Korean Journal Of Agricultural Science

pISSN : 2466-2402
eISSN : 2466-2410

ANIMAL

Adverse effect of IL-6 on the *in vitro* maturation of porcine oocytes

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Abstract

Cytokines are protein mediators that possess the ability to assist cell-to-cell communication in immune system-related activities. In general, pathogen endotoxins activate the release of inflammatory mediators, and with time, there is an increase in the cytokine levels in the body. Interleukin (IL)-6 mediates the acute-phase inflammatory response, and elevated IL-6 levels have been reported in peritoneal fluids of women with pelvic inflammation and endometriosis, thereby associating it with oocyte quality and infertility. To overcome subfertility or infertility in humans and animals, the present study was done to examine the effect of recombinant IL-6 on porcine oocytes matured *in vitro* and subsequently to determine the fertilization rate and embryo development. Porcine oocytes were incubated with varying concentrations of IL-6 (0 - 2 μg·mL^{-1}) for 44 h followed by *in vitro* fertilization and culturing of the oocytes. The oocytes or embryos were fixed with 3.7% paraformaldehyde (PFA) and stained with fluorescence dyes, and the meiotic spindle, chromosome organization, fertilization status and embryo development were subsequently assessed under a fluorescence microscope. We observed induction of an abnormal meiotic spindle alignment in the oocytes incubated with IL-6 compared to the control oocytes incubated without IL-6. Moreover, significantly decreased fertilization rates and embryo development were observed for oocytes incubated with IL-6 ($p < 0.05$). Thus, an increased IL-6 level during oocyte maturation could be associated with fertilization failure due to an aberrant chromosomal alignment and a disruption of the cortical granules. Taken together, our results indicate that successful assisted reproduction can be achieved by controlling the levels of inflammatory cytokines.

Keywords: embryo, IL (interleukin)-6, *in vitro* fertilization, oocyte maturation, pig

Citation: Yi YJ, Adikari AADI, Moon ST, Heo JM, Lee SM. Adverse effect of IL-6 on the *in vitro* maturation of porcine oocytes. Korean Journal of Agricultural Science 48:607-615. https://doi.org/10.7744/kjoas.20210051

Received: July 13, 2021

Revised: August 10, 2021

Accepted: August 18, 2021

Introduction

Cytokines are soluble small mediators derived from proteins, having important functions in autocrine, paracrine, and endocrine signaling. They normally last for a short period and are involved in coordinating almost all developments and activities of the immune system (Gandhi et al., 2016). Cytokines are classified into different families, depending on their specificity, structure and composition of receptor complexes. Some examples of cytokine families include interferons, interleukins, chemokines, mesenchymal growth factors, tumor necrosis factor family, and adipokines. They can further be classified into functional classes such as lymphocyte growth factors, pro-inflammatory molecules, anti-inflammatory molecules, and cytokines capable of polarizing the immune response to antigens (Dinarello, 2007; Spangler et al., 2015; Gandhi et al., 2016).

Following cytokines are grouped in the interleukin-6 (IL-6) family: IL-6, IL-11, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin 1 (CT-1), cardiotrophin-like cytokine (CLC), and IL-27. All mediators belonging to the IL-6 family have two or more molecules of signaling receptor subunit gp 130 in their receptor complexes (Rose-John, 2018). IL-6 is a multi-functional pleotropic cytokine, and lymphoid as well as non-lymphoid cells are known to produce IL-6 (Kishimoto, 1988; Le and Vilček, 1990). IL-6 encompasses a wide range of biological activities in the body, such as immune regulation, hematopoiesis, inflammation and oncogenesis. Moreover, it is important in the activation of B-cells and production of immunoglobulin, and in regulation of the skeleton, cardiovascular system, nervous system, endocrine system and placenta (Kishimoto et al., 1995; Kishimoto, 2010).

When an emergent situation occurs in the body, IL-6 is activated and sends warning signals to the entire host body. Thereafter, pathogen-associated molecular patterns of the exogenous pathogens get recognized by the pathogen recognition receptors present in cells associated with the immune system, such as macrophages and monocytes (Kumar et al., 2011; Tanaka et al., 2014). Considering the female, endometriosis and pelvic inflammations are common conditions resulting in increased cytokine levels in the body. Endometriosis is the outcome when growth of endometrial cells occurs at sites outside the uterus. Moreover, the pelvic inflammatory process is the result of altered functions of immune-related cells and changes in the cytokine levels in the peritoneal cavity. All these activities promote development of endometriosis in women, ultimately leading to impaired fertility (D'Hooghe et al., 2003; Khan et al., 2008). Several studies have reported increased interleukin family cytokines, especially IL-6, in the peritoneal fluid (PF) and serum of women with endometriosis (Gazvani and Templeton, 2002a, 2002b; Kang et al., 2014; Kashanian et al., 2015). Numerous studies have also identified that macrophages are prominently involved in the increment of IL-6 in the peritoneal fluid of women afflicted with endometriosis (Boutten et al., 1992; Harada et al., 2001). Literature findings state that altered IL-6 levels in females are detrimental for oocyte fertilization and development of embryos (Pellicer et al., 2000; Wu et al., 2001; Banerjee et al., 2012).

However, the mechanistic link between elevated levels of IL-6 and fertility impairment remains unclear. Thus, more *in vitro* studies are required for a better understanding, as well as for a better interpretation of the effect of IL-6 on fertility impairments in females with associated health conditions like endometriosis. Moreover, most previous studies were conducted on small laboratory animal models, such as mouse. *In vitro* system using porcine oocytes is well-established technique to indirectly test the reproductive phenomena of mammals (Yi et al., 2021). This study was therefore undertaken to evaluate the adverse effect of IL-6 on the *in vitro* maturation of porcine oocytes.

Materials and Methods

Collection and *in vitro* maturation (IVM) of porcine oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse, and transported to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from the antral follicles (3 - 6 mm in diameter), washed three times in HEPES-buffered Tyrode lactate (TL-HEPES- PVA) medium supplemented with 0.01% (w/v) polyvinyl alcohol (PVA), followed by three washes with oocyte maturation medium (Abeydeera et al., 1998). A total of 50 COCs were transferred to 500 μL maturation medium, and layered with mineral oil in a 4-well multi-dish equilibrated at 38.5℃ in 5% CO_2 atmosphere. The oocyte maturation medium used was tissue culture medium (TCM) 199 supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg·mL^{-1} luteinizing hormone (Sigma-Aldrich Chemical Co., St. Louis, USA), 0.5 μg·mL^{-1} follicle-stimulating hormone (Sigma-Aldrich Chemical Co., St. Louis, USA), 10 ng·mL^{-1} epidermal growth factor (Sigma-Aldrich Chemical Co., St. Louis, USA), 75 μg·mL^{-1} penicillin G, and 50 μg·mL^{-1} streptomycin. The oocytes were cultured in TCM199 for 44 hrs at 38.5℃ and 5% CO_2 in air, and exposed to varying concentrations of recombinant IL-6 (0 - 2 μg·mL^{-1}; IL-6 recombinant swine protein, Gibco^{TM}, ThemoFisher Scientific, Seoul, Korea) included in the IVM medium. Unless otherwise noted, all other reagents used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA).

In vitro fertilization (IVF) and culture (IVC) of porcine oocytes

After IVM, cumulus cells were removed by treating with 0.1% hyaluronidase in TL-HEPES-PVA medium (Abeydeera et al., 1998). Thereafter, in a 35-mm polystyrene culture dish, oocytes were placed into four 100 μL drops of modified Tris-buffered medium (mTBM) and covered with mineral oil. One milliliter of liquid semen preserved in BTS was washed twice in phosphate-buffered saline (PBS) containing 0.1% PVA (PBS-PVA), at 800 × g for 5 min. The washed spermatozoa were resuspended in mTBM, appropriately diluted, and 1 μL of the sperm suspension was added to medium containing oocytes to give a final sperm concentration of 1 × 10^5 spermatozoa·mL^{-1}. Oocytes were co-incubated with spermatozoa for 5 hrs at 38.5℃ in an atmosphere containing 5% CO_2. After IVF, oocytes were transferred to 500 μL porcine zygote medium (PZM-3) (Yoshioka et al., 2002) supplemented with 0.4% bovine serum albumin (BSA, A0281, Sigma-Aldrich Chemical Co., St. Louis, USA), and cultured for an additional 20, 48, or 144 hrs. The IVM, IVF and IVC studies were repeated five times for each treatment regimen.

Evaluation of oocyte maturation, fertilization and embryonic development

Oocytes and embryos were fixed with 2% formaldehyde for 40 minutes at room temperature (RT), washed twice with PBS, permeabilized with PBS-Triton X-100 for 30 min, and stained with 2.5 mg·mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI; DNA staining; Molecular Probes, Eugene, OR, USA) for 40 min. The fertilization status of the zygotes (unfertilized, fertilized-monospermic, or fertilized-polyspermic), cleaved embryo number, blastocyst formation, and cell number per blastocyst, were assessed under a fluorescence microscope (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Tokyo, Korea). For examining oocyte maturation, migration of cortical granules (CGs), and chromosome alignment, oocytes were fixed in 2% formaldehyde for 40 min at room temperature (RT), washed with PBS, permeabilized in PBS with 0.1%

Triton-X 100 (PBS-TX), and blocked for 25 min in PBS-TX supplemented with 5% normal goat serum. Oocytes were then incubated with mouse anti-β-tubulin antibody (Abcam, Seoul, Korea; 1 : 100 dilution) for 40 min, washed with PBS-TX, followed by incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit (GAR) IgG (1 : 200 dilution), FITC-conjugated *Lens culinaris* agglutinin (LCA-FITC; 1 : 200 dilution) and 2.5 μg·mL^{-1} 4'6'-diamidino-2-phenylindole (DAPI) for 40 min, and subsequently observed under fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis

Values are exhibit as the mean ± standard error of the mean (SEM). Data analyses were conducted using one-way analysis of variance (ANOVA) with GraphPad PRISM® (GraphPad software, San Diego, CA, USA). The completely randomized design was applied, and Tukey's multiple comparison test was performed to compare values of individual treatments. Results are considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results and Discussion

IL-6 interferes with normal oocyte maturation during IVM

As presented in Fig. 1, oocytes that matured without IL-6 (Fig. 1A and 1B) showed general cortical granule migration (Fig. 1A' and 1B') and meiotic spindle formation (Fig. 1A" and 1B") after IVM, while abnormal cortical granule migration (Fig. 1C'and Fig. 1D') and meiotic spindle alignment (Fig. 1C"and 1D") were observed in oocytes matured in the presence of 1 μg·mL^{-1} IL-6. Comparing the microscopic images of the stained cortical granules, uneven halo distribution was observed in the plasma membrane, and cortical granule distribution was greater in the cytoplasm than in the plasma membrane of oocytes matured with IL-6 (Fig. 1B' and 1D'). Normal barrel-shaped and well-aligned meiotic spindle apparatus were barely detected in oocytes matured with IL-6 (Fig. 1D"). Examination of Fig. 2 reveals a significant decrease in the rate of oocytes reaching metaphase II (MII) when cultured in the presence of IL-6 (52.2 - 68.3%), as compared to oocytes matured without IL-6 (88%, $p < 0.01$; Fig. 2A). Abnormal rate of CG (27.8 - 68.2%), chromosome (21.7 - 35.4%) and spindle alignment was significantly increased with increasing concentrations of IL-6 during IVM ($p < 0.05$ and $p < 0.01$; Fig. 2B, 2C, and 2D, respectively). Pathological conditions, such as endometriosis and pelvic inflammation, increase oxidative stress in the cells, and promote the generation of reactive oxygen species (ROS). This results in increased cytokine levels (including IL-6) in the biological system. The combination effect of increased ROS and IL-6 levels results in deterioration of the oocyte spindle structure (Punnonen et al., 1996; Harada et al., 1997; Saito et al., 2002). Similarly, Banerjee et al. (2012) also reported that elevated levels of IL-6 impairs the micro-spindles and chromosomal alignment in the oocytes.

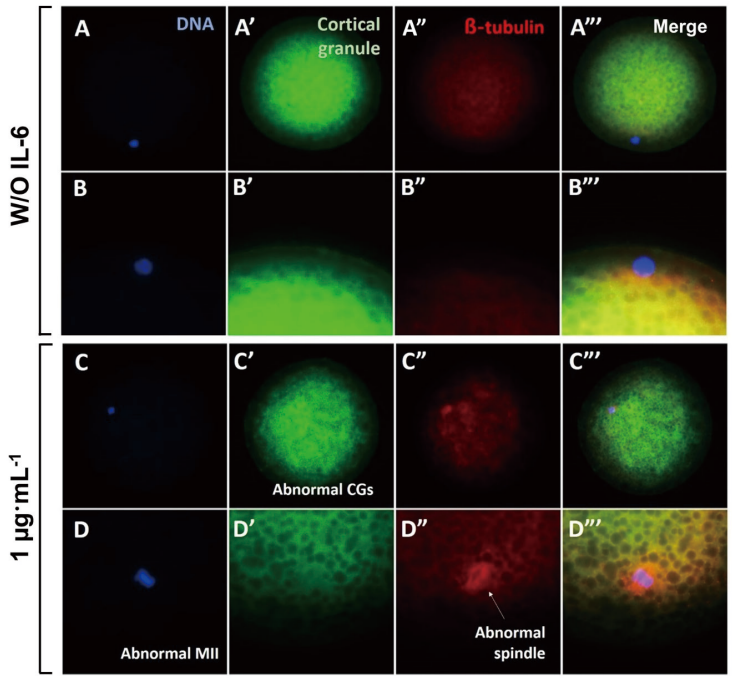

Fig. 1. Representative patterns of cortical granule (CGs) migration and meiotic spindle formation in oocytes matured in the absence (A-A"' and B-B"'; absence [W/O]) or presence (C-C"' and D-D"') of 1 μg·mL^{-1} interleukin-6 (IL-6) during *in vitro* maturation (IVM). DNA: 4'6'-diamidino-2-phenylindole (DAPI) (blue), cortical granules (CGs): FITC-conjugated *Lens culinaris* agglutinin (LCA) (green), spindle: ß-tubulin (red). A-A"' and C-C"': × 400, B-B"' and D-D"': × 1,000 magnification.

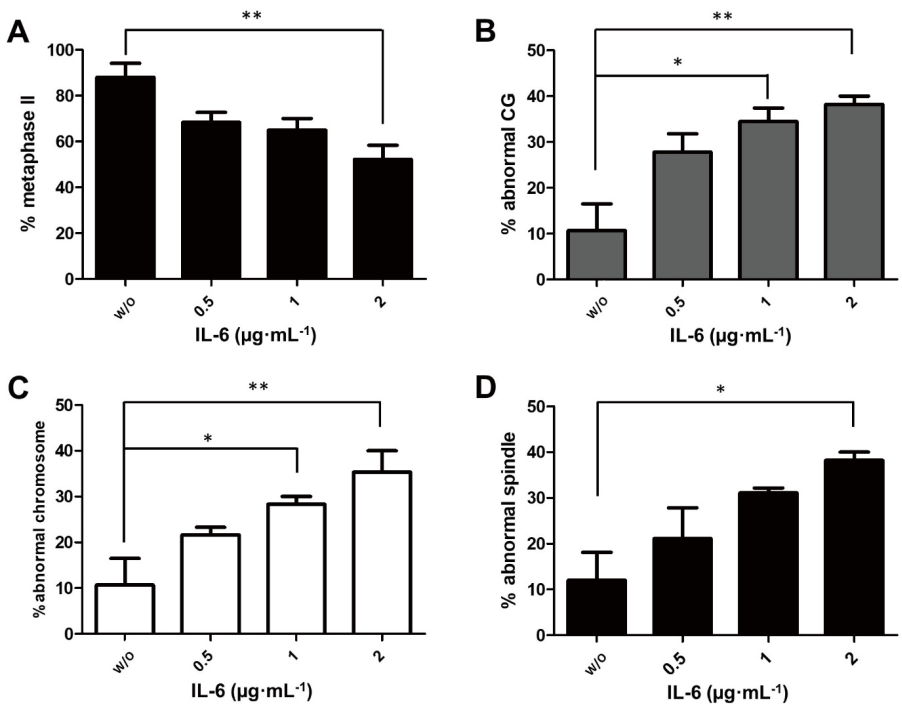

Fig. 2. *In vitro* maturation (IVM) of pig oocyte in the absence (W/O) or presence of interleukin-6 (IL-6). The percentage of metaphase II formation (A), abnormal cortical granule (CG) migration (B), abnormal chromosome formation (C), and abnormal spindle alignment (D) were examined after IVM 44 hrs. All experiments were repeated 5 times. Values are expressed as the mean ± standard error of the mean (SEM). The different superscripts in each group of columns denote a significant difference at *$p < 0.05$ and **$p < 0.01$.

Fertilization failure increases in oocytes matured in the presence of IL-6

Oocytes were matured in the absence or presence of IL-6 for 44 hrs, after which oocytes reaching MII were selected for fertilization. Sperm penetration, embryo cleavage and blastocyst formation were examined after IVF and IVC, at 20, 48, and 144 hrs, respectively (Fig. 3). Total fertilization rate was observed to decrease in oocytes matured with IL-6 (49.4 - 65.6%) as compared to oocytes matured without IL-6 (83%; $p < 0.05$ and $p < 0.01$; Fig. 3A). No significant difference was obtained in the rate of polyspermy, but a higher rate of polyspermy was observed in oocytes matured without IL-6 (62.7% *vs*. 46.7 - 37.7%; Fig. 3B). A lower percentage of embryo cleavage was observed in oocytes matured with IL-6 (50.1 - 63.1%) than oocytes matured without IL-6 (67.1%; $p < 0.01$ and $p < 0.001$; Fig. 4A), which subsequently resulted in decreasing the percentage of blastocyst formation (6.2 - 16.3% *vs.* 26.7%; $p < 0.05$; Fig. 4B).

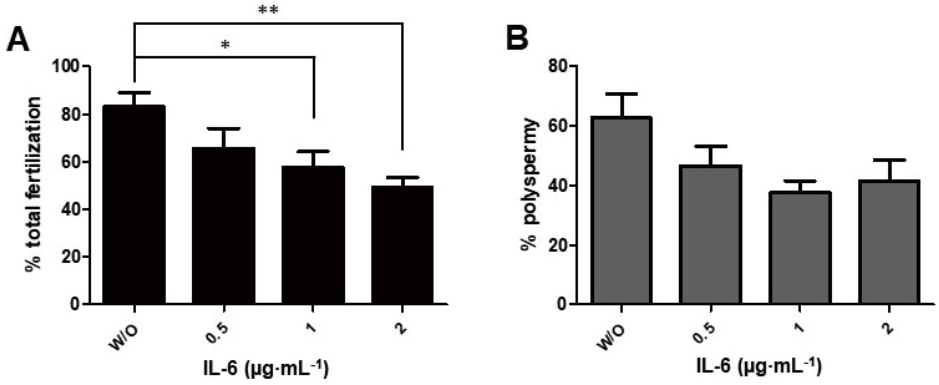

Fig. 3. *In vitro* fertilization (IVF) of pig oocytes matured in the absence (W/O) or presence of interleukin-6 (IL-6). The percentage of total fertilization (A) and polyspermy (B) were examined after IVF 20 hrs. All experiments were repeated 5 times. Values are expressed as the mean ± standard error of the mean (SEM). The different superscripts in each group of columns denote a significant difference at *p < 0.05 and **p < 0.01.

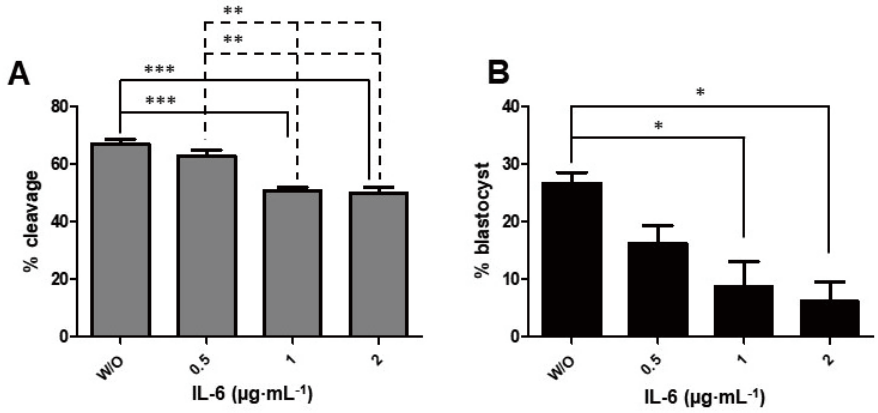

Fig. 4. Subsequent embryo development derived from pig oocytes matured with or without (W/O) interleukin-6 (IL-6). The percentage of cleaved embryos (A) and blastocyst formation (B) were examined after IVF 144 hrs. All experiments were repeated 5 times. Values are expressed as the mean ± standard error of the mean (SEM). The different superscripts in each group of columns denote a significant difference at *p < 0.05, **p < 0.01, and ***p < 0.001.

Pellicer et al. (1998) have reported that MII oocytes obtained from patients with inflammatory disorders are exposed to higher peritoneal fluid concentrations of IL-6. Along with an altered follicular environment, these factors hamper the oocyte quality before the ovulation process. Moreover, a higher concentration of IL-6 has the ability to alter the ciliary beat frequency in the fallopian tube, which affects the ovum pick up and implantation process (Papathanasiou et al., 2008). The possibility of IL-6 to arrest embryo growth to blastocyst development has been reported by Wu et al. (2001). Abnormally elevated levels of IL-6 soluble receptor (sIL-6R) in peritoneal fluid induces the development of endometriosis by promoting the bioactivity of IL-6, ultimately resulting in infertility in females (Li et al., 2017). A study of endometriosis-associated infertility stated that a higher level of IL-6 was found in the follicular fluid, resulting in abnormal functional changes in the follicular genesis and reduced ability to implant (Pellicer et al., 2000). Our results also show that IL-6-exposed oocytes have reduced maturation and embryo development, which is similar to previous reports. Taken together, these results indicate that the high level of cytokines produced by inflammatory reactions interfere with normal fertilization. Therefore, it is necessary to find direct methods to overcome infertility by controlling the inflammatory response.

Conclusion

Compared to the control, abnormal meiotic spindle alignment was induced in oocytes incubated with recombinant IL-6. A significant dose-dependent decrease was obtained for the fertilization rate and embryo development in the presence of IL-6 ($p < 0.05$). Thus, increased IL-6 level during IVF could be associated with fertilization failure due to aberrant chromosomal alignment and cortical granules disruption. Our results indicate that controlling inflammatory cytokines is required for successful assisted reproductive technology, and further studies may be conducted to consider the use of substances to mediate immune responses in reproductive techniques.

Conflict of Interests

No potential conflict of interest relevant to this article was reported.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF- 2013R1A6A3A04063769 and NRF-2020R1A2C1014007).

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References

Abeydeera LR, Wang WH, Prather RS, Day BN. 1998. Maturation *in vitro* of pig oocytes in protein-free culture media: Fertilization and subsequent embryo development *in vitro*. Biology of Reproduction 58:1316-1320.

Banerjee J, Sharma R, Agarwal A, Maitra D, Diamond MP, Abu-Soud HM. 2012. IL-6 and mouse oocyte spindle. PloS ONE 7:e35535.

Boutten A, Dehoux M, Edelman P, Seta N, Menard A, Madelenat P, Durand G. 1992. IL6 and acute phase plasma proteins in peritoneal fluid of women with endometriosis. Clinica Chimica Acta 210:187-195.

D'Hooghe TM, Debrock S, Hill JA, Meuleman C. 2003. Endometriosis and subfertility: Is the relationship resolved? Seminars in Reproductive Medicine 21:243-254.

Dinarello CA. 2007. Historical insights into cytokines. European Journal of Immunology 37:34-45.

Gandhi NA, Bennett BL, Graham NM, Pirozzi G, Stahl N, Yancopoulos GD. 2016. Targeting key proximal drivers of type 2 inflammation in disease. Nature Reviews Drug Discovery 15:35-50.

Gazvani R, Templeton A. 2002a. New considerations for the pathogenesis of endometriosis. International Journal of Gynecology and Obstetrics 76:117-126.

Gazvani R, Templeton A. 2002b. Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. Reproduction 123:217-226.

Harada T, Iwabe T, Terakawa N. 2001. Role of cytokines in endometriosis. Fertility and Sterility 76:1-10.

Harada T, Yoshioka H, Yoshida S, Iwabe T, Onohara Y, Tanikawa M, Terakawa N. 1997. Increased interleukin-6 levels in peritoneal fluid of infertile patients with active endometriosis. American Journal of Obstetrics and Gynecology 176:593-597.

Kang YJ, Jeung IC, Park A, Park YJ, Jung H, Kim TD, Lee HG, Choi I, Yoon SR. 2014. An increased level of IL-6 suppresses NK cell activity in peritoneal fluid of patients with endometriosis via regulation of SHP-2 expression. Human Reproduction 29:2176-2189.

Kashanian M, Sariri E, Vahdat M, Ahmari M, Moradi Y, Sheikhansari N. 2015. A comparison between serum levels of interleukin-6 and CA125 in patients with endometriosis and normal women. Medical Journal of the Islamic Republic of Iran 29:280.

Khan KN, Kitajima M, Hiraki K, Fujishita A, Sekine I, Ishimaru T, Masuzaki H. 2008. Immunopathogenesis of pelvic endometriosis: role of hepatocyte growth factor, macrophages and ovarian steroids. American Journal of Reproductive Immunology 60:383-404.

Kishimoto T. 1988. B-cell differentiation-interleukins and receptors for the antibody-response. Isi atlas of Science-Immunology 1:149-154.

Kishimoto T. 2010. IL-6: From its discovery to clinical applications. International Immunology 22:347-352.

Kishimoto T, Akira S, Narazaki M, Taga T. 1995. Interleukin-6 family of cytokines and gp130. Blood 86:1243-1254.

Kumar H, Kawai T, Akira S. 2011. Pathogen recognition by the innate immune system. International Reviews of Immunology 30:16-34.

Le J, Vilček J. 1990. Interleukin 6: A multifunctional cytokine regulating immune reactions and the acute phase protein response. Pathology Reviews 1990:97-111.

Li S, Fu X, Wu T, Yang L, Hu C, Wu R. 2017. Role of interleukin-6 and its receptor in endometriosis. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research 23:3801-3807.

Papathanasiou A, Djahanbakhch O, Saridogan E, Lyons RA. 2008. The effect of interleukin-6 on ciliary beat frequency in the human fallopian tube. Fertility and Sterility 90:391-394.

Pellicer A, Albert C, Garrido N, Navarro J, Remohi J, Simon C. 2000. The pathophysiology of endometriosis-associated infertility: Follicular environment and embryo quality. Journal of Reproduction and Fertility 55(Supplment):109-119.

Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohı́J, Simón C. 1998. The follicular and endocrine environment in women with endometriosis: Local and systemic cytokine production. Fertility and Sterility 70:425-431.

Punnonen J, Teisala K, Ranta H, Bennett B, Punnonen R. 1996. Increased levels of interleukin-6 and interleukin-10 in the peritoneal fluid of patients with endometriosis. American Journal of Obstetrics and Gynecology 174:1522-1526.

Rose-John S. 2018. Interleukin-6 family cytokines. Cold Spring Harbor perspectives in Biology 10:a028415.

Saito H, Seino T, Kaneko T, Nakahara K, Toya M, Kurachi H. 2002. Endometriosis and oocyte quality. Gynecologic and Obstetric Investigation 53:46-51.

Spangler JB, Moraga I, Mendoza JL, Garcia KC. 2015. Insights into cytokine–receptor interactions from cytokine engineering. Annual Review of Immunology 33:139-167.

Tanaka T, Narazaki M, Kishimoto T. 2014. IL-6 in inflammation, immunity, and disease. Cold Spring Harbor Perspectives in Biology 6:a016295.

Wu MY, Chen SU, Chao KH, Chen CD, Yang YS, Ho HN. 2001. Mouse embryo toxicity of IL-6 in peritoneal fluids from women with or without endometriosis. Acta Obstetricia et Gynecologica Scandinavica 80:7-11.

Yi YJ, Adikari AADI, Moon ST, Lee SM, Heo JM. 2021. Effects of lipopolysaccharides on the maturation of pig oocytes. Korean Journal of Agricultural Science 48:163-170.

Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S. 2002. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biology of Reproduction 66:112-119.