1998; Edgar *et al.*, 1999; Mercier *et al.*, 2004; Fumagalli *et al.*, 2005; Cammarota *et al.*, 2008; Qi *et al.*, 2008). Moreover, the effect of fluoxetine through these signaling molecules could affect functions in many different types of cells, including germ cells. In particular, cells expressing serotonergic receptors are more likely to respond to fluoxetine treatment. The main function of fluoxetine is to selectively block re-absorption of serotonin (5-hydroxytryptamine, 5-HT) in the central nervous system, thereby maintaining 5-HT levels at proper levels (Lemberger *et al.*, 1985; Goodnick, 1991; Morrison *et al.*, 2005; Collier *et al.*, 2012). 5-HT acts locally in rodent ovaries and the female genital tract, where it may influence granulosa and cumulus cells as well as oocytes and preimplantation embryos (Dube and Amireault, 2007; Amenta *et al.*, 1992; Vesela *et al.*, 2003). Bovine mammary tissues and mammary epithelial cells express the receptors for 5-HT (5-HTR1B, 5-HTR2A, 5-HTR2B, 5-HTR4 and 5-HTR7) (Collier *et al.*, 2012). However, little information about the expression and function of 5-HTR is available for bovine embryos. These serotonergic networks, which may be present in the bovine genital tract, could participate in different fluoxetine response due to difference of expression levels or mechanisms in different stages of eggs. Different stages of eggs could work through their own complex signaling pathways induced by fluoxetine. Immature oocytes and zygotes are likely to respond differently following long-term exposure to fluoxetine. Normally, short-term exposure to chemicals, including fluoxetine, may activate a variety of signaling molecules, therefore, giving rise to a positive effect on cell physiological functions, whereas long-term exposure to chemicals might induce cytotoxicity to cells. However, the differences in cell response by exposure time could differ depending on particular cell types because cells have their own characteristics that are affected by their microenvironment and intracellular signaling molecules. On the other hand, long-term exposure of zygotes to fluoxetine could compensate for inhibitory signaling, such as K^+ channel blockage and internal signaling, as shown with IVC.

In our previous study, fluoxetine showed dual effects on mouse embryonic development dependent on both concentration and exposure time (Kim *et al.*, 2012). Fluoxetine at high concentrations and long-term exposure during IVC inhibited mouse early embryonic development through the blockage of TREK channels, but fluoxetine treatment during IVC showed an enhancing effect on bovine embryonic development. These results demonstrate that fluoxetine has a species-specific effect. In addition to differences in the serotonergic networks, the microenvironment, and the signaling pathways, K^+ channel expression levels could be different between mice and cattles.

Bovine cell size is larger than that of mice. Furthermore, bovine embryos have greater lipid contents when compared to mouse embryos (Genicot *et al.*, 2005; Paczkowski *et al.*, 2013). The characteristic lipid profiles of eggs could change in response to the developmental stages and IVC conditions, species-specificity and the microenvironment (Ferreira *et al.*, 2010). The lipid contents could also be a factor in the different effects of fluoxetine treatment on bovine embryonic development. Interestingly, peripheral serotonin enhances lipid metabolism (Watanabe *et al.*, 2010). In addition, serotonin binds to the lipid membrane and produces nonspecific effects (Peters *et al.*, 2013).

In conclusion, fluoxetine could affect a variety of signaling pathways at plasma membrane, thereby affecting bovine embryonic development. These results suggest that serotonin may be a target for controlling bovine embryonic development. Fluoxetine treatment during IVF and IVC is helpful for bovine embryonic development.

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