Effects of Oxytocin and IL-1 α on *In Vitro* Development of Bovine Embryos Cultured with Uterine Cells

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ABSTRACT

The purpose of this study was to determine effects of oxytocin and interleukin-1 α on *in vitro* development of bovine embryo cultured with endometrial epithelial and stromal cells isolated from bovine uterus. The expressions of COX-2 mRNA in bovine endometrium were also studied. When embryos were cultured with epithelial cells, the rate of blastocysts was significantly ($p<0.05$) higher in embryos treated with oxytocin than that of control group. The rate of hatched blastocysts was also significantly ($p<0.05$) higher in embryos treated with oxytocin than those of two control groups. On the other hand, when the embryos were cultured with stromal cells, the rate of blastocysts were significantly ($p<0.05$) higher than those of groups treated with IL-1 α, oxytocin and control with stromal cells than that of control group without stromal cells. The rate of blastocysts hatched were also significantly ($p<0.05$) higher in group treated with IL-1 α than those of control group without stromal cells and oxytocin group. In another experiment, COX-2 gene was expressed in embryo group treated with oxytocin during the co-culture of embryos with epithelial cells. In contrast, COX-2 mRNA was expressed in group treated with IL-1α when the embryos were cultured with stromal cell. This result shows that oxytocin and IL-1 α were stimulate embryo development *in vitro* when embryos were cultured with epithelial and stromal cells, and can affect the development of bovine embryos in the uterus.

(Key words : Uterine cells, Oxytocin, IL-1α, *In vitro* development, Bovine embryos)

INTRODUCTION

Prostaglandins are important regulators of reproductive events including ovulation, implantation, parturition, luteolysis and recognition of pregnancy (Dubois *et al.*, 1998). Prostaglandins are members of the eicosanoid family of molecules. They are derived from open chain, 20-carbon polyunsaturated fatty acids, typically arachidonic acid (Crofford., 2001). Prostaglandin E_2 (PGE_2) is one of the major secretory products in the uterine endometrium of ruminants. The PGE_2 has been shown to block Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced luteolysis when both PGs were infused simultaneously into the ovarian artery or into the ovarian vascular pedicle of non-pregnant ewes (Kennedy, 1980; Keys *et al.*, 1985; Emond *et al.*, 1988). Evidence in humans indicate that follicular ratio of PGE_2 to $PGF_{2\alpha}$ was associated with the pregnancy rate after IVF with increased concentrations of PGE_2 to $PGF_{2\alpha}$ being associated with a greater rate of pregnancy. The ratio of PGE_2 and $PGF_{2\alpha}$ has been implicated in the development and success of early pregnancy in several species (Smith *et al.*, 1991). Concentrations of PGE_2 secreted into the culture media during oocyte maturation, and the hatching rate of ovine embryos increased with treatment of PGE_2 (Sayre and Lewis, 1993). Soto *et al.* (2003) reported $PGF_{2\alpha}$ to inhibit embryonic development only if treatment was during oocyte maturation or fertilization, but it did not affect the development if treatment was at stages after fertilization. In bovine endometrium, epithelial and stromal cells have specific morphological and functional properties. Epithelial cells preferentially produce $PGF_{2\alpha}$, whereas stromal cells produce mainly PGE_2 (Fortier *et al.*, 1988). A small amount of PGE_2 is synthesized by maturing bovine cumulus-oocyte complexes (COCs) *in vitro* (Gurevich and Shemesh, 1996).

The bovine endometrium is a complex tissue and consists mainly of epithelial and stromal cells. Although both kinds of endometrial cells can produce $PGF_{2\alpha}$, PGE_2 they have different morphological and physiological proterties. Oxytocin stimulates $PGF_{2\alpha}$ production only in bovine epithelial cells (Andrew and Daniel, 1993). Oxytocin (OT), from the pituitary and corpus luteum (CL), stimulates the pulsatile release of $PGF_{2\alpha}$ via the OT receptor in the luminal epithelium of

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the endometrium (Flint *et al.*, 1994).

Recent evidence indicates that interleukin-1 (IL-1), which consists of two sparate cytokines called interleukin-1α(IL-$1_α$) and interleukin-β (IL-1β) is produced in the uterus during the periimplantation period (Dinarello, 1989). IL-1α stimulates PG production and COX activity in endometrial stromal cells (Brent and Thoma, 1995).

The product of endometrial PG is mainly governed by the rate-limiting enzyme PG endoperoxidase H synthase (PGHS)-2, also known as cyclooxygenase-2. Because PGHS-1 is not found in bovine endometrium (Arosh *et al.*, 2002), PGHS-2 is responsible for the conversion of arachidonic acid into PG, the common precursor of the various forms of PGs, including $PGF_{2α}$ and PGE_2. The purpose of this study was to determine effects of oxytocin and interleukin-1α on *in vitro* development of bovine embryo cultured with endometrial epithelial and stromal cells isolated from bovine uterus.

MATERIALS AND METHODS

Isolation and Culture of Endometrial Cells

Uteri of Holstein cows were obtained from the slaughterhouse and were transported on ice to the laboratory within 2 hr. The stages of the estrous cycle were determined by macroscopic observation of the ovary and uterus as described previously (Miyamoto *et al.*, 2000). In the present study, uteri of the early estrous cycle (Days 2~5) were used. The epithelial and stromal cells from the endometrium were separated using a modification of procedures described previously (Miyamoto *et al.*, 2000). The uterine horns ipsilateral to the corpus luteum was used for cell culture. A polyvinyl catheter was inserted into the side of the oviduct, and the ends of the horn were tied shut in order to retain the collagenase solution used for solubilizing the epithelial cells as described below. The uterine lumen was washed 3 times with 30~50 ml sterile Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution (HBSS) supplemented with 100 IU/ml penicillin, 100 μg /ml streptomycin and 0.1% BSA. A total of 30~50 ml sterile HBSS containing 0.05% collagenase was then infused into the uterine lumen through the catheter. Epithelial cells were isolated by incubation at 37℃ for 45 min and with gentle shaking for 30 min. The cell suspension was filtered through metal meshes(100 μm and 80 μm) to remove undissociated tissue fragments. The filtrate was washed 3 times by centrifugation at 100 ×g for 10 min with Dulbecco's Modified Eagle's medium (DMEM) supplemented with antibiotics and 0.1% BSA. After the washes, the cells were counted with a hemocytometer. The cell viability was higher than 95% as assessed by 0.5% trypan blue dye exclusion.

After removing the epithelial cells, the uterine lumen was washed with sterile HBSS supplemented with antibiotics and 0.1% BSA. The horns was then cut transversely with scissors into several segments, which were slit to expose the endometrial surface. Intercaruncular endometrial strips were dissected from the myometrial layer with a scalpel and washed once in 50 ml of sterile HBSS containing antibiotics. The endometrial strips were then cut into small pieces (1 mm^3). The minced tissues were digested by stirring for 60 min in 50 ml of sterile HBSS containing 0.05% collagenase and 0.1% BSA. The dissociated cells were filtered, washed and counter as described above. Cell viability was higher than 85%. The cells obtained of consisted of stromal cells and only a few fibroblasts, erythrocytes, and glandular epithelial cells.

Bovine Embryo Production and Culture

Embryos used in the present study were produced by *in vitro* matured and *in vitro* fertilized of bovine oocytes. Oocyte-cumulus complexes (COCs) were matured for 24 hr in tissue culture medium 199 (TCM-199) supplemented with 10% fetal bovine serum, 1 μg/ml estradiol-17β, 5 μg/ml luteinizing hormone, 0.5 μg/ml follicle stimulating hormone. Following the maturation, oocytes were transferred to 50 μl drops of Brackett-Oliphant (BO) medium supplemented with 20 mg/ml of bovine serum albumin (BSA). Frozen-thawed semen were washed two times in BO medium with 10 mM caffeine by centrifugation for 10 min. After centrifuge, approximately $2×10^6$ sperm/ml of frozen/thawed semen was added to each fertilization drop. *In vitro* fertilization was performed for 10~15 hr at 38.5℃ in humidified 5% CO_2 in air atmosphere. And then, zygote were cultured in CR1aa medium with or without bovine uterine endometrial cells that treated by oxytocin (100 nM) and interleukin-1α (10 ng/ml).

RNA Extraction and RT-PCR

Total RNA was extracted from the uterine endometrial cells using TRIzol regent. The yield of extracted total RNA for each sample was determined by ultraviolet(UV) spectrophotometer(optimal density 260 nm). Aliquots of 2 μg of total RNA were used for reverse transcription.

The 2 μg of total RNA was reverse-transcribed using Avian Myeloblastosis Virus (AMV) reverse transcriptase and oligo-dT primer. After addition of PCR mixture, PCR was then carried out for 3 min at 95℃, 50 sec at 57℃, 1 min at 72℃. Primer in the PCR reaction were used COX-2 and β-actin. The sequence of COX-2 primer were 5'-TCCAGAT CACATTTGATTGACA-3' (5' primer, 22 mer) and 5'-TCTTTGACTGTGGGAG GATA

CA-3' (3' primer, 22 mer). The primer for β-actin were 5'-ATCACCAT CTTCCAGGAGCG-3' (5' primer, 20 mer) and 5'-GATGGCATGGACTGTGGTCA-3' (3' primer, 20 mer). PCR products were then separated on 2.0% agarose gel containing 0.5 μg/ml ethidium bromide.

Statistical Analysis

Experiments involving multiple comparisons were studied by analysis of variance followed by Duncan's multiple range test of the means. Means were considered significantly different at $p<0.05$.

RESULTS

As shown in Fig. 1, when embryos were cultured with epithelial cells, the rate of blastocysts was significantly ($p<0.05$) higher in embryos treated with oxytocin than that of control group without epithelial cells. The rate of hatched blastocysts was also significantly ($p<0.05$) higher in embryos treated with oxytocin than that of all experimental groups. Fig. 2 shows the effects of oxytocin and IL-1α on *in vitro* development of bovine embryos co-cultured with stromal cells. The rate of blastocysts were significantly ($p<0.05$) higher in groups treated with IL-1α, oxytocin and control group with stromal cells than that of control group without stromal cells. The rate of blastocysts hatched were significantly ($p<0.05$) in group treated with IL-1α than that of control group without stromal cells and oxytocin group. In another experiment, COX-2 gene was expressed in embryo group treated with oxytocin during the co-culture of embryo and epithelial cells (Fig. 3). In contrast, COX-2 mRNA was expressed in group treated with IL-1α when the embryos were cultured with stromal cells (Fig. 4).

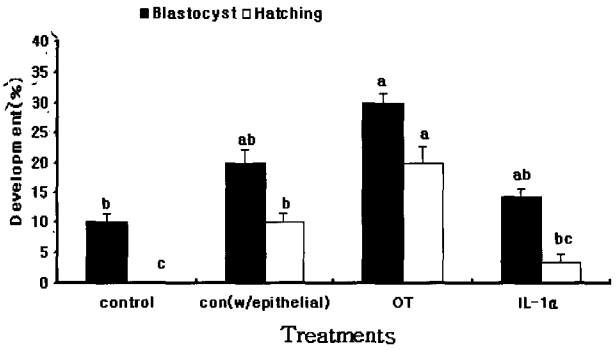

Fig. 1. Effects of oxytocin and IL-1 α on *in vitro* development of bovine embryo cultured with epithelial cells. OT: Oxytocin 100 μM, IL-1 α: Interleukin-1 α 10 ng/ml.

[a-c] Percentages with different superiscripts within same columns indicate significant differences ($p<0.05$).

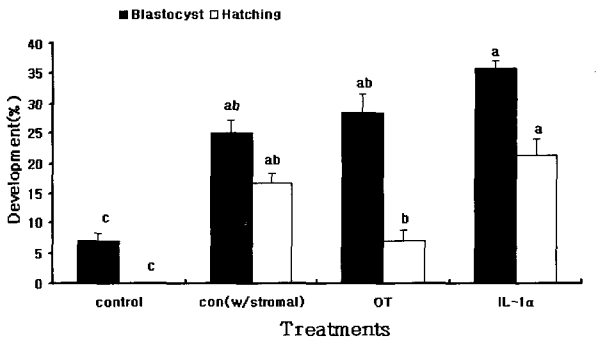

Fig. 2. Effects of oxytocin and IL-1 α on *in vitro* development of bovine embryo cultured with stromal cells. OT: Oxytocin 100 μM, IL-1 α: Interleukin-1 α 10 ng/ml.

[a-c] Percentages with different superiscripts within same columns indicate significant differences ($p<0.05$).

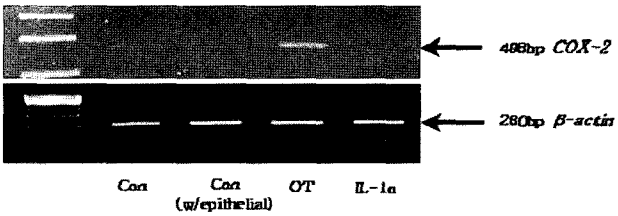

Fig. 3. COX-2 mRNA expression following treatment of oxytocin and interleukin-1 α during the culture of bovine embryos with epithelial cells. OT: 100 μM of oxytocin, IL-1 α: 10 ng/ml of Interleukin-1 α.

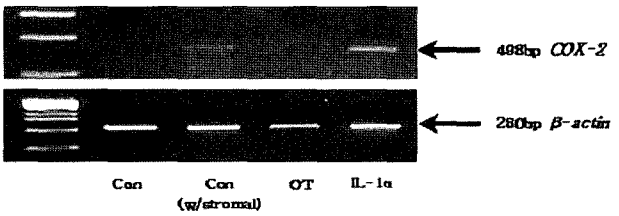

Fig. 4. COX-2 mRNA expression following treatment of oxytocin and interleukin-1 α during culture of bovine embryos with stromal cells. OT: 100 μM of oxytocin, IL-1 α: 10 ng/ml of Interleukin-1 α.

DISCUSSION

The present study was an examination of the effect of oxytocin and interleukin-1α on *in vitro* development during the culture of embryos with endometrial cells. The result of this study shows that oxytocin and interleukin-1α stimulate development *in vitro* of bovine embryos. In mammals, communication between cell types of the uterus is important for regulation of uterine function and implantation. To accomplish this, uterine cells must be capable of releasing biochemical

signals to act on heterologous cell types.

Consistent with the capability of uterine epithelial cells to secrete IL-1α into the uterine lumen, since uterine fluid pooled from mice during the proestrous stage of the cycle contained significant levels of this cytokine. The marked preferential apical secretion of IL-1α by uterus epithelial cells parallels the apical preference for IL-6 secretion by these cells and is in contrast to the basal preference for another proinflammatory molecule, $PGF_{2\alpha}$. Prostaglandins, produced from membrane phospholipids by the action of phospholipase A2, cyclooxygenase, and specific prostaglandin syntheses, are important regulators of ovulation, luteolysis, implantation, and parturition in reproductive tissues (Alan, 2004). Prostaglandin production has been reported from the cumulus-oocyte complex stage (Viggiano *et al.*, 1995), 4~8-cell stage embryo (Sayre and Lewis, 1993), through to the elongated conceptus stage (Lewis and Waterman, 1985). PGs may be also involved in blastocyst hatching since PGs of the E series mediate in the control of water movement across epithelium and hatching occurs due to fluid accumulation in the blastocoele (Yee *et al.*, 1993). In mammal, the pulsatile release of oxytocin by neurohypophysis stimulates the production of uterine $PGF_{2\alpha}$. The ability of oxytocine to stimulate $PGF_{2\alpha}$ release is higher at the time of luteolysis. In the present study, *in vitro* development of embryos was increased by oxytocin during the culture with epithelial cells. This results indicate that oxytocin has affected to development *in vitro* of blastocysts and hatching stages of early embryos.

Oxytocin, from the pituitary and/or the corpus luteum (McCracken *et al.*, 1999) stimulates $PGF_{2\alpha}$ synthesis in the endometrial epithelial cells. This $PGF_{2\alpha}$ is transferred locally to the ovary by a counter-current exchange system and acts on the corpus luteum causing a decline in progesterone secretion and its destruction. The uterine oxytocin receptor concentrations determine the sensitivity of endometrium to oxytocin stimulation (Roberts *et al.*, 1976). Oxytocin preferentially stimulated $PGF_{2\alpha}$ production compared with PGE_2 in bovine endomtrial epithelial cells. Stimulation of the epithelail cells with oxytocin at concentration as known to stimulate prostaglandin production increased the amounts of cyclooxygenase-2 (Parent *et al.*, 2003). However, the hypothesis suggesting that the cyclooxygenase-2 pathway to prostaglandin production be supported conclusively by the results of the present study.

The present studies shows that IL-1α stimulated embryo development *in vitro* during the culture with stromal cells than epithelial cells. Our present studies also showed that the increase in COX-2 activity in the endometrial stromal cells after exposure to IL-1α may have been the result of increased COX-2 gene expression. In support of this, increases in PGs synthesis from stromal cell and PGE_2 accumulation was greater than that of $PGF_{2\alpha}$ and PGFM for both control and IL-1α-treated cells (Brent and Thoma, 1995).

This is further supported by the observation that expression of cyclooxygenase-2 protein in response to oxytocin and interleukin-1α treatment closely matched the production of prostaglandins. Treatment with PGE_2 was reported to increase the hatching rate of ovine embryo (Sayre and Lewis, 1993). Thus, by inducing COX-2 gene expression, the resulting oxytocine and interleukin-1α effects is a net increase of *in vitro* development of early embryos.

In conclusion, this study has demonstrated that coculture with epithelial cells and embryos may express $PGF_{2\alpha}$ and PGE_2 by oxytocin and IL-1α stimulation result to enhance blastocysts and hatching rates. Thus, both cell types may be responsible for embryo development *in vitro* and *in vivo*.

ACKNOWLEDGEMENT

The authors acknowledge Institute of Animal Resources, Kangwon National University, Korea for culture of bovine epithelial cells.

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(Received: 16 November 2006 / Accepted: 18 December 2006)