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Persistent Stimulation of Sex-related Hormones in the Uterus is Associated with Differences in the Igf/Akt Signaling Pathway and Uterine Damage

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ABSTRACT

Background: The health of the female endometrium is determined by a delicate network of hormones and signaling genes. Our study analyzed the effects of continuous excess of major sex-related hormones on insulin-like growth factor/protein kinase B (IGF/Akt) signaling on the functional and morphological changes of the endometrium.

Methods: After estrus synchronization of six mice, human chorionic gonadotropin (hCG), pregnant mare serum gonadotrophin (PMSG), progesterone (P4) andtestosterone (TES) were overstimulated andthe differences in IGF/Akt signal expression were analyzed using an immunoassay method.

Result: The results of this study showed that the morphological changes of the uterus in the hCG and P4 treatment groups had very similar patterns and the expression of genes belonging to the IGF/Akt signaling pathway affected maturation of the endometrium uterine gland and induced expression of pregnancy-associated plasma protein to increase the maturation rate of the uterus. However, the TES treatment group showed increased uterine apoptotic factors and inhibited endometrial development. Therefore, based on the results of this study, hormonal stimulation can have different effects on the functional maturation and morphological development of the uterus andrepeated hormonal excess processes can be predicted through IGF/Akt signaling.

Key words: Difference, Endocrine disruption, IGF signal, Sex, Testosterone, Uterine health.

INTRODUCTION

The uterine function is primarily regulated by the combined stimulation of estrogen and progesterone produced by the ovaries and uterus (Kumar *et al*., 2018; Tahir *et al*., 2022). However, the disruption of finely regulated networks that maintain elevated levels of gonadotropins or mutations in the receptor can lead to various pathological conditions, including premature ovarian failure (POF), ovarian hyperstimulation syndrome, polycystic ovary syndrome (PCOS), compromised oocyte quality, ovulation defects andcancer (Franik *et al*., 2018). Moreover, previous studies indicate that female mice overexpressing the β-subunit of human chorionic gonadotropin (hCGβ+) exhibit elevated prolactin (PRL), progesterone andtestosterone levels throughout their lifespan, along with sexual precocity. As these mice mature, they develop obesity, infertility, mammary tumors and prolactinomas (Ahtiainen *et al*., 2010; Manokaran *et al*., 2023).

Additionally, excess hormones can induce the epithelial growth factor receptor (EGFR) ligand heparin-binding EGF-like growth factor (HB-EGF) activity, leading to estrogen overproduction (Miyata *et al*., 2017; Miyamoto *et al*., 2011). This, in turn, activates the cAMP-PKA-JNK/ERK-Ca2±FOXO1 pathway, potentially causing mitochondrial dysfunction and cell death in granulosa cells (Huang *et al*., 2022).

Excess steroid hormones, including sex hormones, can lead to permanent phenotypic changes in adult women. For instance, excess fetal androgens induce masculinized behavior, neuroanatomical changes and male-like features in the female urogenital tract (Cussen *et al*., 2022). Conversely, excess fetal estrogen contributes to abnormalities in the vagina and cervix, including clear cell carcinoma andincreases the risk of breast cancer (Binay *et al*., 2014).

Androgen excess is clinically or biochemically defined as elevated androgen steroid levels in women and typically presents with features such as hirsutism, alopecia, or acne

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(Lebbe and Woodruff, 2013). PCOS is a common underlying diagnosis in adolescent girls and reproductive-aged women, exhibiting signs of and rogen excess and affecting at least 10% of the general population (Sun *et al*., 2015; Azziz *et al*., 2009).

Ultimately, hormonal excess disrupts endocrine function and impairs the functionality of the reproductive organs, potentially leading to sexual dysfunction. In this study, we aimed to analyze the overall uterine histological and functional differences by assessing the changes in insulin-like growth factor (IGF) and various hormone receptor activities after sustained elevation of testosterone, hCG, PMSG and progesterone levels in sexually mature female mice.

MATERIALS AND METHODS

Preparation and certification of animals

The present study was conducted from 2022 to 2023 at the Animal Developmental Biotechnology Laboratory of Hankyong National University. All experiments were performed as described by Kim *et al*. (2020). All animal procedures followed a protocol approved by the Animal Experimental Ethics Committee of Hankyong National University (Permission No: 2023-1). All surgeries were performed under pentobarbital sodium anesthesia and every effort was made to minimize pain. 20 female mouses were synchronized to estrus according to Oh and Kim (2022) method, then randomly divided into four groups of 6 mouses each. Afterward, human chorionic gonadotropin (hCG), pregnant mare serum gonadotropin (PMSG), progesterone (P4) andtestosterone (TES) were injected into each group at a dose of 5 IU every other day for 14 days. Uterus tissue was collected from mice in each group according to the dissection method.

Histological inspection of the uterus tissue

From each group uterus tissue was collected and fixed in 70% Diethyl pyrocarbonate (DEPC)-ethanol, dehydrated, paraffin-embedded and sectioned at 5 um thickness. Representative sections from each uterine paraffin block in the treatment group were randomly selected and routine hematoxylin and eosin (HandE) and 4′,6-diamidino-2-phenylindole (DAPI) fluorescence (V11324, Thermo Fisher Scientific Solutions, Waltham, MA, USA) staining were performed for histological inspection using an optical microscope (×200×400; Manufacturer, City, Abb. State, Country) (Kim *et al*., 2018).

Immuno-detection of uterus tissue protein

Estrogen (E2)-rQ(ab32063, Abcam, Cambridge, UK), follicle-stimulating hormone (FSH)-β(sc-7797, Santa Cruz Biotechnology, Dallas TX, USA), LH-r(ab76902, Abcam), IGF-Ir (sc-712, Santa Cruz), phosphoinositide 3-kinase (PI3K; sc-365290, Santa Cruz), Akt1 (sc-5298, Santa Cruz), pregnancy-associated plasma protein A (PAPP-A; sc-365226, Santa Cruz), Matrix metalloproteinase-9 (MMP-9; sc-13520, Santa Cruz), proliferating cell nuclear antigen (PCNA; sc-7907, Santa Cruz), tumor necrosis factor (TNF)-α (sc-1070, Santa Cruz), B-cell lymphoma 2 (BCL-2; Cell Signaling Technology, Danvers, MA, USA) andCaspase-3 (Casp-3; ab32351, Abcam) primary antibodies and proteins were applied to 96-well enzyme-linked immunosorbent assay (ELISA) plates and activated at 4°C for one day to evaluate specific proteins in each cell. Next, immune reactions were performed using secondary antibodies (Rabbit, Mouse and Goat IgG-HRP antibodies; Abcam) for 2 h at room temperature and a substrate solution (R and D Systems, Minneapolis, MN, USA) was added. The absorbance was measured at 450 nm.

Alizarin red and Alcian blue stain

Calcium and mucopolysaccharides in uterine tissue sections from each treatment group were analyzed using Alcian Blue and Alizarin Red S (ARS) (Sigma Aldrich, St. Louis, MO, USA). First, the tissues were deparaffinized and hydrated to remove the antigens composed of the tissues and then immersed in 0.5% ARS (w/v in water; pH 6.36-6.4) to induce staining for approximately 30 min at room temperature. Afterward, the sections were immersed in deionized water for approximately 5 min to stop staining, dehydrated in EtOH, fixed and mounted with Permount™. Calcium deposition was visualized using NI2-U (Nikon, Tokyo, Japan) to analyze orange and red spots. Histological analysis was performed using NIS-Elements C software (ver. 3.2; Nikon).

Immunohistochemistry

The expression of specific proteins in the uterine tissues was analyzed by immunostaining following previously reported protocols (Kim *et al*., 2020).

The processed slides were blocked with 5% NHS+1% normal goat serum (NGS) 1×PBS for 1 h. The blocking solution was removed and the primary antibodies E2-rQ, FSH-ß, LH-r, Akt, IGF-I, PI3K and Bcl-2 (#3498, Cell Signaling Technology) were diluted 1:200 in the blocking solution andincubated overnight at 4°C to induce antigen-antibody reactions. The slides were washed with 1×PBS for 5 min andthe secondary antibodies, horseradish peroxidase-conjugated anti-rabbit and anti-mouse (IgG-HRP antibody, Abcam), were diluted 1:200 in the blocking solution and incubated for 1 h at room temperature.

The slides were then reacted with ABC reagent (Vector Laboratories, CA, USA) and DAB (Vector Laboratories, Newark, CA, USA) and counterstained with hematoxylin. The stained slides were mounted with Permount™ and observed under an NI-U microscope (Nikon).

Immunofluorescence

The expression of specific proteins in the uterine tissues was analyzed by immunofluorescence staining following previously reported protocols (Kumar *et al*., 2024). Uterine tissue (14 days) was prepared by deparaffinization, as in immunohistochemistry, washed for 30 min in PBS andpermeabilized with 0.2% Triton X-100 for 30 min at room temperature. After blocking with 3% bovine serum albumin

in PBS, the samples were incubated with antibodies against the active forms of β-actin (sc-69879; Santa Cruz Biotechnology), mTOR (#2972; Cell Signaling Technology), PAPP-A and Dynactin p62 (sc-25730; Santa Cruz Biotechnology) at a 1:300 dilution. Subsequently, the samples were washed and incubated at room temperature for 2 h with the following secondary antibodies: Alexa Fluor™ 488 (A11001, Invitrogen, Waltham, MA, USA) and Goat Anti-Rabbit IgG Dylight™ 594 (35560, Invitrogen), both at a 1:300 dilution. Nuclei were counterstained with DAPI (Sigma Aldrich) and the samples were mounted using a fluorescence mounting medium. Finally, the cells were imaged using a fluorescence microscope (NI2-U; Nikon).

Statistical analysis

ELISA data were tested for significance (Duncan and General Linear Model) using Statistical Analysis System software (SAS Institute, version 9.4, Cary, NC, USA). Statistical significance was set at $p<0.05$.

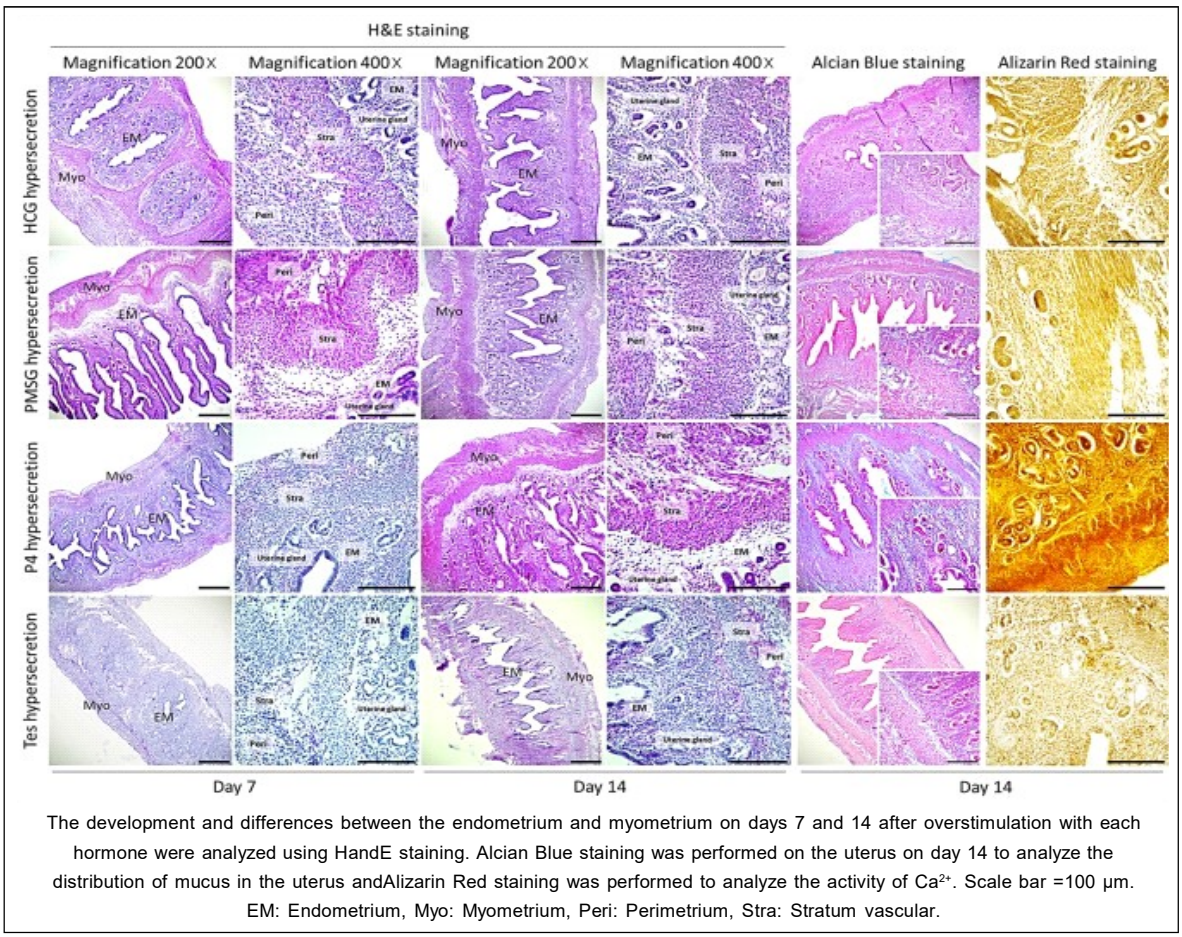
Results and Discussion

Changes in the uterus due to excessive hormone action

The uterus was uniquely shaped when the hormones were administered for 14 days. In the hCG and PMSG groups, the size of the uterus and development of the endometrium were reduced compared to those on day seven, while in the case of progesterone, it was confirmed that it became more enlarged. However, in contrary to the TES group, the uterine size and endometrial cell distribution were atrophied and did not develop as compared to the other treatment groups. In the analysis of the distribution of mucopolysaccharides, a representative component of the viscous substance between the uterus and tissues, hCG and PMSG showed a similar distribution centered in the endometrium after 14 days of hormone treatment. However, for progesterone, a significantly higher distribution pattern was observed in the endometrial and uterine gland sections. Compared to the other groups, the distribution of the mucopolysaccharides in the TES group was very low and the stromal cell zone was densely organized. In the distribution analysis of Ca^{2}+, the P4 group showed a high distribution and hCG and PMSG were distributed throughout the endometrium (Fig 1).

Comparison of expression patterns of hormone receptors

In the hCG treatment group, all hormone receptors showed higher expression patterns than in the other hormone treatment groups andthis was high on days 7 and 14.



The development and differences between the endometrium and myometrium on days 7 and 14 after overstimulation with each hormone were analyzed using HandE staining. Alcian Blue staining was performed on the uterus on day 14 to analyze the distribution of mucus in the uterus andAlizarin Red staining was performed to analyze the activity of Ca^{2+}. Scale bar =100 µm. EM: Endometrium, Myo: Myometrium, Peri: Perimetrium, Stra: Stratum vascular.

Fig 1: Analysis of differences in morphological changes according to hormonal overstimulation.

The expression pattern in the uterine tissue was generally high in the endometrium and a similar expression pattern was observed in the myometrium. hCG showed exceptionally high expression of the LH receptor (Fig 2A). Generally, all hormone receptors are expressed in the uterine glands of the endometrium, with some being expressed in the stratum vasculature. In the TES group, in which morphological changes in the uterus were negative, the FSH receptor was intensively expressed in the uterine gland on days 7 and 14 (Fig 2B).

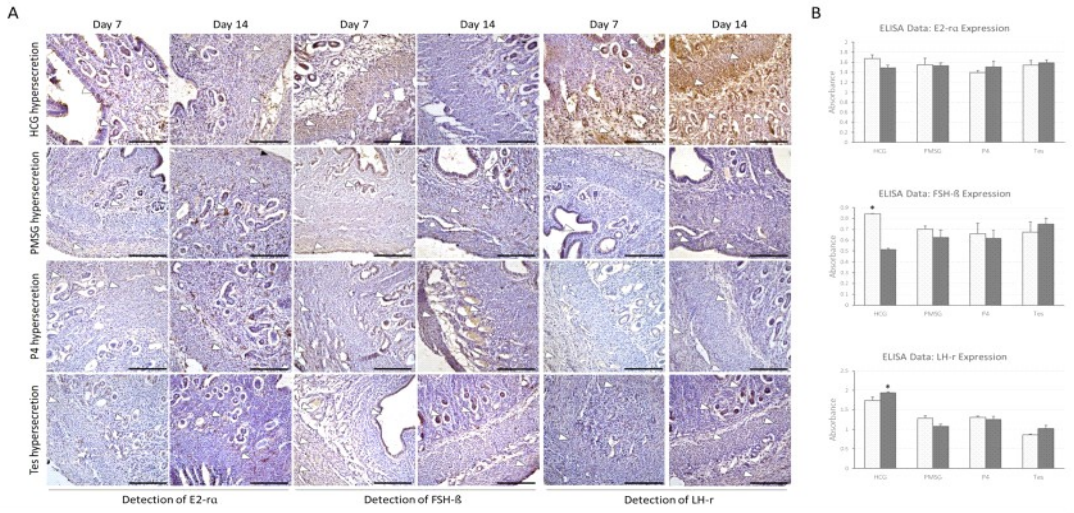
Comparison of expression patterns of IGF signal and tissue restructuring factors

In the PMSG group, the expression of genes (PI3K and Akt proteins) related to IGF signaling was significantly higher compared to other treatment groups and increased in the endometrial tissue (Fig 3A-D). The analysis revealed that the expression of mTOR and PAPP-A in the uterine tissue showed a high increase of PAPP-A in the uterine glands of all treatment groups except the TES group and it was highly expressed in the overall endometrium of the P4 group. The hCG and P4 groups had similar expression patterns, but the mTOR response was higher in these groups than in the PMSG group (Fig 3B). The P4 treatment group exhibited a higher expression pattern of MMP-9 and MT1-MMP on day 14 compared to other treatment groups and MT1-MMP was prominently expressed in the endometrium (Fig 3C). In the PMSG treatment group, MT1-MMP was expressed in the endometrium but at very low levels andMMP-9 expression rapidly decreased on day 14. In the TES treatment group, MT1-MMP was widely expressed throughout the uterine tissue, particularly in the stromal cell zone, while MMP-9 expression was relatively low and increased on day 14 compared to day 7 (Fig 3C, D).

Comparison of expression patterns of apoptotic factors expressed in the uterus according to hormone treatment

The cytoplasmic distribution of tissues was analyzed using Dynactin p62 to observe the distribution of tissues andas a result, hCG and PMSG formed similar cytoplasmic distributions. P4 treatment resulted in a very high cytoplasmic distribution compared to the other treatment groups. However, the cytoplasmic distribution and endometrial formation were very low in the TES group (Fig 4A). In analyzing whether hormone treatment induces apoptosis of uterine tissues, the expression of Casp-3 and TNF-α was sharply higher in the TES treatment group compared to other treatment groups and significantly increased on day 14 (Fig 4B). In the case of PCNA and BCL-2, which inhibit apoptotic factors, the total protein expression was significantly higher on day seven of PMSG treatment, but the tissue expression distribution showed differences (Fig 4B). BCL-2 expression was high in the myometrium sections of all hormone-treated groups; however, expression was prominent in the endometrium of the hCG- and P4-treated groups (Fig 4C).

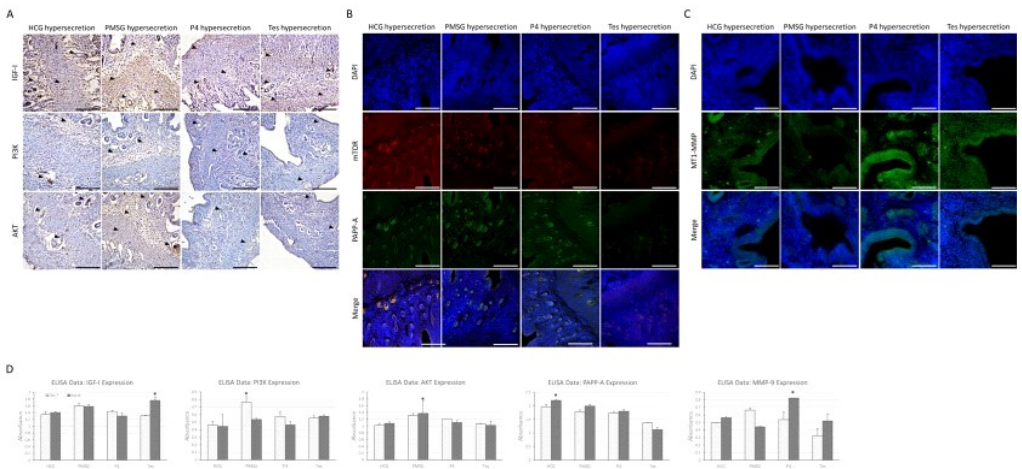
In our study, excessive increase of hCG, PMSG, P4 and TES levels caused morphological changes in the tissues according to the characteristics of the hormones andit was confirmed that the expression patterns of the genes related to the metabolism also changed (Oh and Kim 2022). In fact, unlike the results of most researchers that high hCG causes a rapid increase in the expression of the β-LH subunit and precocious puberty or has a negative



A: The expression of each hormone protein between the 7th and 14th day of hormonal stimulation was analyzed by immunohistochemistry to determine the difference in the endometrium. B: The expression pattern of each hormone-related factor was compared and analyzed in total proteins using ELISA. All experiments were repeated at least three times. Scale bar= 100 µm. *Different letters within the same column represent a significant difference ($p<0.05$).

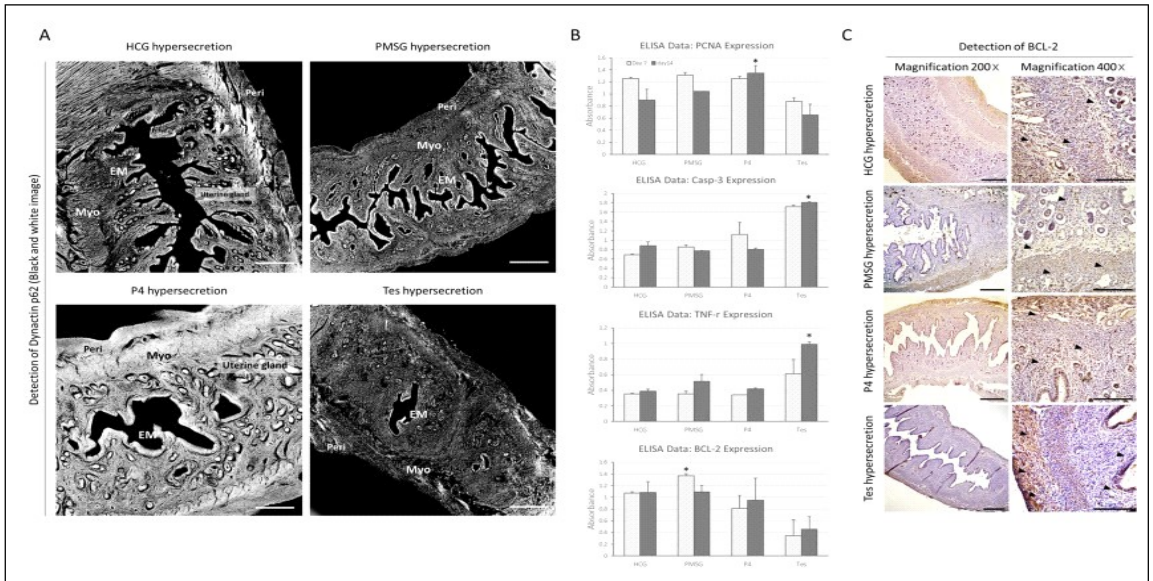
Fig 2: Analysis of E2, FHS andLH protein responses to hormonal stimulation.

effect on the development of the endometrium through morphological changes in the uterus (Rulli *et al*., 2002), our study showed similar IGF signaling in both hCG and PMSG treatments. Endometrial stromal zone and uterine gland development were similar to those observed in the P4 treatment group andexpression of PAPP-A, which is associated with implantation induction, increased significantly in the uterine gland (Kim *et al*., 2020; Oh and Kim, 2022). In particular, the excessive action of P4 in our study showed results similar to those of previous studies (Yusuf *et al*., 2023). However, when continuously overstimulated for 14 days, excessive stress was observed in the uterus. However, the expression of Akt, among the genes belonging to the IGF signaling pathway, was lowered, whereas the response of mTOR was increased (Zhang *et al*., 2023).



A: IGF/Akt protein expression pattern and localization analysis using immunohistochemistry. B: Expression pattern analysis of survival signal and implantation-related proteins using immunofluorescence analysis. C: Expression pattern analysis of MT1-MMP, an MMPs activator. D: Comparative analysis of expression pattern of survival-related proteins using ELISA analysis. *Different letters within the same column represent a significant difference ($p<0.05$).

Fig 3: Expression pattern analysis of IGF/Akt signaling and proteins related to uterine function in the uterus on day 14 of hormone excess.



A: Analysis of tissue distribution through the expression pattern of Dynactin P 62. B: Analysis of differences in the expression of proteins related to apoptosis by ELISA. C: Analysis of the expression pattern and localization of BCL-2 protein, a representative apoptosis inhibitor. *Different letters within the same column represent a significant difference ($p<0.05$).

Fig 4: Analysis of morphological changes and differences in apoptosis-related proteins according to tissue distribution in the uterus treated with each hormone.

mTOR response was lower than that of the hCG treatment group, but considering the development of the stratum vasculare and the concentration of MMP-9 in the uterine gland (Kim *et al*., 2014; Oh and Kim 2022), progesterone seems to induce uterine hypertrophy. Because the expression of PCNA and Dynactin P62 is distributed throughout the uterine tissue, it is believed to induce the rapid differentiation of cells.

In contrast, PMSG stimulation is believed to significantly alter the state of the uterus. In previous studies, PMSG and hCG stimulation have been shown to affect PI3K/Akt signaling in the ovary and uterus (Xie *et al*., 2023), causing damage to the uterus. However, in our study, when PMSG was overstimulated for 14 days, Akt activation occurred at the same location as the expression of IGF protein andit seemed to induce the expression of E2-r in the uterine gland. Thus, hCG and PMSG activate IGF/Akt signaling in the stroma and uterine glands, which are the main sections of the endometrium (Zhang *et al*., 2023; Hu *et al*., 2019). However, PMSG was found to have a significantly passive effect on the expression and spread of PAPP-A and the action of MT1-MMP, unlike the hCG and P4 excess treatment groups. Testosterone stimulation has a very different impact on IGF/Akt signaling and PAPP-A, as well as on the morphological development of the uterus formed by hCG, PMSG and P4, as listed above (Cussen *et al*., 2022). Compared to other hormone treatment groups, TES seemed to inhibit the synthesis of PI3K in the IGF/Akt signaling (Plaza-Parrochia *et al*., 2017; Wang *et al*., 2019). However, unlike previous studies, E2-r and FSH-r were expressed in the uterine gland and formed the activity of MT1-MMP in the uterine tissue. Overall, as reported by Lebbe and Woodruff (2013), excessive testosterone stimulation damages the formation of the endometrium, inhibits the formation of mucus distributed in the uterus andnegatively affects the development of the stratum vasculature for embryo implantation and maturation of the uterine gland (Chen *et al*., 2019; Cussen *et al*., 2022).

However, when we synthesized our research results, we found that testosterone maintained the shape of the uterus, similar to other hormone hypersecretions, by expressing each hormone receptor and maintaining the expression of the IGF gene in the myometrial section (Chen *et al*., 2019; Kim *et al*., 2018; 2020). This suggests that testosterone maintains the shape of the uterus through an unknown mechanism in its action of unconditionally shrinking the uterus and inducing death. Our study analyzed the effect of each stimulating hormone excess on the IGF/Akt signaling of the uterus and tissue restructuring and found that excessive stimulation of hCG and P4 resulted in similar tissue changes and that excess TES did not induce unconditional uterine atrophy (Wang *et al*., 2019). This result was similar to that reported by Zhang *et al*. (2023) andit was concluded that the IGF/Akt signaling can monitor the response of the ovary and uterus to hormonal excess, not only in the ovary but also in the uterus. With this being said, he results partly showed that hormonal excess can cause continuous stress to the uterus, which can disrupt the subtle genetic network.

Conclusion

In conclusion, repeated excess hormones can promote apoptosis along with severe morphological damage to the uterus and induce problems for embryo implantation by disrupting the IGF/Akt signaling pathway. Although additional studies are needed to analyze the absolute effect of hormone stimulation through specific submolecular mechanisms, this study concludes that repeated excess hormones can play a role in selecting the activity of IGF or Casp-3 and disrupt the function of the uterus. Therefore, this study provides a basis for investigation of various uterine problems owing to changes in the activation or inactivation of the IGF signaling caused by repeated hormone excess.

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Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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