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Effect of uterine histotroph on embryo development in pigs*

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ABSTRACT

The aim of this study was to investigate the effect of uterine histotroph on embryo development and the expression of cysteine-rich protein 2 (CRP2), coatomer subunit gamma-2 (G2COP), myoglobin (MYG), vascular endothelial growth factor D (VEGFD), collagen alpha 4 chain (COL4) and galactoside 3-L-fucosyltransferase 4 (FUT4) proteins in porcine embryo during pre-implantation. Uterine histotroph (UH) was collected from uterine horn on corpus albican phase, and embryos were cultured in porcine zygote medium with UH for 168 hours. Cleavage and blastocyst formation of embryo were detected at 168 hours after *in vitro* fertilization. And CRP2, G2COP, MYG, VEGFD, COL4 and FUT4 proteins were observed using confocal laser microscope. In results, embryo cleavage rate was not significantly changed by UH, but blastocyst rate was significantly (P<0.05) decreased in UH-treated embryos. Moreover, CRP2, G2COP, MYG, VEGFD, COL4 and FUT4 proteins were expressed in blastomere. CRP2 in embryo was significantly overexpressed (P<0.05), but not G2COP, MYG, VEGFD, COL4 and FUT4 proteins. In summary, UH on corpus albican phase was increased CRP2 protein in embryo, and inhibited blastocyst formation in preimplantation porcine embryos, suggesting that CRP2 may play an interrupter on embryo development in pigs.

(Key words: uterine histotorph, cysteine-rich protein 2, embryo)

Introduction

The uterus is one of female reproductive tract, which undergoes a repetitive cycle. In addition, intrauterine physiological environments such as endometrium thickness, amount of cytokines and secretion substances are dramatically changed by steroid hormones (De Rensis *et al*., 2012). One of the physiological changes, secretion substances, provide materials to develop and implant embryo and grow fetus which is called uterine fluid or histotroph (Wang *et al*., 2005; Østrup *et al*., 2011). The uterine histotroph (UH) has various substances such as hormones, enzymes, growth factors, cytokines, proteins, adhesion factors, nutrients, and other substance (Lee *et al*., 2016). Thus, the UH provides an important source for embryo development during pre-implantation phase (Tarraf and Knight, 1995).

The uterine physiology is orchestrated by corpus luteum of ovary in mammals and corpus luteum is formed in ovulated site of ovary which secretes progesterone. The progesterone level of blood is increased until day 14 (the day of ovulation = day 0) in pigs, however, if embryos do not implant in endometrium, corpus luteum is regressed, and progesterone level of blood is dramatically decreased and prostaglandin F2 alpha is increased for day 14 to 21 that occurred degeneration of endometrium in pigs. In addition, corpus luteum is changed to corpus albican, which is called 'luteolysis' (Zenclussen *et al*., 2014).

In previous study, we found various proteins in the UH during follicular and luteal phase (corpus albican phase) in the pigs. Forty-nine proteins in UH were identified by matrix-assisted laser desorption/ionization (MALDI-TOF). Cysteine rich protein 2 (CRP2), coatomer subunit gamma-2 (G2COP) and myoglobin (MYG), vascular endothelial growth factor D (VEGFD), collagen alpha 4 chain (COL4) and galactoside 3-L-fucosyltransferase 4 (FUT4) proteins were higher in luteal phase than follicular phase in the UH from sows (Lee *et al*., 2016). Thus, this study was investigated to influence of UH focused on the six proteins in

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porcine embryo.

CRP2 associates with the actin cytoskeleton, cell differentiation and promote smooth muscle cell proliferation (Kong *et al*., 1997), G2COP is related with endoplasmic reticulum (ER) to Golgi transport, (Duden, 2003), which gene was increased in blastocyst compared to early stage of mouse embryo (Cui *et al*., 2007). MYG can be combined with iron, the complex carries oxygen in mammalian cells (Oliveira *et al*., 2012), and MYG in smooth muscle tissue in the human uterine was increased during pregnancy (Jaisle *et al*., 1966). VEGF is an important factor in angiogenesis, and regulates vascular permeability (Ferrara and Davis-Smyth, 1997), also VEGF levels of UH are significantly reduced in infertility compared with fertile women during mid-proliferation phase (Hannan *et al*., 2011). COL4 is one of the extracellular matrix, all of which are present in basement membranes (Hudson *et al*., 2003). COL4 of follicular basal lamina surrounds oocyte-granulosa complex, plays an important role during follicular development in the bovine ovary (Rodgers *et al*., 1998). FUT4 is One of the glycosyltransferases family which mostly observed in some epithelial cells (Homeister *et al*., 2001). FUT4 mRNA and protein in human endometrium tissues were expressed, also progesterone regulated FUT4 transcription and translation. Thus FUT4 may be an important factor in human and animal during implantation (Ponnampalam and Rogers, 2008). Thus, we focused on the functions of six proteins in the fertilization, embryo development, uterine physiology and pregnancy in human and pigs. First, we investigated whether UH at corpus albican phase regulates embryonic development, and observed the pattern of protein expression of CRP, G2COP, MYG, VEGFD, COL4 and FUT4 in porcine embryos.

Materials and Methods

1. Collection of uterine histotorph (UH)

All procedures involving animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139). Uteri were collected from 8 crossbred pigs (Yorkshire × Landrace) in slaughterhouse (Pocheon farm, Korea) and transported to the laboratory at 4°C within 2 hours. Collected uteri were classified into corpus albican (n = 4) phases of the estrous cycle according to ovary morphology. UH samples were collected from uterine horn, and amount of UH was measured using measuring cylinder. The UH samples were centrifugation at 4,500 g for 5 min, then the supernatants were transferred to fresh tube which filtered using 0.2 μm diameter filter (Sartorius, Gottingen, Germany, Lee *et al*., 2016). The samples were stored at -80°C before experiment.

2. *In vitro* fertilization

Ovaries were collected from local slaughterhouse and transported to the laboratory within 2 hours. Cumulus–oocyte complexes (COCs) were aspirated with 18-gauage needle from antral follicles (2–6 mm) and selected COCs of evenly distributed cumulus cells under a microscope. The COCs were cultured with modified TCM-199 with 10% (v/v) porcine follicular fluid (pFF), 0.5 μg/mL follicle stimulating hormone (FSH), 0.01 μg/mL epidermal growth factor (EGF), 10 IU human chorionic gonadotropin (hCG), and 5 μg/mL luteinizing hormone (LH) for 22 hours and COCs were cultured with 5 and 10% pFF without hormones for 22 hours (Lee and Park, 2015). The COCs were transferred to TCM-199 containing 0.1% hyaluronidase to remove the cumulus cells. Modified Tris-buffered medium (mTBM) with caffeine was used for *in vitro* fertilization (IVF). The mature oocytes were washed three times in mTBM and transferred to a 25 μL droplet of mTBM (15 oocytes/droplet). Sperm were collected from Duroc cross boars (Gumbo, Wonju, Korea), washed in modified Modena B semen extender (Lee *et al*., 2015), and re-suspended to 6.0 × 105 sperm/mL in mTBM. The sperm–oocyte were incubated for 6 hours, after the extra sperm and cumulus cells were removed by repetitive pipetting and washed three times using porcine zygote medium 3 (PZM-3). The fertilized oocytes were incubated in PZM-3 supplementation with 5 and 10% UH of corpus albican phase. Cleavage and blastocyst formation in embryos were measured at 168 hours after IVF. All cultures were performed at 38.5°C in 5% CO_2.

3. Immunofluorescence

Immunofluorescence was used to detect the expression of CRP2, G2COP, MYG, VEGF, COL4 and FUT4 proteins in porcine embryos (Huang *et al*., 2015). Briefly, embryos at 120 hours after IVF were fixed in 4% paraformaldehyde based on phosphate buffer saline-polyvinyl alcohol (PBS-PVA) for 15

min at room temperature and permeabilized with 0.1% Triton X-100 based on PBS-PVA for 30 min. Then, embryos were blocked in PBS-PVA supplementation with 5% bovine serum albumin for 1 hour. All primary antibodies and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The embryos were incubated with goat polyclonal primary antibodies raised against IgG anti-G2COP (sc-23204, 1:200), CRP2 (sc-167547, 1:200) and COL4 (sc-9302, 1:200), and rabbit polyclonal antibody targeting IgG anti-MYG (sc-25607, 1:200), VEGF (sc-25784, 1:200) and FUT4 (sc-292247, 1:200) for 1 hours at room temperature. Then, embryos were washed three times in PBS-PVA, treated with a secondary antibody (1:1000, goat IgG anti-alexa 488 and rabbit IgG anti-Alexa 546) for 1 hours in a dark room and 1mg/mL hoechst 33342 was used for embryo nuclear staining. The embryos were visualized by confocal laser microscopy (LX70 FV300, Olympus, Tokyo, Japan). The proteins intensity of embryo was calculated using ImageJ software (NCBI, Bethesda, MD).

4. Statistical analysis

All data were analyzed using 9.4 SAS program and presented as means ± standard error. Treatment groups were compared respectively using the least significant difference (LSD) test. All data were analyzed using analysis of variance (ANOVA) and *P*-value was considered significant.

Results

1. Effect of UH on embryo development and blastocyst in pigs

Amount of UH is 2.12 ± 0.74 mL in uterine horn when ovary formed corpus luteum, however UH volume (86.4 ± 3.76 mL) was increased when ovary formed corpus albican (Table 1). Table 2 is shown that influence of UH of corpus albican phase on cleavage and blastocyst formation in pre-implantation porcine embryos. The cleavage was no significantly difference between 5 and 10% UH without PZM-3 group (0%) and PZM-3 groups at 48 hours after IVF (data not shown). However, cleavage rate and ratio of 2-16 cell embryos were no significantly difference between UH-treated groups, but blastocyst rate was significantly decreased in 5 and 10 % UH-treated groups ($P < 0.05$).

2. Effect of UH on the expression of CRP2, G2COP, MYG, VEGFD, COL4 and FUT4 proteins in porcine embryos

The proteins of CRP2, G2COP, MYG, VEGFD, COL4 and FUT4 in porcine embryos at 120 hours were expressed after IVF (Fig. 1, 2 and 3). We used >16 cell stage embryos for detecting proteins. The embryo morphology was not changed at embryo morphology between 0 and 10% UH-treated groups (Top on Fig. 1AB, 2AB and 3AB). The six proteins were expressed in all blastomere. CRP2 protein was significantly ($P < 0.05$) higher at 10% UH-treated group (Fig. 1C), but not G2COP, MYG, VEGFD, COL4, and FUT4 proteins.

Discussion

The uterine undergoes a repetitive cycle, UH is secreted from uterine gland, have various nutrients, cytokines, hormones and others which compositions are changed during estrous cycle (Ashworth and Bazer, 1989; Gray *et al*., 2000). Biological process of UH proteins are cell proliferation, responses, translation, transport, and metabolism, and major molecular functions associated with nucleic acid binding, oxygen activity, enzymatic activity, growth activity, iron binding and redox binding in endometrium (Lee *et al*., 2016). Practically, high level of glucose, total protein and inorganic ions in uterine flushing fluid negatively affect embryo quality (Wiebold, 1988). In addition, macroglobulin, serum albumin, antitrypsin were decreased for mid-proliferative and mid-secretory phase in women, while antithrombin-III protein was increased at infertile compared to fertile women in UH (Hannan *et al*., 2010). Thus, UH substances may be directly involved with not only uterine physiology but also embryonic development in human and animals.

Optimal UH substances level such as nutrients, cytokine, growth factors, hormones, atmosphere, osmotic level and others are very important to develop embryos in human and animals (Van Loendersloot *et al*., 2010). Especially, growth factors, hormones and antioxidant were known to regulate embryo development (Wilson *et al*., 1981; Paria and Dey, 1990; Ali *et al*., 2003), however, abnormal steroid hormones and growth factor level decreased embryonic development in human (Yovel *et al*., 1995; Hannan *et al*., 2011). In addition, uterine secretion of different phase of estrous cycle influenced embryo development in human (Hannan *et al*., 2011). Practically,

mid-proliferation phase UH improved blastocyst formation, but mid-secretory phase UH decreased embryo development in human (Hannan *et al*., 2011). Also, UH dramatically inhibited embryo development and blastocyst development in women with unexplained infertility (Hannan *et al*., 2011). Optimal levels of chemokines, growth factors, receptors and cytokines of UH had positive effect to embryo development, but lack of the substances and excessive levels decreased embryo development in human (Hannan *et al*., 2011). Thus, UH of optimal estrus phase is beneficial for embryo development. We observed that

Table 1. Change of uterine histotorph volume during estrous cycle in pigs

Estrous cycle	Corpus luteum phase	Corpus albican phase
Uterine histotroph volume (mL)	2.12 ± 0.74	86.4 ± 3.76

Table 2. Effect of uterine histotroph of corpus albican phase on cleavage and blastocyst formation in porcine embryos.

Uterine histotroph (%)	No. of oocytes	Cleavage (%)	No. of embryo developed to (%)	
			2-16 cells	Blastocyst
0	196	154 (79.1 ± 3.2)	126 (63.6 ± 4.4)	28 (19.5 ± 4.4)[a]
5	216	167 (75.8 ± 5.2)	161 (73.1 ± 4.8)	6 (3.4 ± 1.5)[b]
10	222	166 (76.8 ± 3.2)	163 (75.1 ± 3.1)	3 (2.1 ± 1.1)[b]

Data are presented as the mean ± SEM. a,b: values in brackets with different superscripts differ significantly from each other ($p < 0.05$). n=4

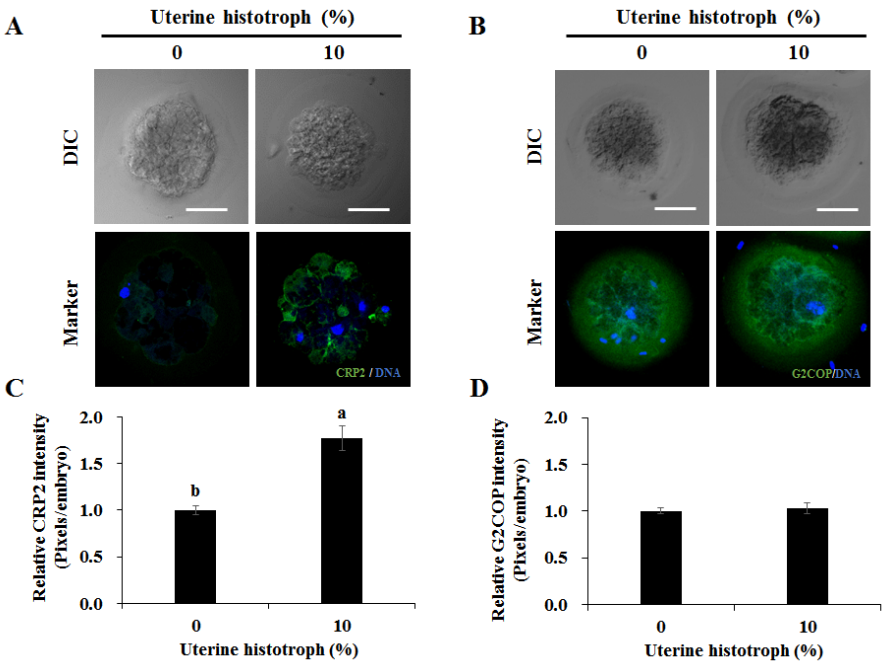

Fig. 1. Expression of cysteine-rich protein 2 (CRP2) and coatomer subunit gamma-2 (G2COP) in uterine histotroph of corpus albican phase treated porcine embryos, morphology and CRP2 (A) and G2COP (B) protein expression and relative expression of CRP2 (C) and G2COP (D) in embryo, embryo was used at 120 hours after *in vitro* fertilization, green and blue color indicate respectively CRP2 and G2COP protein and nucleus, bars represent means ± standard error. [a,b] Within the histogram, similarly shaded bars with different letters are significantly different (P < 0.05). White scale bar: 20 μm.

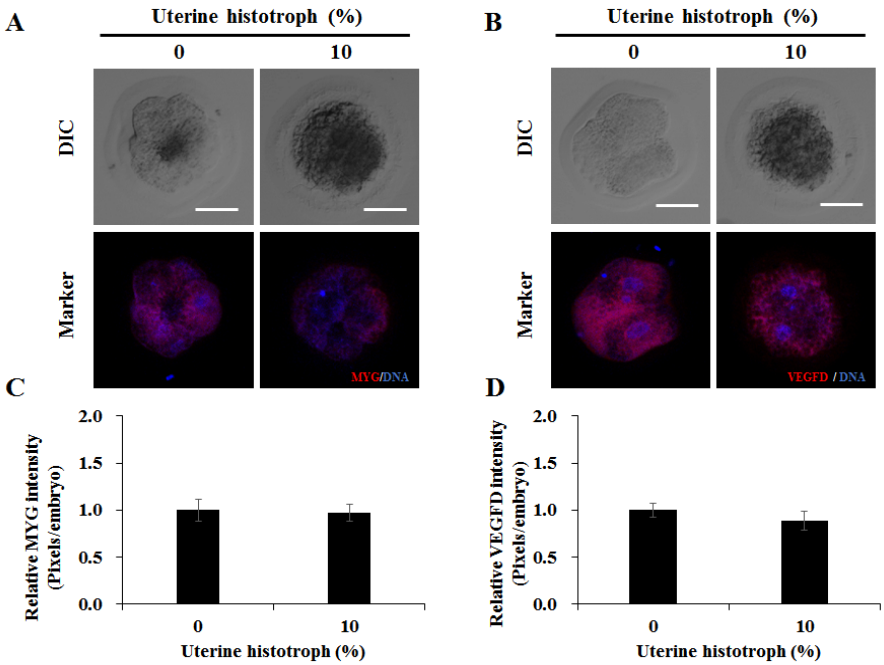

Fig. 2. Expression of myoglobin (MYG) and vascular endothelial growth factor D (VEGFD) in uterine histotroph of corpus albican phase treated porcine embryos, morphology and MYG (A) and VEGFD (B) protein expression and relative expression of MYG (C) and VEGFD (D) in embryo, embryo was used at 120 hours after *in vitro* fertilization, red and blue color indicate respectively MYG and VEGFD protein and nucleus, bars represent means ± standard error. White scale bar: 20 μm.

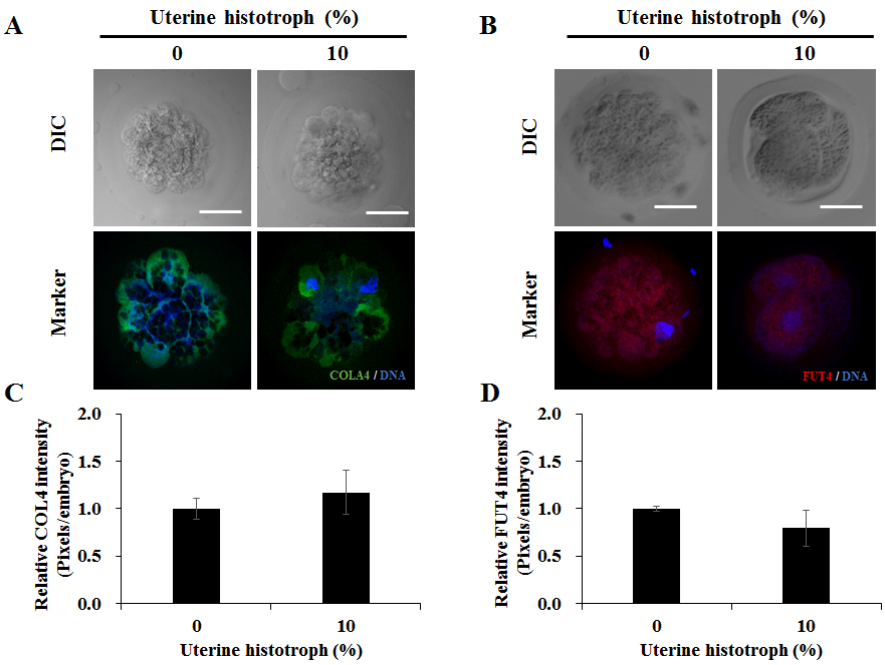

Fig. 3. Expression of collagen alpha 4 chain (COL4) and galactoside 3-L-fucosyltransferase 4 (FUT4) in uterine histotroph of corpus albican phase treated porcine embryos, morphology and COL4 (A) and FUT4 (B) protein expression and relative expression of COL4 (C) and FUT4 (D) in embryo, embryo was used at 120 hours after *in vitro* fertilization, green/red and blue color indicate respectively COL4/FUT4 protein and nucleus, bars represent means ± standard error. White scale bar: 20 μm.

amount of UH in corpus albican phase of estrous cycle, the UH was dramatically increased when corpus albcian is formed (Table 1). In this time, endometrium was degenerated by decreased progesterone level and was occurred luteolysis (Quirk *et al*., 1986). The abnormal fertilization time such as delayed ovulation and sperm transfer into female reproductive tract and too late sperm injection to female reproductive would be impeded embryo production compared to normal fertilization, the embryos may be located in corpus albican phase intrauterine environment, the embryo would be exposed to UH of corpus albican phase. In this study, UH of corpus albican phase did not influence embryo cleavage, however, negative effect for blastocyst formation in pigs. UH of corpus albican phase may contain unsuitable chemokines, growth factor, cytokines compared to corpus luteum phase which is negative effect to embryo development in pigs. Therefore, we suggested that many groups need to study about the role of growth factors and hormones and the function of cytokines in UH in human and animals.

Previous study, we examined protein expression patterns of UH during the follicular phase and luteal (corpus albican) phase, found that differentially-expressed proteins using MALDI-TOF, the biological and molecular function of proteins were investigated focused on physiological change of endometrium during estrous cycle in pigs. Kayser *et al*. (2006) reported that various proteins were analyzed using tandem mass spectrometry in uterine fluid of early pregnancy pigs and Lee *et al*. (2016) found twenty UH proteins using MALDI-TOF in follicular and luteal phase uterine. However, study of this proteins change in UH exposed embryo is not reported in pigs. The CRP2 was mostly studied in vascular smooth muscle cells, could regulate actin cytoskeleton and smooth muscle gene (Lin *et al*., 2008), and CRP2 gene is regulated by progesterone (Kong *et al*., 1997). In this study, only CRP2 protein increased by UH of corpus albican phase in porcine embryo. We suggest that UH of corpus albican phase influences CRP2 protein expression, it may induce excessive cell differentiation and unnatural cytoskeleton structure in porcine embryo.

Therefore, these results provide understanding of the role of UH on embryonic development and interaction between embryos and intrauterine environments. Also, UH may be a factor for pondering the causes of infertility in domestic animal and human.

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