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Morphological changes in mouse ovary due to hormonal hypersecretion and matrix metalloproteinase-2 activity

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Summary. We analyzed whether aberrant gonadotropin secretion affects the morphological remodeling of murine ovarian tissues facilitated by activated matrix metalloproteinase (MMP) enzymes. Six mice were intraperitoneally injected with 5 IU of pregnant mare serum gonadotropin (PMSG) or human chorionic gonadotropin (HCG) every two days after estrus synchronization. Morphology and expression of various MMPs were assessed following the successful induction of hormonal secretion in these tissues. HCG treatment, but not PMSG treatment, resulted in the expanded production of granulose second follicular cells. In addition, the number of developing follicular cells in the HCG group increased compared with that in the PMSG group. Ovarian diameters were also very small in the PMSG group. Immunohistochemistry revealed decreased MMP-2 protein activity in the HCG group and increased MMP-2 activity in the PMSG group. Activity was particularly high in theca and granulose cells of the PMSG group, but only partial activity was observed in the theca cells of the HCG group. Vascular endothelial growth factor activity was increased in both the external and internal theca cell walls in the PMSG group while the HCG group showed high overall expression of this protein in the internal theca cells. These data indicate that follicular cell activity and remodeling of the ovaries differ based on the type of secretory hormone signals they receive. Inappropriate gonadotropin secretion may induce functional changes in the ovaries, and follicular remodeling may be facilitated by the activity of various MMPs.

Key words: Hormonal Hypersecretion, MMPs, TIMPs, Ovary, Mouse

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Introduction

Production of oocytes by the ovaries facilitates important exocrine and endocrine functions pivotal to reproductive physiology (Adashi 1994; Eppig 2001). Specifically, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play important roles in restoring the estrous cycle during the development of follicular cells in the ovary and influence physiological activity (Hirshfield 1991; Adriaens et al., 2004). Hormonal imbalances in pregnant mare serum gonadotropin (PMSG) and/or human chorionic gonadotropin (HCG) may lead to abnormal protein expression in the ovaries of mice resulting in functional and structural changes within the ovary (Satoh et al., 2009). These may affect mouse ovulation, as both PMSG and HCG are used to control the corpus luteum and induce ovulation during synchronized estrus. In addition, changes in the dose or timing of PMSG or HCG expression may induce superovulation (Luo et al., 2011), fundamentally altering the positive and negative regulatory feedback ultimately resulting in morphological changes in the ovary (Rotterdam, 2004; Vrbikova and Hainer, 2009; Goodarzi et al., 2011). This suggests that the normal functioning of the ovarian tissues requires that these organs undergo a series of dramatic changes in their tissue structure controlled by multiple factors over the course of the reproductive cycle. However, aberrant hormonal stimulation can result in drastic tissue remodeling and angiogenesis via the abnormal production and degeneration of the corpus luteum and follicular atresia. Over time, several lines of indirect evidence have suggested that both the MMP systems are important in generating the proteolytic activity needed to manage these tissue remodeling processes (Tsafriri, 1995; Curry and Osteen, 2001). This aligns with the fact that cellular turnover and renewal throughout the body is regulated by changes in the activation status of the matrix metalloproteinases (MMPs). MMP-2 activity has been linked to tissue remodeling of the ovary and reorganization of cells in

the follicle facilitating the breakdown of intercellular basal membranes and elimination of aging and abnormal cells (Thomas et al., 2001; Goldman and Shalev 2004). In addition, changes of MMP activity is critical to the process of apoptosis with abnormal hormone secretion causing perturbations in this process as well, often resulting in a number of abnormalities in the ovary (Visse and Nagase 2003; Kim et al., 2012). In this study, we analyzed changes in murine follicular cells following the hypersecretion of PMSG and HCG hormones. The expression of MMP-2, effect of MMP-2 inhibitors, and expression patterns of VEGF were assessed to clarify the underlying morphological remodeling taking place in the ovarian tissues following aberrant hormone expression.

Materials and methods

Animals

Female ICR mice (Institute of Cancer Research; 6-week-old female mice) were obtained from Dahan Bio Link (Eumseong, Korea) and maintained in light-controlled and air-conditioned rooms. Animals were kept on a 12-hour dark/light schedule at a constant temperature of 21°C and 50% relative humidity. All animal procedures followed the protocol approved by the Animal Experimentation Ethics Committee at Hankyong National University (permission number: 2018-1). All surgeries were performed using pentobarbital sodium anesthesia, and every effort was made to minimize pain. All ovaries were treated to stimulate ovulation and then used for further experiments. Estrus was synchronized using the method described by Kim et al., 2020b. Stimulation involved injecting 5 IU PMSG (Serotropin; Teikoku Zoki, Tokyo, Japan) into the abdominal cavity and injecting 5 IU HCG (Puberogen®; Sankyo, Tokyo, Japan) 48 hours later (Luo et al., 2011), adjusting the estrus cycle of all mice to commence simultaneously at day 0. The hormone treatments were performed 24 hours after estrus synchronization was completed.

Preparation of ovarian tissues

Eighteen female mice were randomly divided into three groups of six, as follows: the control group (n=6), PMSG group (n=6), and HCG group (n=6). Each group was treated with PMSG, PBS or HCG every 2 days for up to 14 days; the animals were euthanized, and ovarian tissues were harvested.

Hematoxylin and eosin staining

At the end of the hormone treatments, each group's ovaries were collected and fixed in 70% diethyl pyrocarbonate (DEPC)-ethanol, dehydrated, paraffin-embedded, and sectioned at 5 um thickness. After representative sections from each ovary paraffin-block were randomly selected, routine hematoxylin and eosin

(H&E) staining was performed to facilitate histological examination using an optical microscope (x40).

Hormone enzyme-linked immunosorbent assays (ELISA)

For the ELISAs, ovarian protein samples were diluted in 100% assay buffer. LH (8G9A2, Abcam, Cambridge, UK), FSH (sc-7797 Santa Cruz Biotechnology Inc., Texas USA), E2 (Anti-17 beta Estradiol antibody, ab54122, Abcam, Cambridge, UK), VEGF (sc-7269, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and MT-MMP (sc-373908, Santa Cruz Biotechnology, Santa Cruz, CA, USA) levels were measured using a quantitative sandwich ELISA (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions. All samples were evaluated in duplicate, and the mean levels were calculated for data analysis. The levels of each hormone were determined using a standard curve, which was constructed using the following four parameters: $(y=(A-D)/(1+(x/C)^B)+D)$. The standard curve was based on seven known values, and all hormone average fold values were expressed as the mean ± standard deviation.

Immunofluorescence (IF)

Sections of paraffin embedded ovary tissues were used for IF. Dehydration and permeabilization were performed by freezing the slides at -20°C in 5 mM 0.1% Triton X-100 in PBS. After blocking with 3% bovine serum albumin (BSA) slides were incubated with a β-actin (sc-47778, Santa Cruz Biotechnology Inc., Texas USA) at a 1:150 dilution. After washing, the slides were incubated with anti-rabbit IgG conjugated to Alexa-488 (Molecular Probes). Images were acquired using an Olympus AX70 fluorescence microscope fitted with a CCD-colour camera.

In-situ zymography

In order to perform the in-situ zymography, sections were deparaffinized and rehydrated using xylene, 100% ethanol and 95% ethanol, washed in ddW for 5 minutes, and then boiled in 10 mM sodium citrate for 10 minutes. This was repeated 20 times; then, these emulsions were (ddW, 10% SDS, 2% Glycerol) mixed in a 1:2 ratio with the zymography reaction buffer and allowed to react at 37°C for 48 h in a slide box filled with 1 M Tris. After these reactions were complete, we performed routine hematoxylin and eosin (H&E) staining for histological inspection using an optical microscope (Kim et al., 2014).

Immunohistochemistry (IHC)

Ovarian tissues were embedded in paraffin and sectioned. These samples were then sequentially treated with xylene, 100% ethanol, 95% ethanol, and ddW and then boiled in 10 mM sodium citrate for 5 minutes.

Antigen retrieval was accomplished by heating each sample in 10 mM sodium citrate buffer (pH 6.0) to 95°C. After rapid cooling to room temperature, endogenous peroxidase activity was validated using a 0.3% hydrogen peroxide solution. After three washes in 1× phosphate buffered saline (PBS), each slide was blocked using 1% goat serum and 3% horse serum. Primary antibodies against MMP-2 (sc-13595, Santa Cruz Biotechnology) and TIMP-2 (sc-9905, Santa Cruz Biotechnology) were then applied and left to incubate overnight at 4°C. Following a 5-minute wash using 1×PBS, secondary antibody was added, and the slides were incubated for another 4 hours at room temperature (25°C). Each sample was washed five times for 3 minutes each with 1×PBS, and then 300 μl of reaction substrate (3,3'diaminobenzidine, DAB) was added, and the reaction was allowed to proceed for up to 10 minutes before washing with ddW. Counterstaining with periodic acid–Schiff (PAS) reagents and a hematoxylin solution containing 4% acetic acid completed the processing, and each sample was covered with a dehydration, clarification, and permount solution (Thermo Fisher Scientific, Waltham, MA, USA) and observed using an optical microscope (Nikon, Tokyo, Japan) at x200 and x400 magnification. Protein expression patterns were analyzed using the Live Image Histogram function in SPOT 5.6 Microscope Imaging Software (SPOT Image, Michigan, USA).

Zymography

To analyze the enzymatic activity of the MMPs expressed within the ovarian tissues, 20 mg of total protein in a solution of 5% Bromo Phenol Blue, 10% SDS, and 2% glycerol was added to 10 μl FOZ loading buffer (5% Bromo phenol blue, 10% SDS, and 2% Glycerol) and allowed to react on ice for 5 minutes. The proteins were resolved using electrophoresis, and the gel was induced twice using protein renaturation buffer (2.5% Triton X-100, 1xPBS) and then washed with sterile water. Proteins were then allowed to react at 37°C for 18 h in a zymography reaction buffer (1 M Tris-HCL pH 7.5, 5 M NaCl, 1 M $CaCl_2$, 0.2 mM $ZnCl_2$, 0.2% Triton X-100, 0.02% NaN_3). After these reactions were complete, the zymography-induced proteins were stained using Coomassie Brilliant Blue (Bio-Rad, Hercules, CA,
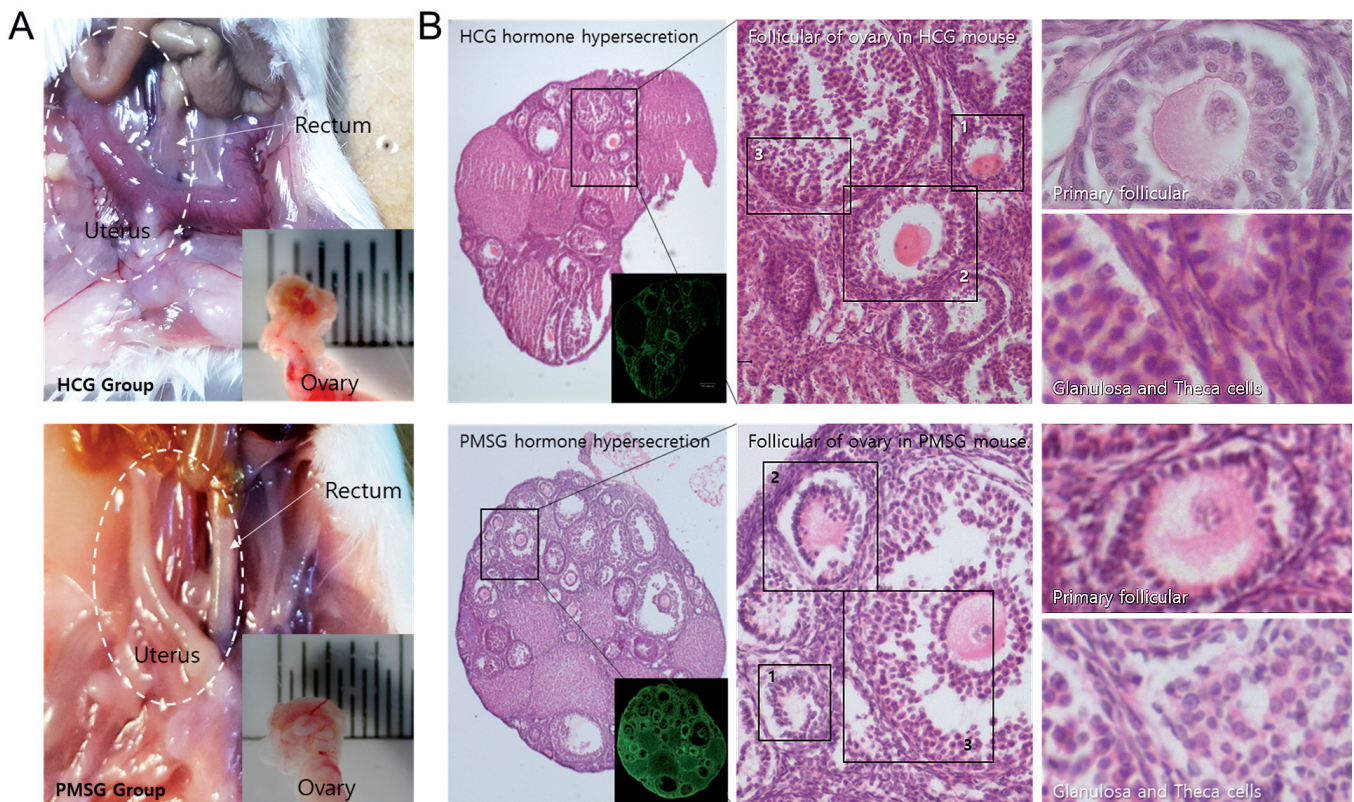

Fig. 1. Morphological analysis of murine follicular cells and ovarian tissues. **A.** Mouse anatomy; there were differences in the size of the uterus in the HCG and PMSG groups. The length of the ovaries was similar, but their width was different between these two groups. The small picture shows the length of the ovary. **B.** Hematoxylin and eosin staining; The inset shows β-actin IF in the ovarian tissues. 1: Primary follicular point, 2: First follicular point, 3: Secondary follicular point. The picture on the left of panel **B** highlights the important morphological features (primary follicular cells, glanulosa theca cell zone) of the ovaries.

USA) for 1 h, and the stained portions were analyzed as previously described (Balasingam and Yong 1996). We used the Kim et al., 2020a method to evaluate MMP activation.

Statistical analysis

All data were collected, tabulated, and analyzed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA) and Med Calc 21 for windows (Med Calc Software bvba, Ostend, Belgium). Statistical significance was established at $P<0.05$.

Results

Morphological and cell density remodeling of ovarian tissues following aberrant hormone secretion

We investigated the morphological changes associated with prolonged abnormal hormonal signaling in murine ovarian tissues. We noted a significant change in the size of the ovaries (Fig. 1), with the length of the ovaries remaining similar in all the treatment groups (HCG group: 250 μm; PMSG group: 300 μm), However, we noted significant changes in the width of these organs in the PMSG group (about 150 μm) when compared to the HCG group (about 200 μm) (Fig. 1A). The number of corpus luteum was increased, follicular atrophy was observed in the HCG, dense spots were present in the cell cytoplasm, oocyte maturation was delayed, and cumulus cell density was also reduced in these animals. Interestingly, an IF assay revealed that β-actin is differentially expressed between the HCG and PMSG groups, with β-actin being more strongly expressed in the HCG animals, which may explain the differences in the size of these tissues (Fig. 1B).

Hormone, VEGF, and MT-MMP expression in ovarian tissues

The results from the hormone and target protein expression analyses in each group are shown in Fig. 2. Expression of 17-beta estradiol (E2) was highest in the PMSG group. However, follicular stimulation hormone (FSH) was significantly higher in the Control and HCG group, while luteinizing hormone (LH) was higher in the PMSG group (Fig. 2A). The expression levels of VEGF were similar to FSH. In addition, the expression of MT1-MMP was relatively low in the PMSG group and was significantly higher in the HCG group (Fig. 2C). Mice in the PMSG group saw a rapid reduction in their body weight between 5 and 10 days, with the HCG animals gaining weight between days 5 and 9 and then losing weight after day 10. In addition, both groups experienced weight gains at day 11 (Fig. 2B).

MMP activation in the ovaries

MMP activity in the hormone-treated ovaries is shown in Fig. 3. While the MMP-2 activities were similar between ovaries within the same group, its activity was significantly upregulated in the PMSG group and significantly downregulated in the HCG group. However, MMP-9 expression was much higher in
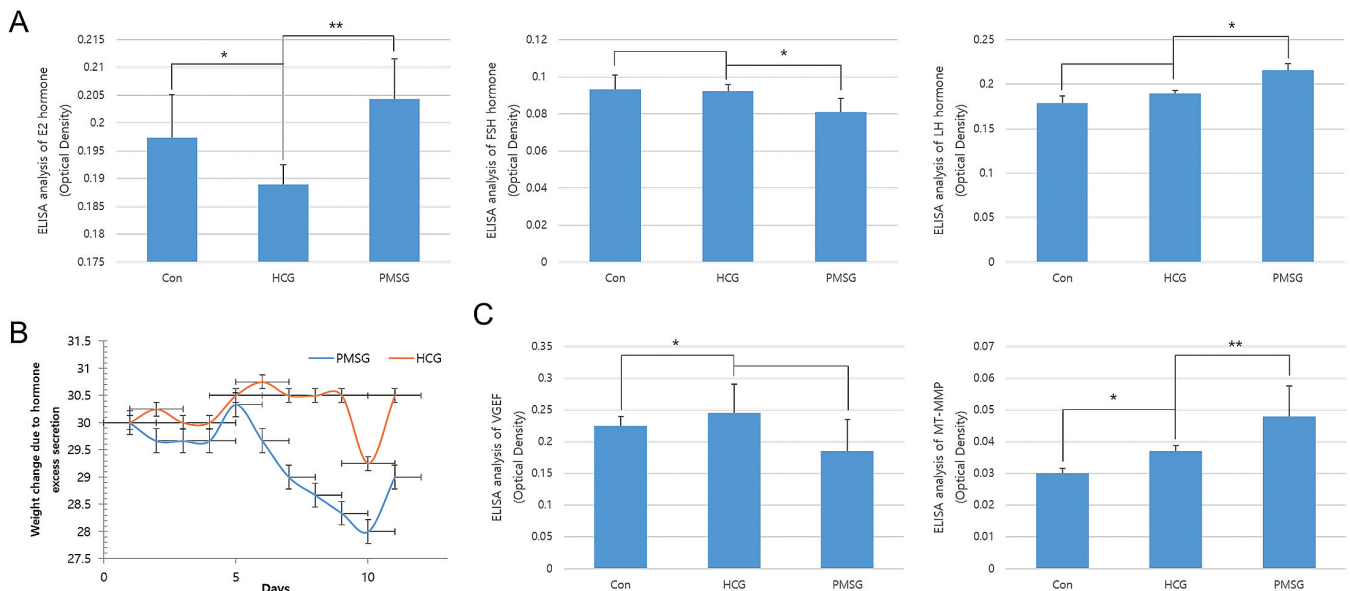

Fig. 2. ELISA-based analysis of E2, FSH, LH, VEGF and MT1-MMP in mouse ovary tissues following various hormone treatments. ELISA experiments were repeated three times, and data are expressed as the average fold change (mean ± SD). **A.** Hormone ELISA analysis. **B.** Changes in body weight in different hormone treatment groups. **C.** Protein ELISA analysis. *,** Different letters within the same column represent significant differences between groups (P<0.05).
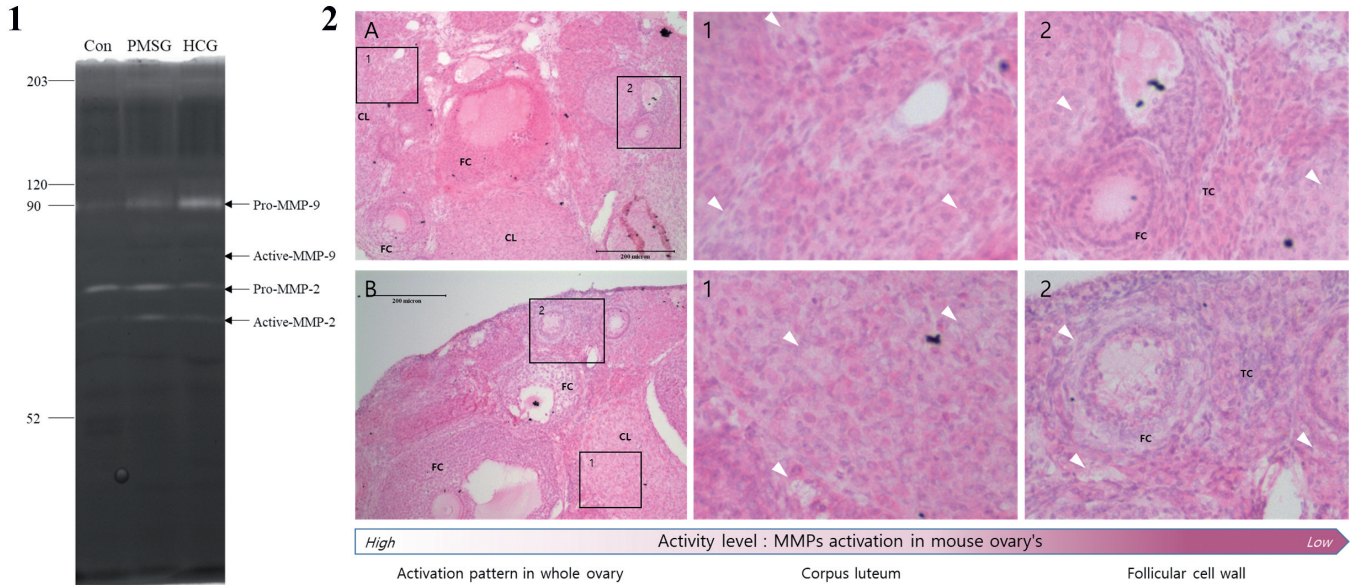
Fig. 3. MMP activation and localization in mouse ovaries following different hormone treatments. White arrows denote MMP activation. Follicular cells were counterstained with hematoxylin. **1.** Zymography. **2.** In-situ zymography. **A.** PMSG treatment. **B.** HCG treatment, 1) corpus luteum cell zone, 2) follicular cell zone. The activity of MMPs can be confirmed based on the concentration of the red signal in the cytoplasm. The lower is the concentration, the higher is the activity of MMPs. A, B, × 200; other panels, × 400.
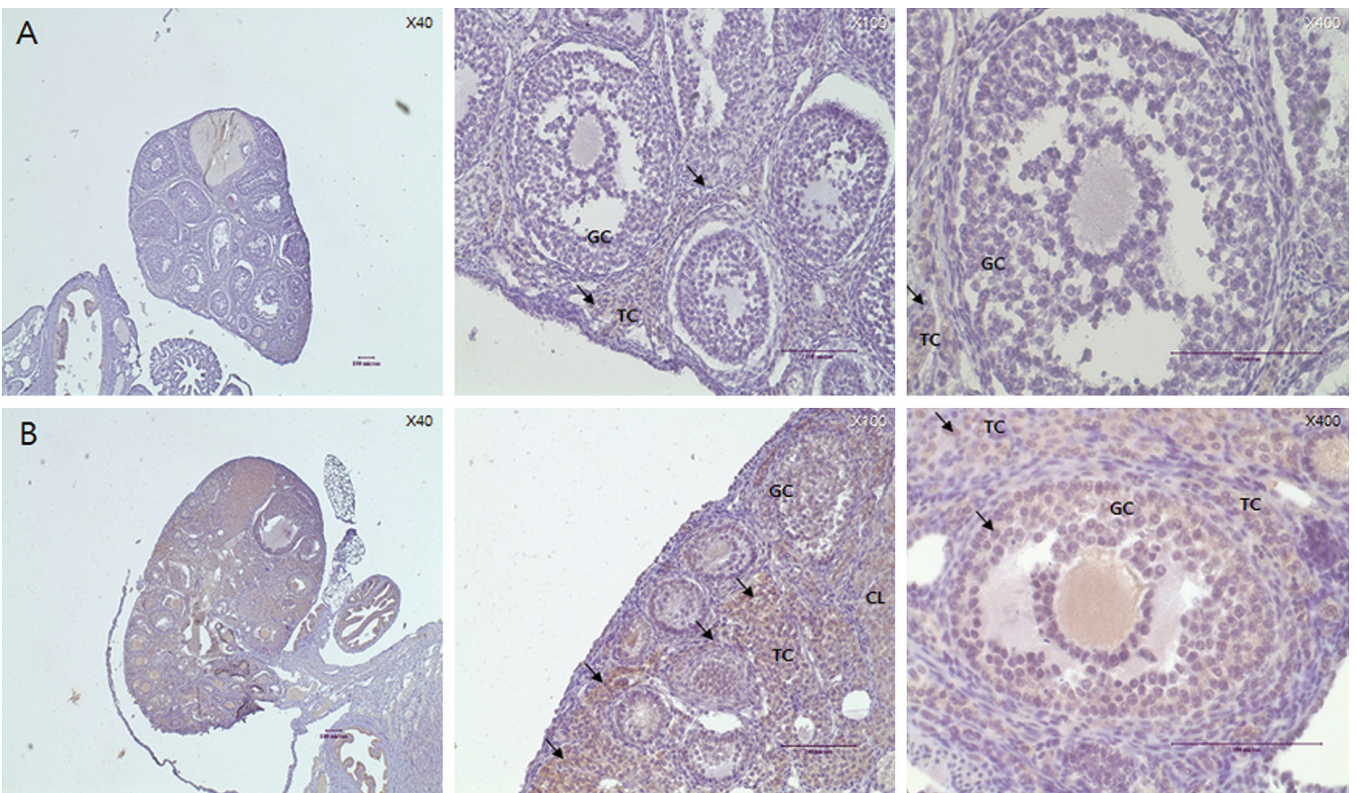

Fig. 4. Immunolocalization of MMP-2 proteins in mouse ovarian tissues following hormone treatment. Black arrows denote areas positive for MMP expression. **A.** PMSG group. **B.** HCG group.

the HCG group than in either the control or PMSG groups (Fig. 3-1). MMP activation was relatively low across the whole ovary but was significantly upregulated in the corpus luteum and decreased in the follicular cells. The PMSG group displayed increased activation of MMPs across the ovarian tissues when compared with HCG (Fig. 3-2).

Analysis of localized gelatinase expressiont

MMP-2 was actively expressed in ovary tissues from the PMSG group and was upregulated in comparison to the HCG group. Expression was prominent in the follicular cells in the PMSG group and in the theca cell layer in the HCG group (Fig. 4). TIMP-2 expression was not different between the groups, but its expression was increased in the internal theca cell layer of the HCG group compared to the PMSG group; however, its expression could not be observed in the glanulosa cell sections (Fig. 5).

Discussion

This study evaluated the effects of hormone secretion on the morphological remodeling of the ovarian tissues by analyzing the expression of MMPs after treatment with PMSG or HCG hormone over a 14-day period. Hormones are very important in the development of the ovaries and regulation of the reproductive cycle, especially in the reconstruction of the uterus and in the maintenance of normal pregnancy (Hirshfield, 1991; Adashi, 1994; Eppig, 2001). However, hypersecretion or abnormal secretion of hormones can lead to serious problems in the ovaries, which can in turn result in various hormone-related pathologies (Satoh et al., 2009; Casarini et al., 2015). The key hormones in ovarian development and ovulation are FSH and LH; these hormones act antagonistically (Choi and Smitz, 2014; Casarini et al., 2015). Unregulated FSH expression results in the development of excessive numbers of ovarian follicles, polycystic ovaries or ovarian cysts (Dierich et al., 1998; Drummond and Fuller, 2012), which can delay ovulation and affect ovarian development. Hypersecretion of LH interferes with corpus luteum regression in the ovary, which affects the development of the uterus (Kim et al., 2018; Banaszewska et al., 2003). Our results suggest that the continuous expression of PMSG or HCG after the formation of the corpus luteum in super-ovulating mice induces hypersecretion of FSH and LH. PMSG
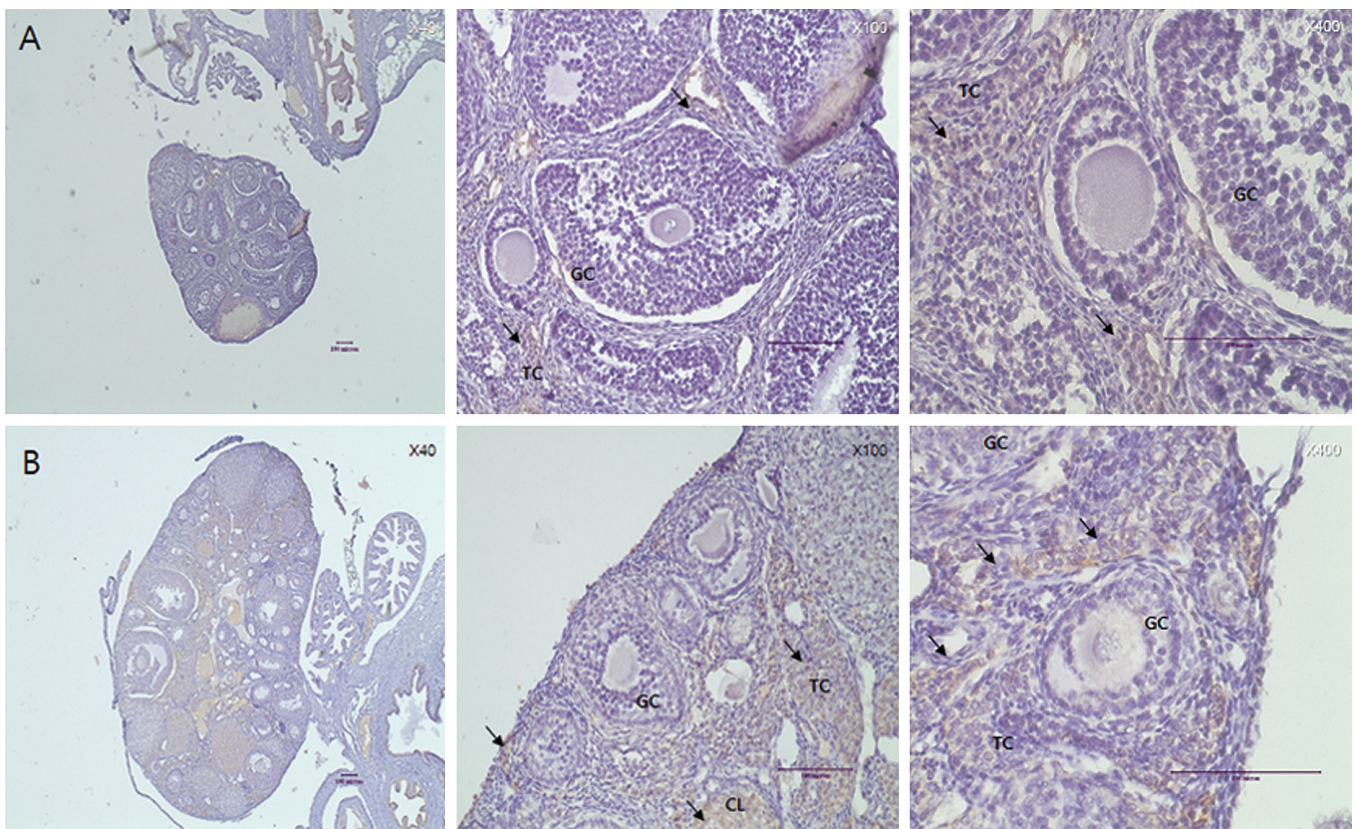

Fig. 5. Immunolocalization of TIMP-2 proteins in mouse ovarian tissues following hormone treatment. Black arrows indicate areas that are positive for TIMP-2 expression. **A.** PMSG group. **B.** HCG group.

hypersecretion was associated with morphological remodeling in the ovaries and a higher number of follicular cells when compared to HCG overexpression (Ilgaz et al., 2015). However, these findings are different from those described in a previous study where FSH overexpression resulted in the production of polycystic follicular cells and not follicular cysts (Wang et al., 2017). This suggests that this hormone specifically stimulates the development of the follicular cells (Hirshfield, 1991; Adashi, 1994; Eppig, 2001). Suggesting that the expression of MMP could be very pronounced (Kim et al., 2014). A previously reported, we observed that the activation of MMPs is critical for follicular development, with simultaneous expression in the theca and follicular cell layers, with very high expression levels in the latter following the hypersecretion of PMSG (Kim et al., 2012, 2014). In other words, the continuous secretion of PMSG after ovulation leads to the creation of polycystic follicular cells, and the activation of MMPs accelerates the reconstruction of the ovaries. These results suggest that the increase of MT-MMP proteins in the PMSG animals results in increased MMP activity. This result was different from the observations in the HCG group. Hypersecretion of LH results in the continuous retention of the corpus luteum or lutein cysts and affects follicular development (Baird, 1992; Kawate et al., 2000). However, follicular development was shown to occur along with the extensive remodeling of the corpus luteum suggesting that the retention of the corpus luteum could be confirmed, and the development of the corpus luteum is also considered to be maintained. However, follicular development proceeded to some extent when animals were treated with PMSG, here initial follicular formation was low, but follicular development did occur, which would likely increase the expression of other genes that can inhibit HCG or affect the return of estrus. Following the development of the granular membrane cells, the follicle undergoes extensive morphological rearrangements. According to Fujibe et al., 2019, follicle development and granular membrane cell development are the result of androgen stimulation. However, according to a recent study, even without E2 stimulation, granular membrane cells increase hormone receptor expression based on their FSH or LH hormone stimulation (Kim and Yoon, 2020a). As such, our study showed that HCG treatment reduced E2 stimulation but increased FSH and LH stimulation. In comparison, the PMSG group had a higher concentration of E2, but the concentration of FSH was significantly lower and the concentration of LH was significantly higher. Therefore, PMSG increases the utilization efficiency of estrogen, causing changes in FSH and LH concentration, and seems to be involved in follicular development (Kumar et al., 2018). Although we did not study the influence of the hypersecretion of other hormones, morphological analysis of the ovaries may provide further insight. The expressions of MMPs expression was different. Stimulation of MMPs was minimized in the HCG

treatment group (Light and Hammes, 2015; Kim and Yoon, 2020b), and their expression was very low in the follicular cell layer and increased in the theca cell layer. In other words, hypersecretion of HCG influences the development of follicular cells and can help to retain the morphology of the corpus luteum. There was little change in weight of the mice in the HCG group where MMP expression was low, and the morphological remodeling of the corpus luteum showed low LH hormone expression but was accelerated following injection of HCG. In addition, the study by Quintana et al., 2004, demonstrated that a significant increase in VEGF protein increases the development of an enhanced vascular network promoting follicular development. However, as previously reported, ovaries are very rarely reconstructed (Norambuena et al., 2017). Our results indicate that there is some follicular development even during hormone imbalance, following HCG hypersecretion. Our data suggests that further studies on the physical response of ovarian tissues to hormone hypersecretion may uncover new mechanisms of hormone regulation which may advance the treatment of ovarian disease.

Conclusion

In this study, hypersecretion of PMSG and HCG during follicular development affected morphological remodeling of ovaries by maximizing or minimizing the utilization of MMPs, suggesting that these enzymes are involved in reproductive development. We confirmed that mouse ovaries according to hypersecretion of PMSG and HCG were markedly different in morphology, among which PMSG increased the use of E2, and increased the expression of MMPs in both external and internal theca cell walls to induce change in the ovaries. On the other hand, the HCG group had less effect of E2, but increased the expression of FSH and LH, and increased the expression of MMPs in the internal theca cell and glanulosa cell sections, affecting the ovaries. Therefore, this research suggests that PMSG and HCG perform critical functions in ovarian tissue development and remodeling and that evaluation of ovarian remodeling may be a useful tool in understanding the clinical effects of various treatment strategies in the future.

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