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IL-10 and TNF-α level changes in Oviduct and Endometrium at the Preimplantation Period in Sows

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돼지 착상초기 단계에서 난관 및 자궁내막 내 IL-10 및 TNF-α의 변화

전다연, 사수진, 박현주, 진현주, 김조은, 민예진, 최요한, 정용대, 정학재*
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Abstract After implantation, dramatic shifts in immune cell populations occur in the maternal reproductive tract, and these changes are key to the implantation and pregnancy process in sows. Immune cells, including T lymphocytes, are recruited during the early implantation period, and an accurate balance between Th1 and Th2 is essential for successful implantation. In this study, the concentration of TNF-α (tumor necrosis factor-alpha) and IL-10 (interleukin-10) in early pregnancy were investigated using an enzyme-linked immunosorbent assay (ELISA) to determine Th1/Th2 ratios in oviduct (OVI), endometrium (ENDO), and the uterine mucosal layer (UML) before implantation. Significant differences in IL-10 levels were observed in OVI, ENDO, and UML tissues on day 7 post-implantation ($p < 0.01$), whereas TNF-α levels were no different. As regards IL-10 and TNF-α ratios, a significant difference was found on day 2 in OVI tissues ($p < 0.05$). The result of this study supports the notion that the maternal reproductive system accepts the embryo through changes in preimplantation reproductive system-specific pro-/anti-inflammatory mechanisms.

요 약 수정란이 착상하는 동안 모체의 생식 기관에서 면역 세포 군집의 변화가 발생하며 이는 임신 과정의 핵심이다. 착상 전·후 기간동안 T 림프구를 포함한 면역 세포는 이식 초기에 생식 기관으로 불러들여지며, T 림프구 중 보조T세포인 Th1과 Th2 사이의 균형은 착상의 성공에 중요한 영향을 미친다. 본 연구에서는 돼지 수정란 착상 전 모체의 난관 (OVI), 자궁내막 (ENDO), 자궁 점막층 (UML)에서 Th1, Th2의 균형을 확인하고자 종양괴사인자-알파 (TNF-α)와 인터루킨-10 (IL-10)의 농도를 효소결합 면역흡착 분석법 (ELISA)을 이용해 분석하였다. IL-10의 경우 7일차에 OVI, ENDO, UML에 유의한 차이가 있었으나 ($p < 0.01$), TNF-α의 경우 모두 유의한 차이가 없었다. IL-10:TNF-α 비율은 OVI에서만 7일째에 유의한 차이가 나타났다 ($p < 0.05$). 본 연구의 결과는 착상 전 생식 기관별 염증-항염증 기전의 변화를 통해 모체의 생식계가 배아를 수용하는 과정의 일부를 뒷받침할 수 있다.

Keywords : Preimplantation, IL-10, TNF-α, Oviduct, Endometrium, Sow

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1. Introduction

During implantation and pregnancy, dramatic changes in the immune cell populations occur in the maternal genital tract with the vascular remodeling of the endometrium, and this is the key to the implantation and pregnancy process [1]. The immunological aspects of animal reproduction, especially in viviparous

mammals, when it comes to pregnancy, it has been known as immunological tolerance in regard that the female tolerated the intrusion of allogeneic cells such as male spermatozoa and semi-allogeneic fetuses [2]. T lymphocytes, dendritic cells, macrophages, and natural killer lymphocytes are the recruited cells that can be found in uterine tissue early in implantation [3,4]. Particularly in T lymphocytes, previous studies have shown that the total T cell population is more abundant in the pregnant than in the non-pregnant endometrium, as are other T cell subsets [5]. Recently it was demonstrated that on day 16 of pregnancy, most T cells presented in the endometrium are T-helper cells [6]. The accurate balance between Th1 cytokines, mainly TNF-α, and Th2, particularly IL-10, is essential to achieve good obstetric outcomes [7].

Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory Th1-cytokine, plays a major role in the inflammatory mechanisms regulating implantation, placentation, and eventually in pregnancy outcome. Several reproductive disorders, particularly recurrent pregnancy loss, early and severe pre-eclampsia, and recurrent implantation failure syndrome, may be due to an increase in Th1-dependent cytokines, especially TNF-α [7].

Interleukin-10 (IL-10) is one of the anti-inflammatory cytokines which plays a crucial role in the maternal-embryo immune tolerance mechanism, which is the way the mother can be pregnant [8]. Transitioning to an immunosuppressive state by decreasing the inflammatory response at the uterine level is necessary for semi-allogenic conceptus acceptance [9]. According to Wang, although it is associated with increased IL-10 production with successful embryo implantation [10], low IL-10 expression is associated with a decrease in endometrial receptivity resulting in blastocyst implantation failure [11].

Although the immune mechanisms are considered important, the information about the immune status in the reproductive tract at very early pregnancy stages is limited. Therefore, the aim of this study was to expand our knowledge of maternal immune mechanisms during the preimplantation period. Specifically, we focused on the IL-10 (anti-inflammatory cytokine) and TNF-α (a pro-inflammatory cytokine) profile at the oviduct (OVI), endometrium (ENDO), and uterus mucosal layer (UML) on day 2, 4, and 7 after insemination in sows.

2. Materials and Methods

2.1 Animals

In this study, 11 sexually matured female Duroc (1~2 years) were used and reared in Cheonan-si, Chungcheongnam-do, Korea. All sows were allocated into individual crates. All animals had access to water ad libitum and were fed commercial diets according to their nutritional requirements.

Sows were fed Altrenogest (Regumate®, MSD Animal Health, Korea) for 21 days to synchronize their estrus. The detection of estrus was performed by snout-to-snout contact of sows and mature boars and back-pressure testing twice a day with an experienced operator. Sows showing a clear stance on pressure were considered in estrus. The sperm donors were three sexually mature boars, and all semen was mixed and diluted 1:1 with a diluted semen extender (Seminark™ Gold, Noah Biotech, Korea) to eliminate the male influence. The mixture was stored under 17℃. Post-cervical insemination was performed at 12, 24, and 36 hours after the onset of estrus. Day 0 was defined as the first day of artificial insemination (AI). Pigs were euthanized 2, 4, and 7 days after AI. The embryos were recovered by washing the oviduct and uterus.

2.2 Sample Preparation

One side of the oviduct samples was collected from the ampulla for OVI sampling, and for protein extraction, it was rapidly frozen in liquid nitrogen and stored at -80℃. The middle part of one side of the uterine horn was cut and scraped. UML samples were collected by scraping the endometrium, mixed 1:1 with TCM-199 (Medium 199, Sigma-Aldrich, USA), heated at 38℃ for 1 hour, cooled at 4℃ for 3 hours, and then stored at -80℃. To collect the ENDO specimen, the endometrial samples were cut and snap-frozen in liquid nitrogen and stored at -80℃, like other tissue samples. Cut OVI and ENDO into smaller pieces, add 100 μl RIPA buffer containing 1% protease inhibitor per 100mg of tissues, and ground with mortar and pestle. A total of 33 samples were used. Three samples, OVI, ENDO and UML, were collected per sow, and three, four and four sows were used on day 2, day 4 and day 7, respectively.

2.3 Cytokine Analysis

Measurement of IL-10 and TNF-alpha concentration in tissues were performed using porcine IL-10, TNF- alpha quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). ELISA assays were carried out according to the manufacturer's procedures. Measurements were repeated twice and the average was used. Absorbance measurements were performed at two wavelengths, 450 nm, and 570 nm.

2.4 Statistical Analysis

Statistical analysis was performed using one-way ANOVA and Tukey-Kramer adjustments in R software. Data were presented as mean. Statistical significance was considered for the p-value $<$ 0.05.

3. Results and Discussion

3.1 IL-10 and TNF-α concentration

Fig. 1 and Fig. 2 shows the TNF-α and IL-10 concentrations in OVI, ENDO, and UML, analyzed by ELISA, respectively. For IL-10, there were significant differences on day 2, 4, and 7 in OVI, ENDO, and UML ($p < 0.01$). In OVI, the IL-10 concentrations were higher on day 2 and 4 than on day 7. In contrast, in ENDO and UML, IL-10 concentration on day 7 was higher than on day 2 and 4. For TNF-α, there were no significant differences on day 2, 4, and 7 in OVI, ENDO, and UML. However, in UML, there was a significant difference between day 2 and 4 ($p < 0.05$). Immune cells act to recognize and normally eliminate invaders, preventing disease and contamination of host genetic materials. However, embryos that are semi-allogenic to their mother are protected from assault by the maternal immune system. The continued development of the embryo requires a careful balance of the maternal immune system and successful implantation can be reached. Therefore, understanding the complex molecular conversation, also known as cross-talk between maternal and embryo, is crucial for successful implantation.

Cytokines and chemokines are a family of secreted immune modulators that control the function and differentiation of immune and non-immune cells [12]. During pregnancy, cytokines and chemokines have been shown to be essential for the implantation and immune

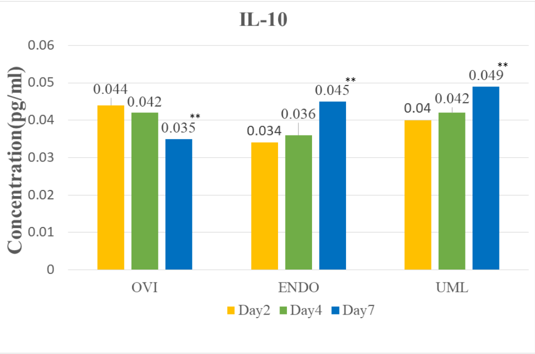

Fig. 1. IL-10 concentration in OVI, ENDO and UML. Values are mean(** $p < 0.01$). OVI, oviduct; ENDO, endometrium; UML, uterus mucosal layer.

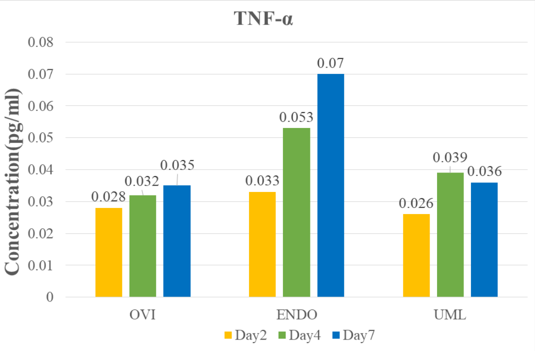

Fig. 2. TNF-α concentration in OVI, ENDO and UML. Values are mean. OVI, oviduct; ENDO, endometrium; UML, uterus mucosal layer.

regulation of the maternal immune system [13-15]. As shown in Table 1, the genital environment during pregnancy changes immediately after fertilization and is converted into a so-called 'post-mating inflammatory cascade [16,17]. The main function of this inflammatory response is to remove sperm debris, bacteria, and prime the maternal immune system to accommodate the presence of paternal antigens for implantation [18-20]. Then, when the embryo enters the uterine (day 4-5 after insemination), the anti-inflammatory response is initiated and a favorable environment for conceptus development has been established [21,22]. Several studies have shown an increase in anti-inflammator cytokine signaling factors

Table 1. Schematic overview of some immunological events to achieve successful pregnancy in pigs. E2, estrogen; P4, progesterone; PG, prostaglandin (Adapted from "Immunological uterine response to pig embryos before and during implantation" by Parrilla et al., 2022, *Reproduction in Domestic Animals*, Vol.57, No.5, pp.14-13)

Event (day after mating)	Uterine response
Inflammatory cascade (day 0~4)	· Eliminating bacteria and sperm debris · Priming maternal immune system to paternal antigens · ↑Pro-inflammatory factors, Neutrophil, Macrophages, Dendritic cells, T lymphocytes · ↑E2/↓P4 to ↓E2/↑P4
Preimplantation period (day 5~11)	· Establishment of immuno tolerant mechanisms for allowing free floating embryo survival and implantation · ↑Anti-inflammatory factors, Macrophage, Dendritic cells, Th cells, T reg cells · ↓Pro-inflammatory factors, immune related genes · ↓E2/↑P4
Implantation period (day 12~18)	· Establishment of adequate endometrial receptivity allowing conceptus and development · Endometrial vascular remodeling and angiogenesis initiation of placentation · Day12: ↑Pro-inflammatory factors, E2, factors related to PG, NK cells · Day13-18: ↑Pro-inflammatory factors, Th and Treg cells, NK cells, IL-18, ↓IL-1b2 · Day20-28: ↑Anti-inflammatory factors

and a decrease in the expression of pro-inflammatory in pregnant females [23,24]. After fertilization, the embryos move in the oviduct (from infundibulum to isthmus) for 4 or 5 days, then gradually move to the uterus (implantation site) and are attached to the epithelium. The whole process of this action requires 7 to 11 days. On day 0 to 4 after mating, the pro-inflammatory cascade occurs and the pro-inflammatory factors dominate, and day 5 to 11, anti-inflammatory cytokines, the so-called preimplantation period, lead [17]. Also, it is well known that progesterone concentration in uterus increases after fertilization and this high level of progesterone contribute to making a proper endometrial environment for implantation and keeping the pregnancy state by inducing the production of the Th2 cytokines and inhibiting

Th1 cytokines [25]. Therefore, the results support that IL-10, the anti-inflammatory cytokine, is higher in OVI on day 2 and 4, and higher in ENDO and UML on day 7 due to the embryos' existence and increased uterus progesterone level.

3.2 IL-10 and TNF-α ratio

Fig. 3 shows the IL-10 (anti-inflammatory cytokine) and TNF-α (pro-inflammatory cytokine) ratio in OVI, ENDO, and UML. There were no significant differences in ENDO and UML on day 2, 4, and 7. However, in OVI day 2 and 4 is higher than day 7 ($p < 0.05$).

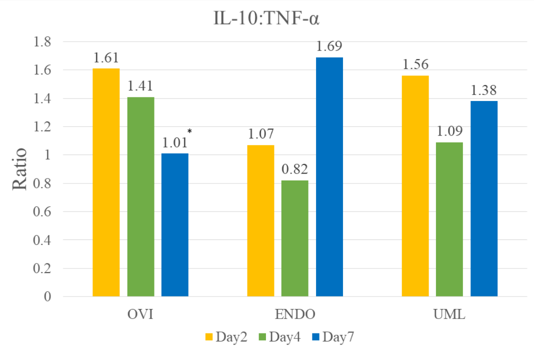

Fig. 3. IL-10(anti-inflammatory cytokine) and TNF-α (pro-inflammatory cytokine) ratio in OVI, ENDO and UML. Values are mean(* $p < 0.05$). OVI, oviduct; ENDO, endometrium; UML, uterus mucosal layer.

In a normal pregnancy, pro-inflammatory cytokines are followed by a clear shift toward an anti-inflammatory cytokine immediately after implantation. It can be characterized by an increase in IL-10, a decrease in TNF-α, and an increase in the ratio of IL-10:TNF-α. However, there were no significant differences in our findings except for OVI. In OVI, the anti-/pro-inflammatory ratio has been shown to decrease over time. Immediately after fertilization, the embryo gradually moves from the oviduct to the uterus, and the oviduct (first place for fertilization) returns to the pro-inflammatory state, which can be explained as so-called homeostasis [26]. However, the absence of significant differences in TNF-α could have affected the ratio and if there was a cytokine balance disruption, it could be explained that insufficient receptivity of the oviduct and endometrium could lead to inadequate fetal development [4]. Reproductive immunologists have hypothesized that some infertility may be due to a lack of growth factors and inadequate immune protection mechanisms in the oviduct and endometrium [27,28].

Previous studies have shown that IL-10 and TNF-α are predictive biomarkers contributing to the knowledge of immune responses in early implantation. In humans, IL-10 was relatively low and TNF-α relatively high in patients diagnosed with miscarriage and with threatened miscarriage compared to normal pregnancies [29,30]. After 5 days of breeding, the epithelium secretes protease inhibitors and pro-inflammatory cytokines [31-33] to have sensitivity to the invading trophoblast and is known to indirectly stimulates Th2 cytokine production in response [34].

An obvious weakness of this study was the lack of samples. We were unable to get the data in non-pregnant gilt or sow. Instead, in a previous study, Parrilla et al. (2022) demonstrated that cytokine levels in endometrial explants of blastocyst-bearing and uninseminated cyclic sow were different, particularly in IL-1ra, IL-10, IL-10, TGF-β1, TGF-β2, IL-1α, IL-2, INF-γ [17].

Most of the previous studies have focused on immunological responses in normal and abnormal pregnant humans [35-37]. Here we show that the immunological profiles were different on day 2, 4, and 7 after fertilization. Further studies about the signaling pathway between the reproductive system and embryo are needed to clarify the mechanism of implantation in early pregnancy. In addition, additional researches about finding the precise location of cytokines and chemokines expression are crucial as they may contribute to fetal and placental development and elucidate the exact causes of pregnancy failure both *in vivo* and *in vitro*. Furthermore, comparisons of cytokines released from in *in vitro* medium, endometrium and embryos during *in vivo* and *in vitro* embryonic development may facilitate further studies of *in vitro* embryonic production.

4. Conclusion

The embryos, which are semi-allogenic to their mother, require careful balance in the maternal immune system thus, they can reach successful implantation. In the present study, the concentration of TNF-α and IL-10 in early pregnancy was investigated in OVI, ENDO, and UML. In our study, the anti-inflammatory cytokine IL-10 was higher in OVI on day 2 and 4, and higher in ENDO and UML on day 7. However, there were no significant differences in the ratio of IL-10:TNF-α among days. The result may support the changes in pro-/anti-inflammatory mechanisms during the preimplantation period of each genital tract. Further studies on the signaling pathway between the reproductive system and embryo are needed to clarify the implantation mechanism of implantation in early pregnancy. In addition, studies are needed to find the exact location where cytokines and chemokines are expressed.

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