# The effect of growth hormone on ovarian function recovery in ovarian insufficiency in a mouse model

## 난소부전 마우스 모델에서 성장호르몬에 의한 난소 기능회복 효과

February 2023

Graduate School of Medicine

Seoul National University

Obstetrics and Gynecology major

Su Mi Kim

# The effect of growth hormone on ovarian function recovery in ovarian insufficiency in a mouse model

Advised by Professor Jung Ryeol Lee

Submitting a Ph.D. Dissertation of Medicine

October 2022

Graduate School of Medicine
Seoul National University
Obstetrics and Gynecology major

Su Mi Kim

Confirming the Ph.D. Dissertation written by

Su Mi Kim

January 2023

Chair _____(Seal)

Vice Chair _____(Seal)

Examiner _____(Seal)

Examiner _____(Seal)

Examiner _____(Seal)

# Abstract

# The effect of growth hormone on ovarian function recovery in ovarian insufficiency in a mouse model

Su Mi Kim
Department of Obstetrics and Gynecology
The Graduate School of Medicine
Seoul National University

**Objectives**

The present study was designed to evaluate the effects and the mechanisms of action of growth hormone (GH) on ovarian function recovery in ovarian insufficiency induced by the administration of cyclophosphamide (CP) in a mouse model.

**Materials and Methods**

After inducing ovarian insufficiency by administering a single intraperitoneal injection of 400 mg/kg of CP to six-week-old ICR mice, mice were divided into four groups (control group, CP administration group, GH 1 mg/kg administration group, and GH 2 mg/kg administration group). There were 10 mice in each group. GH was administered a week later for 7 days. Five mice from each group were sacrificed the next day, and their ovaries were collected for histological examination. The remaining mice were superovulated for *in vitro* fertilization (IVF). The following experiments were conducted to explore the mechanism of action of GH on ovarian

function recovery. The terminal deoxynucleotidyl transferase dUTP-nick end labeling (TUNEL) assay was used to detect apoptosis. Masson's trichrome staining was used to analyze the degree of fibrosis. To quantify angiogenesis, CD31 immunohistochemistry was performed. Angiogenesis-related gene expression profile was assessed using quantitative reverse transcription polymerase chain reaction (RT-qPCR).

**Results**

Administration of CP induced the loss of non-growing (primordial and primary) follicles. GH significantly protected primordial follicles and increased follicular quality. The CP group showed a decrease in fertilization rate and blastocyst formation rate in IVF. In contrast, the GH treatment group enhanced IVF outcomes in a dose-dependent manner. GH treatment also decreased apoptosis, stromal fibrosis, and increased angiogenesis. Many genes involved in angiogenesis are upregulated, especially the upregulation of leptin (*Lep*), platelet endothelial cell adhesion molecule 1 (*Pecam-1*), and angiogenin (*Ang*) have been observed in GH treatment groups.

**Conclusion**

GH treatment may promote ovarian function recovery in ovarian insufficiency induced by administration of CP via decreasing apoptosis, stromal fibrosis, and upregulation of *Lep*, *Pecam-1*, and *Ang* genes involved in angiogenesis.

Keywords: Growth hormone, Ovarian insufficiency, Cyclophosphamide, Breast cancer, Ovarian regeneration, Fertility preservation

*Student Number*: 2018-37972

# Contents

# List of Tables

# List of Figures

# List of Supplementary Figures

# 1. Introduction

## 1.1. Background

Breast cancer is the most commonly diagnosed cancer in women worldwide (1), and approximately 10% of breast cancer cases occur in women younger than 45 years (2). With the improved survival rate among breast cancer patients, the demand for improving the quality of life is also increasing. Fertility preservation is one of the important issues for breast cancer survivors (3). Considering the rising trend in the postponement of marriage and childbearing, the demand for fertility preservation among breast cancer survivors is expected to increase.

Cyclophosphamide (CP) is an alkylating agent widely used as adjuvant chemotherapy for breast cancer (4). CP is ranked as a high risk of gonadotoxicity as it can cause apoptosis, overactivation of primordial follicles, and atresia of growing follicles (5). Administration of CP could induce premature ovarian insufficiency, and it causes not only infertility but cognitive dysfunction, osteopenia, and cardiovascular disease (6).

Oocyte and embryo cryopreservation are considered the standard practice of fertility preservation, but those methods cannot recover the ovarian endocrine function (7). Ovarian tissue cryopreservation is an option that could resume the ovarian endocrine function (8), but there is concern about the possibility of re-entrance of tumor cells after transplantation of cryopreserved tissue in cancer patients (9). Those cryopreservation methods are not feasible in places where assisted reproductive centers do not exist. Gonadotropin-releasing hormone agonists (GnRHa) could be offered to young women with breast cancer when proven fertility preservation

methods are not feasible. The evidence for using GnRHa to preserve function is conflicting, so it cannot replace established methods (10). Research on therapeutic agents to restore damaged ovarian function has been conducted recently. A representative therapeutic agent is a stem cell. However, the safety of stem cells has not yet been proven, and the methods for injecting stem cells are not standardized (11).

Growth hormone (GH) is a monomeric peptide hormone secreted mainly by the anterior part of the pituitary gland (12). In the classical view of GH, pituitary GH acts on its hepatic receptors to produce hepatic insulin-like growth hormone I (IGF-I). However, this view has been revised because not only the liver but also the peripheral organs, including the ovary, can produce IGF-I by GH stimulation. Moreover, it has been found that reproductive cells can produce GH itself to control the signaling pathway by paracrine or autocrine actions (13). GH plays essential roles in cell growth, development, and metabolism throughout the body. In female reproduction, GH is required for the onset of puberty, follicular development, steroidogenesis, and oogenesis in the ovary (14). Since GH is involved in the regulation of female infertility, GH as adjuvant therapy in *in vitro* fertilization (IVF) has received the most attention, especially in patients with a poor ovarian response (POR) (15). In a recent systematic review and meta-analysis, GH supplementation to POR increased clinical pregnancy rate, number of oocytes retrieved, number of MII oocytes, and live birth rate (16, 17).

GH has already been administered during IVF clinically, so it seems safer than other therapeutic agents, such as stem cell therapy; however, it is unclear what effect it has in restoring ovarian function. Only a few studies were conducted to explore the effects of GH on ovarian function recovery. Mahran et al. demonstrated that GH

showed a radioprotective effect and rescued ovarian function (18). Liu et al. showed that GH promoted ovarian tissue repair, estrogen release, and oocyte maturation via activation of the Notch-1 signaling pathway (19). Nevertheless, little information was found on the mechanisms by which GH restores ovarian function. It is necessary to evaluate the effects and the mechanisms of GH on ovarian function recovery more diversely.

## 1.2. Objectives

The present study is designed to evaluate the effects and mechanisms of action of GH on ovarian function recovery in ovarian insufficiency induced by administration of CP in a mouse model.

# 2. Materials and Methods

## 2.1 Animals

The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (BA-2009-304-087-01). Six-week-old female ICR mice (Orient Bio Inc., South Korea) were acclimated for one week before experimentation and housed in ventilated cages at room temperature, humidity, and a light-controlled environment with 12-hour light-dark cycles. They were provided food and water ad libitum.

## 2.2 Study Design

**Figure 1** shows the experimental scheme of the present study. Cyclophosphamide (CP) (Sigma-Aldrich, Burlington, MA, USA) was administered to induce ovarian insufficiency (OI). A preliminary study was conducted to determine the CP dose to be administered (**Supplementary Figures 1~3**). The mice were divided into three groups (4 mice per group) as follows: 1) control group: mice only receiving 200 μL of 0.9% normal saline injection; 2) low-dose CP group: mice receiving 400 mg/kg of CP by a single intraperitoneal injection; 3) high-dose CP group: mice receiving 400 mg/kg of CP by an intraperitoneal injection every two days for three times. A total of 1200 mg/kg of CP was injected. Three days after the last injection, ovaries were collected to perform hematoxylin and eosin (H&E) staining, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, and Masson's trichrome staining. The high-dose CP group showed a high mortality rate (50%). Furthermore, the primordial follicle damage (**Supplementary Figure 1**), apoptosis rate (**Supplementary Figure 2**), and fibrosis rate (**Supplementary Figure 3**) were

too high to be analyzed. We concluded that the low-dose CP group achieved the desired degree of ovarian damage with no harm to animal life but the deterioration of ovarian function. The mice were randomly divided into four groups (10 mice per group) as follows: 1) control group: mice only receiving 0.9% normal saline injection 200 μL; 2) CP group: OI mice receiving 200 μL of 0.9% normal saline injection; 3) low-dose GH treatment group: OI mice injected with 1 mg/kg GH (Growtropin, DONG-A ST, Seoul, Korea); 4) high-dose GH treatment group: OI mice injected with 2mg/kg GH. Normal saline or GH was administered intraperitoneally a week after induction of OI for 7 days. The dose (18, 19) and route of GH administration (20, 21) were determined after reviewing published studies. Five mice from each group were utilized for histological examination, and five mice per group were superovulated for IVF. Subsequently, all mice were sacrificed a day after the last injection of GH.

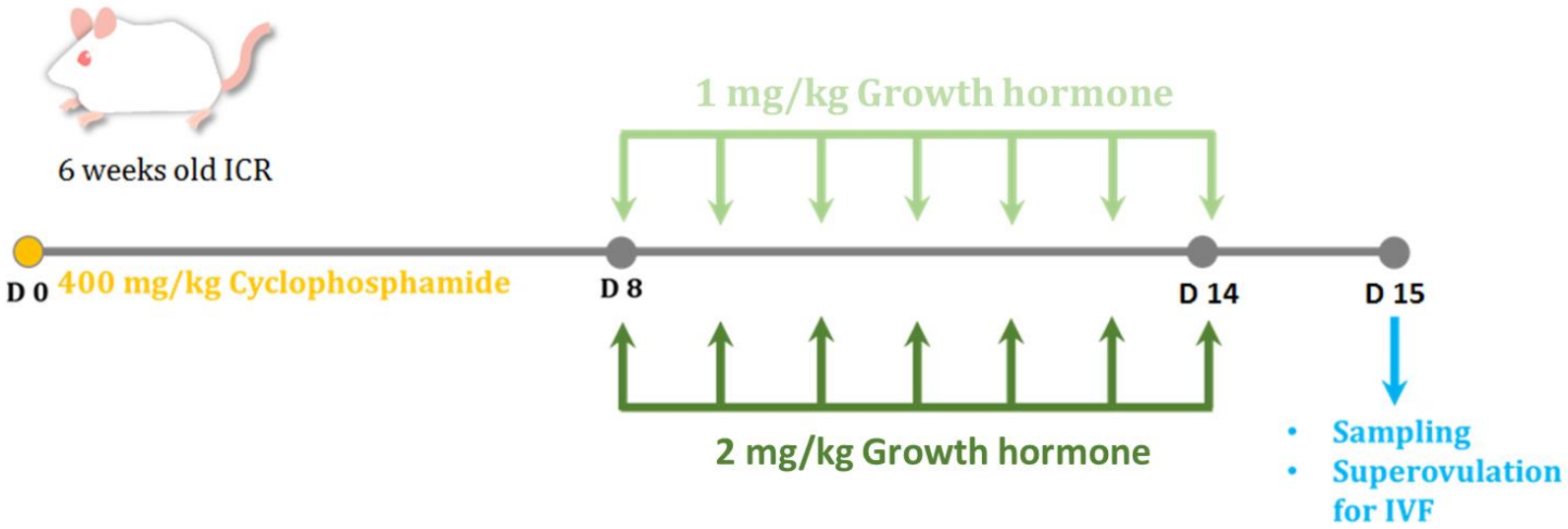



**Figure 1.** The experimental scheme for the present research. To induce ovarian insufficiency (OI), 400mg/kg of cyclophosphamide (CP) was administered by a single intraperitoneal injection. The mice were randomly divided into four groups: the control, CP, GH 1 mg/kg treated, and GH 2 mg/kg treated groups. Normal saline or GH was administered for a week after induction of OI for 7 days. All mice were sacrificed a day after the last injection of GH.

## 2.3 Histological analysis

Ovaries were fixed in formalin and embedded with paraffin. To count the number of follicles and evaluate follicular development and quality, serial sections at 5 μm thickness were stained with H&E solution (Cancer Diagnostics, Durham, NC, USA) and visualized by light microscopy (Nikon, Tokyo, Japan). The total number of follicles less than 48 μm in diameter was counted in at least eight selected non-overlapping fields. The developmental stages of follicles were classified by the following categories (22): (1) primordial follicles: a single layer of flattened granulosa cells; (2) primary follicles: a single layer of granulosa cells, one or more of which are being cuboidal; (3) secondary follicles: two or more layers of cuboidal granulosa cells lacking an antral space; (4) antral follicles: multiple layers of cuboidal granulosa cells with the antrum present. The follicular quality was assessed by morphological criteria as follows (23, 24): (1) primordial and primary follicles: grade 1, spherical with even distribution of granulosa cells; grade 2, granulosa cells pulled away from the edge of the follicle but with the oocyte still spherical; grade 3, granulosa cells with pyknotic nuclei and misshapen oocyte; (2) secondary and antral follicles: grade 1, evenly distributed granulosa and theca cells with small spaces between cells and a spherical oocyte; grade 2, intact theca cells, apparent loss of granulosa cells but with a spherical oocyte; grade 3, disrupted granulosa cells with theca cells pulled away from the edge with pyknotic nuclei and misshapen vacuolated oocyte.

## 2.4 In Vitro Fertilization

Five mice from each group were hyperstimulated by intraperitoneal injection of 7.5 IU pregnant mare's serum gonadotropin, followed by 7.5 IU human chorionic gonadotropin after 48 hours. After 16 hours, the mice were sacrificed, and cumulus-oocytes complexes (COCs) were harvested by fallopian tube puncture. The epididymal sperm were retrieved from 10-week-old male ICR mice, and the sperm suspensions were incubated at 37℃ in humidified 5% $CO_2$ in air for an hour. The metaphase II oocytes were inseminated with sperms and incubated at 37℃ in humidified 5% $CO_2$ in air for 4 hours. Inseminated oocytes were washed with HTF medium (FUJIFILM Irvine Scientific, Santa Ana, CA, USA) and incubated in a fresh HTF medium. Fertilization was assessed by the formation of two cells 24 hours after insemination. Then, cleaved embryos were transferred to KSOM media (MilliporeSigma, Burlington, MA, USA). The blastocyst development was assessed 72 hours after insemination.

## 2.5 Analysis of follicular apoptosis

Apoptosis of the ovarian follicles was analyzed using the TUNEL assay (Promega, Madison, WI, USA). Briefly, after deparaffinization and rehydration, the slides were briefly treated with proteinase K solution (Promega) for 20 minutes at room temperature, followed by incubation with a TUNEL reaction mixture for an hour at 37℃. After washing with D-PBS, the slides were mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI, ImmunoBioSience Corp., Mukilteo, WA, USA) and examined under an inverted Zeiss AX10 microscope. Apoptotic cells were visualized as green fluorescence and DAPI-stained

cells were visualized as blue fluorescence. Follicles containing more than 30% apoptotic cells were regarded as apoptotic follicles (25). Quantification of the apoptotic area was analyzed by the Image J software (National Institutes of Health, Bethesda, MD, USA).

## 2.6 Analysis of follicular fibrosis

Masson's trichrome staining was performed to analyze follicular fibrosis using the Roche Trichrome III Blue Staining Kit (Roche, Basel, Switzerland). After deparaffinization and rehydration, the slides were treated with Bouin solution (Sigma-Aldrich) for an hour at 60℃. After washing with distilled water, the slides were stained in Weigert iron Hematoxylin solution for 10 minutes. After washing with distilled water, the slides were placed in Biebrich-Scarlet Acid fuchsin solution for 5 minutes. After washing with distilled water, the slides were placed in Aniline Blue Solution for 8 minutes. After washing with distilled water, the slides were treated with 0.5% acetic acid for a minute. The slides were dehydrated in ethanol and treated with xylene for a minute. Finally, the slides were mounted with the Mounting Medium (Dako). Stained slides were analyzed using the Image J software (National Institutes of Health).

## 2.7 Immunohistochemistry for CD 31

The density of the blood vessel was evaluated by immunohistochemistry with anti-CD 31 antibodies. After deparaffinization and rehydration, target antigen retrieval was performed using citrate buffer. The slides were put in the microwave oven and heated for 20 minutes at 700W, followed by cooling for 30 minutes at room

temperature. The slides were placed in the peroxidase-blocking solution for 10 minutes and incubated overnight at 4 ℃ with anti-31 antibody (1:200 dilution, Abcam, Cambridge, UK). After washing, the slides were treated with EnVision+HRP solution (Dako) for 60 minutes and substrate-chromogen solution (1:50, Dako) for 10 minutes. The slides were counterstained with hematoxylin and dehydrated with ethanol and xylene. The CD31- positive areas were determined using the Image J software (National Institutes of Health).

## 2.8 Western blot for CD 31

Protein samples obtained from mouse ovaries were incubated with PRO-PREP™ Protein extraction solution (iNtRON Biotechnology, Seongnam, South Korea). Twenty micrograms of proteins were separated by 10 % SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a Mini Trans-Blot cell and criterion blotter (Bio-rad, Hercules, CA, USA). After blocking in 5% scheme milk in 1x TBST with 0.1% Tween 20 (Sigma-Aldrich) at room temperature for 1 hour, the blots were probed at 4°C overnight with primary antibodies 1:300 dilution of anti-CD 31 (BS-0195R; Bioss, Woburn, MA, USA) and 1:1000 dilution of anti-β-tubulin (s2128; Cell Signaling Technology, Danvers, MA, USA). Secondary incubation of a 1:2000 dilution of mouse anti-rabbit IgG (sc-2357; Santa Cruz Biotechnology, Dallas, TX, USA) was performed at room temperature for 1 hour. Band intensity was quantified using Image J and normalized by β-tubulin.

## 2.9 Quantitative reverse transcription polymerase chain reaction and data analysis

Angiogenesis-related gene expression profile was assessed using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Total RNA was extracted from the ovarian tissues using a TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA synthesis and the subsequent real-time PCR were carried out using AccuTarget™ qPCR Screening Kit (Bioneer, Daejeon, South Korea). The real-time PCR reaction contained a final volume of 20 μL, 1 μg reverse-transcribed total RNA, and a 2× PCR master mix. All qRT-PCR amplification and analyses were conducted using an Accupower qPCR premix kit in an Exicycler 96 real-time quantitative thermal block (Bioneer). The PCR conditions were as follows: initial denaturation of the template DNA at 95°C for 10 minutes, followed by 40 cycles of amplification of the template DNA, in which each cycle consisted of denaturation at 95°C for 5 seconds and primer annealing and extension at 58°C for 25 seconds. All reactions were done in triplicate. The details of the analyzed genes are listed in **Table 1**. Data analysis was based on the relative quantitative method, and the ΔΔCT value was used to determine the relative fold change in expression. Scatterplots of the mRNA expressions that were up or down-regulated by 1-fold compared to the control group were created. Heatmap results were generated using the R program. All the data were normalized to the reference beta-actin (ACTB) gene expression level.

## 2.10 Statistical analysis

The data were presented as mean ± standard error of the mean (SEM). Multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's methods as a post-hoc test by GraphPad Prism, version 8.4.0 (Graph-Pad Software Inc., San Diego, CA, USA). $P$ <0.05 was considered statistically significant.

**Table 1.** List of genes for Real-time Polymerase Chain Reaction

| Gene Symbol | Gene ID (NCBI) | Gene full name |
|---|---|---|
| Adgrb1 | 107831 | adhesion G protein-coupled receptor B1 |
| Akt1 | 11651 | thymoma viral proto-oncogene 1 |
| Ang | 11727 | angiogenin |
| Angpt1 | 11600 | angiopoietin 1 |
| Angpt2 | 26360 | angiopoietin 2 |
| Anpep | 16790 | Alanyl aminopeptadase, membrane |
| Ccl11 | 20292 | chemokine (C-C motif) ligand 11 |
| Ccl2 | 20296 | chemokine (C-C motif) ligand 2 |
| Cdh5 | 12562 | cadherin 5 |
| Col18a1 | 12822 | collagen, type XVIII, alpha 1 |
| Col4a3 | 12828 | collagen, type IV, alpha 3 |
| Csf3 | 12985 | colony stimulating factor 3 (granulocyte) |
| Ctgf | 14219 | cellular communication network factor 2 |
| Cxcl1 | 14825 | chemokine (C-X-C motif) ligand 1 |
| Cxcl2 | 20310 | chemokine (C-X-C motif) ligand 2 |
| Cxcl5 | 20311 | chemokine (C-X-C motif) ligand 5 |
| Edn1 | 13614 | endothelin 1 |
| Efna1 | 13636 | ephrin A1 |
| Efnb2 | 13642 | ephrin B2 |
| Egf | 13645 | epidermal growth factor |
| Eng | 13805 | endoglin |
| Epas1 | 13819 | endothelial PAS domain protein 1 |
| Ephb4 | 13846 | Eph receptor B4 |
| Erbb2 | 13866 | erb-b2 receptor tyrosine kinase 2 |
| F2 | 14061 | coagulation factor II |
| F3 | 14066 | coagulation factor III |
| Fgf2 | 14173 | fibroblast growth factor 2 |
| Fgf6 | 14177 | fibroblast growth factor 6 |

| | | |
|---|---|---|
| Fgf1 | 14164 | Fibroblast growth factor 1 |
| Fgfr3 | 14184 | fibroblast growth factor receptor 3 |
| Figf | 14205 | vascular endothelial growth factor D |
| Flt1 | 14254 | fms related receptor tyrosine kinase 1 |
| Fn1 | 14268 | fibronectin 1 |
| Hgf | 15234 | hepatocyte growth factor |
| Hif1a | 15251 | hypoxia inducible factor 1, alpha subunit |
| Ifng | 15978 | interferon gamma |
| Igf1 | 16000 | insulin-like growth factor 1 |
| Il1b | 16176 | Interleukin 1 beta |
| Il6 | 16193 | Interleukin 6 |
| Itgav | 16410 | integrin alpha V |
| Itgb3 | 16416 | integrin beta 3 |
| Jag1 | 16449 | jagged 1 |
| Kdr | 16542 | kinase insert domain protein receptor |
| Lect1 | 16840 | chondromodulin |
| Lep | 16846 | leptin |
| Mapk14 | 26416 | mitogen-activated protein kinase 14 |
| Mdk | 17242 | midkine |
| Mmp14 | 17387 | matrix metallopeptidase 14 (membrane-inserted) |
| Mmp19 | 58223 | matrix metallopeptidase 19 |
| Mmp2 | 17390 | matrix metallopeptidase 2 |
| Mmp9 | 17395 | matrix metallopeptidase 9 |
| Nos3 | 18127 | nitric oxide synthase 3, endothelial cell |
| Nrp1 | 18186 | neuropilin 1 |
| Nrp2 | 18187 | neuropilin 2 |
| Pdgfa | 18590 | platelet derived growth factor, alpha |
| Pecam1 | 18613 | platelet/endothelial cell adhesion molecule 1 |
| Pgf | 18654 | Placenta growth factor |
| Plau | 18792 | plasminogen activator, urokinase |
| Plg | 18815 | plasminogen |
| Ptgs1 | 19224 | prostaglandin-endoperoxide synthase 1 |

| | | |
|---|---|---|
| Ptk2 | 14083 | protein tyrosine kinase 2 |
| S1pr1 | 13609 | sphingosine-1-phosphate receptor 1 |
| Serpine1 | 18787 | serine (or cysteine) peptidase inhibitor, clade E, member 1 |
| Serpinf1 | 20317 | serine (or cysteine) peptidase inhibitor, clade F, member 1 |
| Smad5 | 17129 | SMAD family member 5 |
| Sphk1 | 20698 | Sphingosine kinase 1 |
| Tbx1 | 21380 | T-box 1 |
| Tek | 21687 | TEK receptor tyrosine kinase |
| Tgfa | 21802 | transforming growth factor alpha |
| Tgfb1 | 21803 | transforming growth factor, beta 1 |
| Tgfb2 | 21808 | transforming growth factor, beta 2 |
| Tgfb3 | 21809 | transforming growth factor, beta 3 |
| Tgfbr1 | 21812 | transforming growth factor, beta receptor I |
| Thbs1 | 21825 | thrombospondin 1 |
| Thbs2 | 21826 | thrombospondin 2 |
| Tie1 | 21846 | tyrosine kinase with immunoglobulin-like and EGF-like domains 1 |
| Timp1 | 21857 | tissue inhibitor of metalloproteinase 1 |
| Timp2 | 21858 | tissue inhibitor of metalloproteinase 2 |
| Tnf | 21926 | Tumor necrosis factor |
| Tnfsf12 | 21944 | tumor necrosis factor (ligand) superfamily, member 12 |
| Tymp | 72962 | thymidine phosphorylase |
| Vegfa | 22339 | vascular endothelial growth factor A |
| Vegfb | 22340 | vascular endothelial growth factor B |
| Vegfc | 22341 | vascular endothelial growth factor C |

# 3. Results

## 3.1 Gross observation and ovarian weight changes

A day after the GH injection, ovaries were harvested, and the weight of the ovaries was determined. When grossly observed, the size of ovaries appeared to be increased in the GH-treated group when compared to the CP group (**Figure 2A**). As indicated in **Figure 2B**, ovarian weight was significantly decreased after CP administration. In the GH-treated group, ovarian weight increased but did not reach statistical significance.

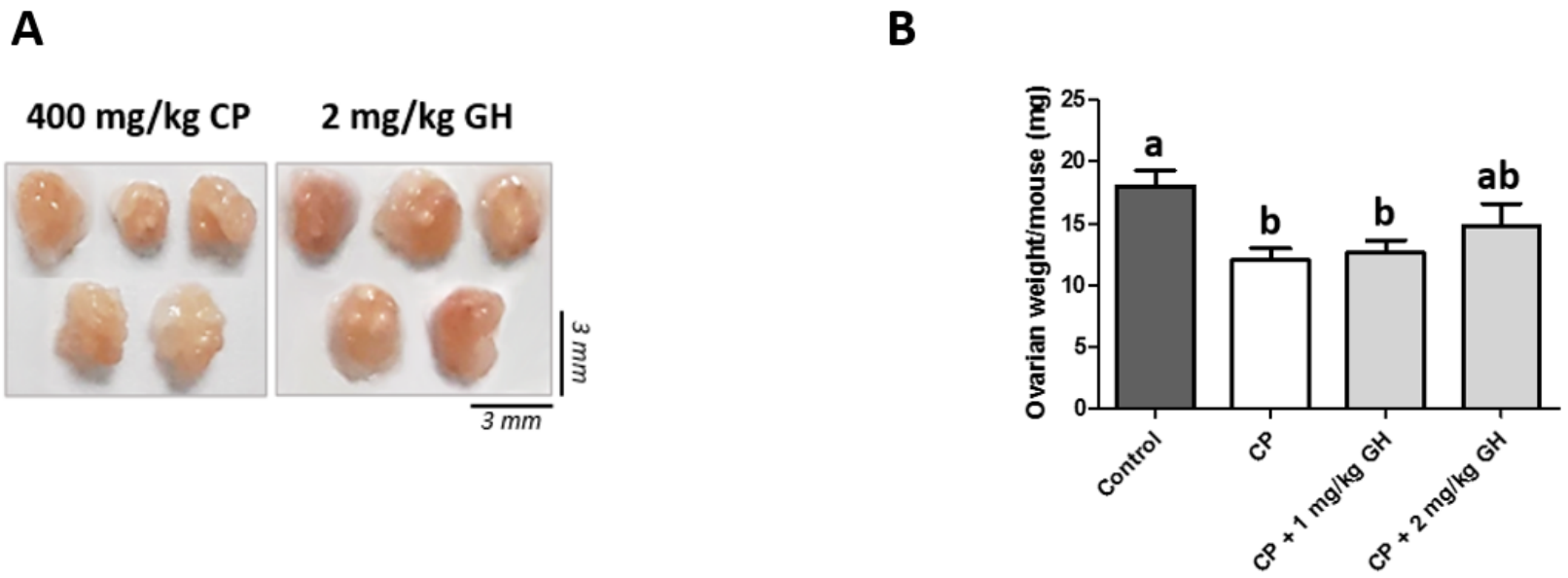



**Figure 2.** (A) The gross appearance of the ovaries. (B) The average ovarian weight. Graphs are presented as mean ± SEM, and different superscript letters indicate statistically significant differences ($p < 0.05$). CP: cyclophosphamide; GH: growth hormone.

## 3.2 Evaluation of ovarian follicle morphology

The number and structural quality of follicles were evaluated using H&E stained ovaries (**Figure 3**). The stromal density and normality of ovarian follicles were decreased in the CP group. However, the density of stromal cells in GH-treated groups appeared higher than that in the CP group, and relatively high-graded follicles were observed in the GH-treated groups. The total number of follicles and grade 1 (G1) follicles decreased in the CP and GH-treated groups compared to the control group (**Figure 4A, B**). A statistical significance was not observed in the total number of follicles between the CP and GH-treated groups (**Figure 4A**), but the number of G1 follicles increased significantly in the GH 2 mg/kg treated group than in the CP group ($p$ <0.05) (**Figure 4B**). **Figure 4C** represents the mean G1 follicle number according to the follicular development stages. The primordial and primary follicles were dramatically decreased after CP administration ($p$ <0.05), indicating that CP induces non-growing follicle loss. The proportion of G1 primordial follicles was significantly increased in the GH 2 mg/kg treated group compared to the CP group. The G1 follicle ratio was increased in the primary and secondary follicles in the GH-treated groups compared to the CP group, but with no statistical significance. Thus, GH treatment prevented primordial follicle loss induced by CP administration and improved the follicular quality.

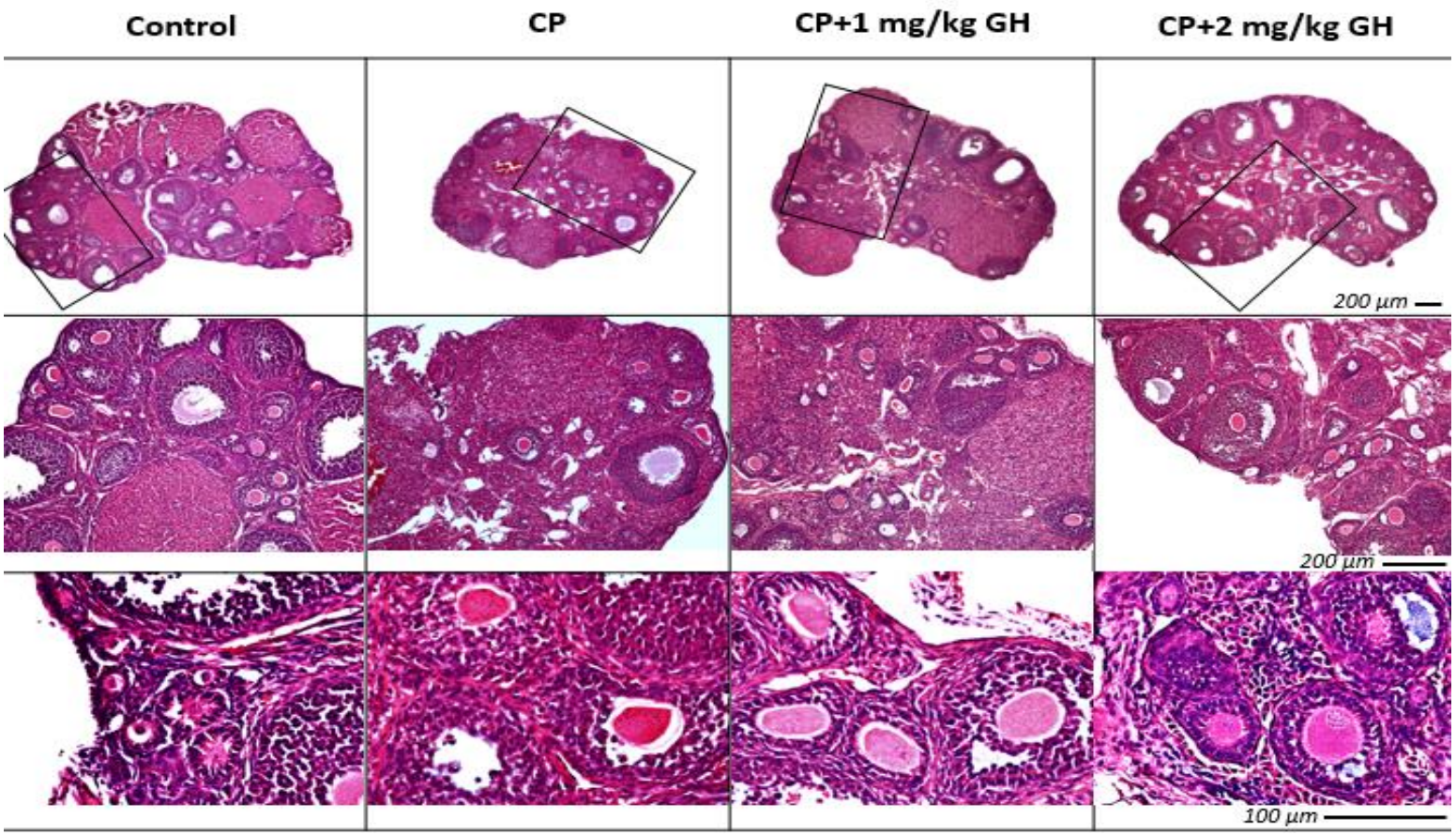



**Figure 3.** Representative images of hematoxylin and eosin-stained ovarian tissue. CP: cyclophosphamide; GH: growth hormone.

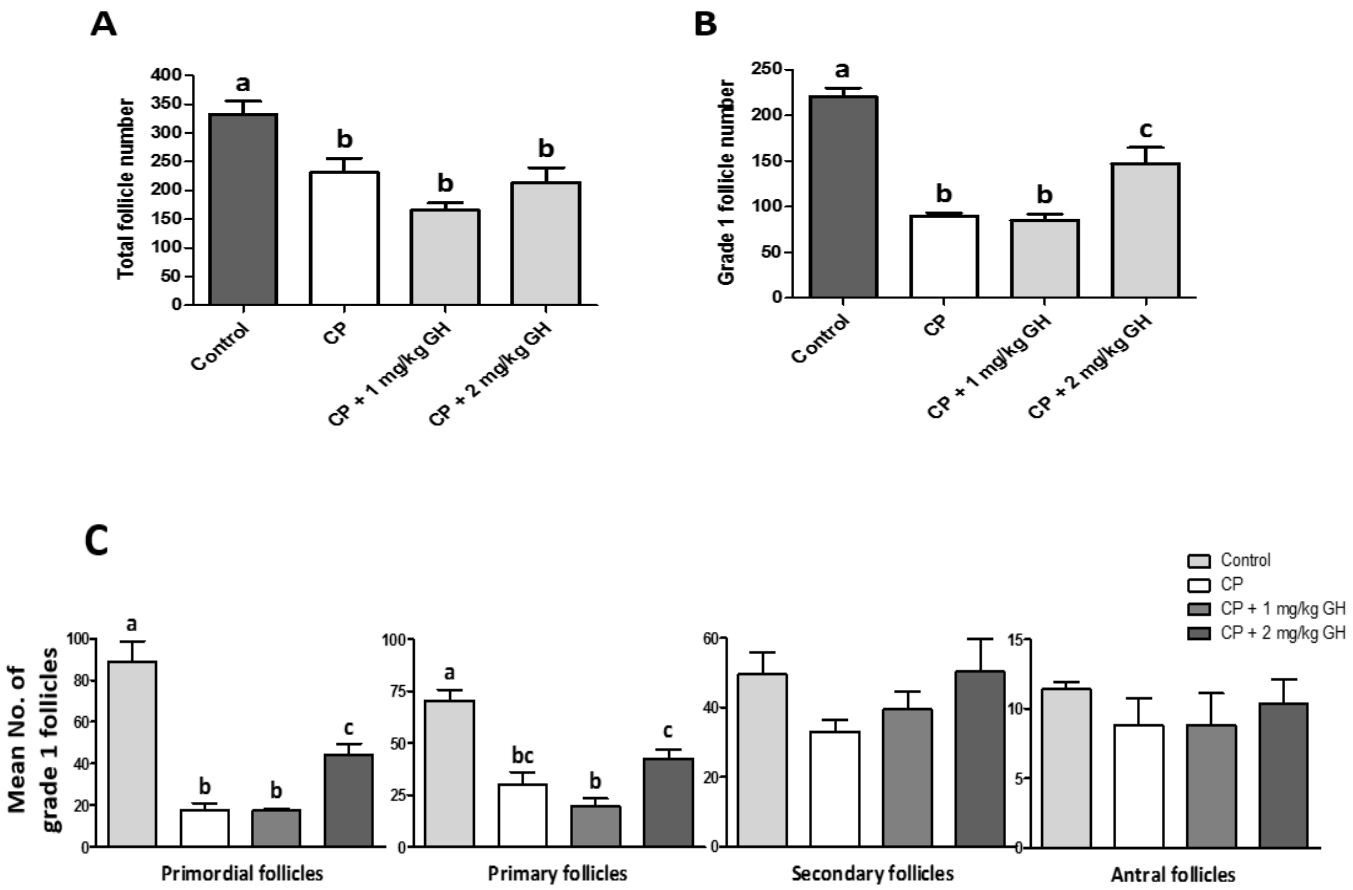

A
Total follicle number
400
350
300
250
200
150
100
50
0
a
b
b
b
Control
CP
CP + 1 mg/kg GH
CP + 2 mg/kg GH
B
Grade 1 follicle number
250
200
150
100
50
0
a
b
b
c
Control
CP
CP + 1 mg/kg GH
CP + 2 mg/kg GH
C
Mean No. of grade 1 follicles
Control
CP
CP + 1 mg/kg GH
CP + 2 mg/kg GH
Primordial follicles
Primary follicles
Secondary follicles
Antral follicles

**Figure 4**. Histological analysis of ovaries. (A) Total follicle number (B) Grade 1 follicle number, and (C) Mean numbers of grade 1 follicles according to the follicular development stages of ovarian tissue. Graphs are presented as mean ± SEM; n=5 and different superscript letters indicate statistically significant differences ($p < 0.05$). CP: cyclophosphamide; GH: growth hormone.

## 3.3 *In Vitro* Fertilization (IVF)

**Table 2** shows data for IVF. MII oocyte rate was 88.9% in the control group but declined to 78.8% in the CP group. It increased to 81.1% and 89.4% in the GH 1 mg/kg and GH 2 mg/kg treated groups, respectively. After insemination, the fertilization rate (81.7%) and blastocyst formation rate (26.9%) also decreased in the CP group, whereas those values were 88.3% and 81.3% in the control group. The fertilization and blastocyst formation rates were increased in the GH-treated groups: 91.0% and 40.5% in the GH 1 mg/kg treated group and 92.3% and 50.0% in the GH 2 mg/kg treated group. The blastocyst formation rate was far more decreased in the CP group, but it increased in the GH-treated group in a dose-dependent manner.

**Table 2. IVF outcomes with the control group, CP group, and GH-treated groups.**

| Group | No. of mouse | No. of normal oocyte | Mean No. of normal oocyte | No. of MII oocyte | Maturation rate (%) | Fertilization rate (%) | Blastocyst formation rate (%) |
|---|---|---|---|---|---|---|---|
| Control | 5 | 135 | 27 | 120 | 88.9 | 88.3 | 81.3 |
| CP | 5 | 104 | 20.8 | 82 | 78.8 | 81.7 | 26.9 |
| CP+GH 1mg/kg | 5 | 164 | 32.8 | 133 | 81.1 | 91.0 | 40.5 |
| CP+ GH 2mg/kg | 5 | 160 | 32 | 143 | 89.4 | 92.3 | 50.0 |

CP: cyclophosphamide; GH: growth hormone.

## 3.4 Evaluation of apoptosis

**Figure 5A** shows a representative image of TUNEL-positive areas in the ovary of each group. Apoptosis was significantly increased in the CP groups and remarkably decreased in the GH-treated group in a dose-dependent manner (**Figure 5B**).

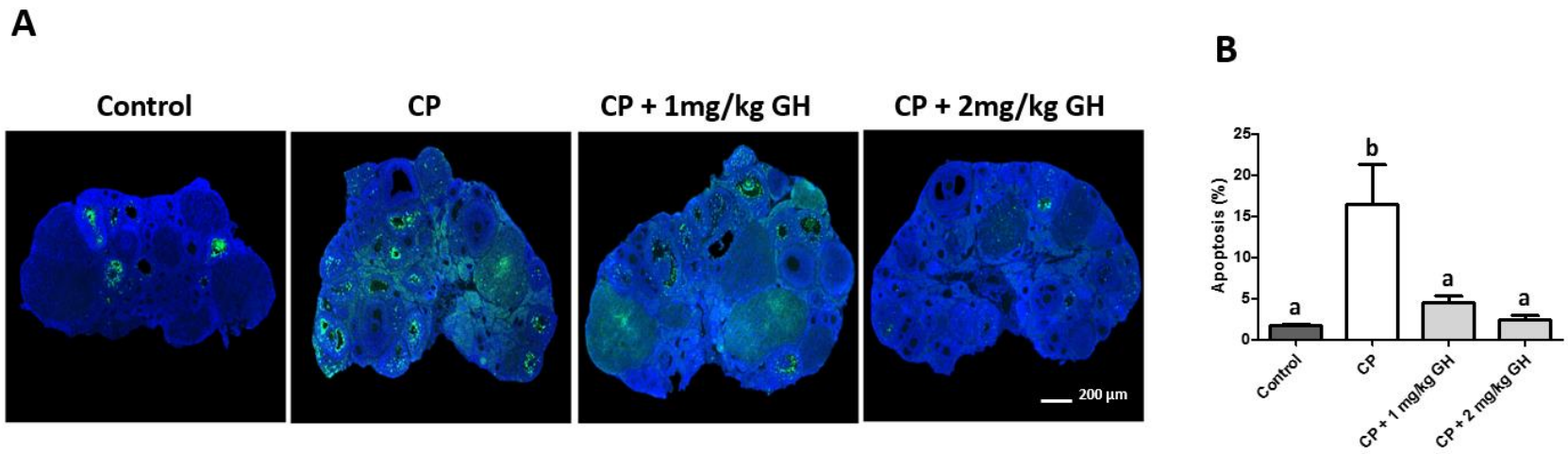



**Figure 5.** Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. (A) Green fluorescence indicates apoptotic cells and blue fluorescence (DAPI) indicates intact cell nuclei. (B) Quantification of the TUNEL positive area. Graphs are presented as mean ± SEM; n=5 and different superscript letters indicate statistically significant differences ($p < 0.05$). DAPI: 4',6-diamidino-2-phenylindole CP: cyclophosphamide; GH: growth hormone.

## 3.5 Evaluation of fibrosis and angiogenesis

**Figure 6A** shows the representative image of Masson's trichrome stain. This staining is used to detect collagen fibers and is widely used to measure tissue fibrosis (26). The CP group shows an extensive fibrotic area, and its proportion was significantly reduced in the GH-treated groups (**Figure 6B**).

A representative figure of CD-31 immunostained ovaries is given in Figure 7A. CD31 is expressed on the surface of endothelial cells and is well established as the maker of vessel density (27). The CD-31 positive area was significantly decreased in the CP group compared to the control group (**Figure 7B**). The CD-31 positive area was increased in the GH-treated group with significant differences compared to the CP group, and there were no significant differences from that of the control group, indicating restoration of blood vessel density as much as the control group (**Figure 7B**).

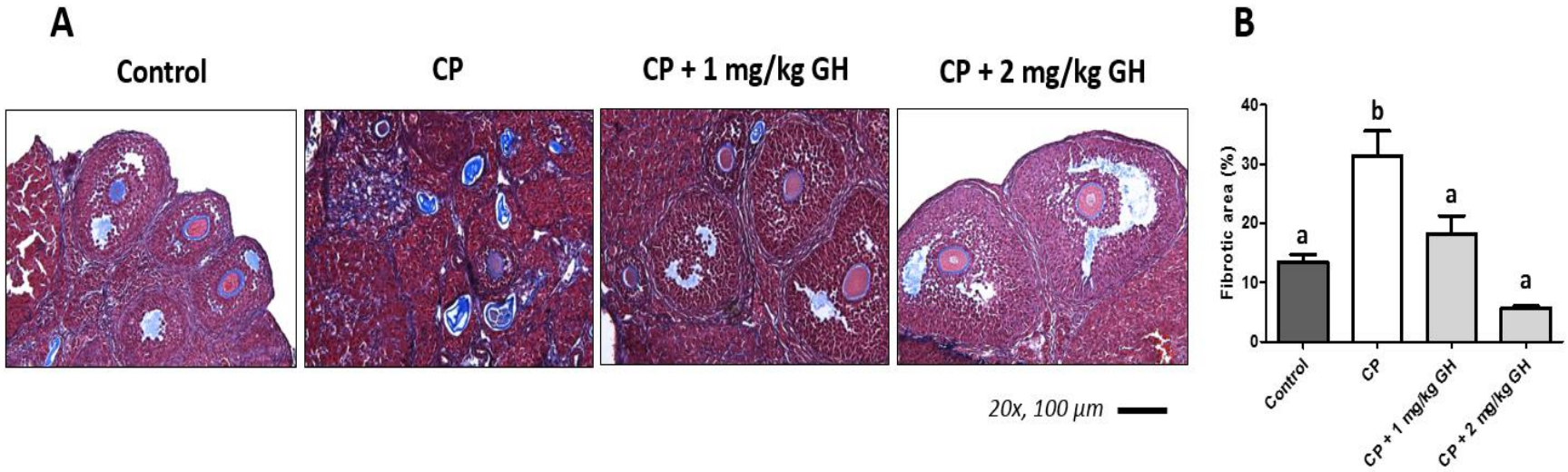



**Figure 6.** Masson's trichrome stain. (A) The fibrotic surface, nuclei, and cytoplasm were stained blue, black, and red, respectively. (B) Quantification of the fibrotic area. Graphs are presented as mean ± SEM; n=5 and different superscript letters indicate statistically significant differences ($p < 0.05$). CP: cyclophosphamide; GH: growth hormone.

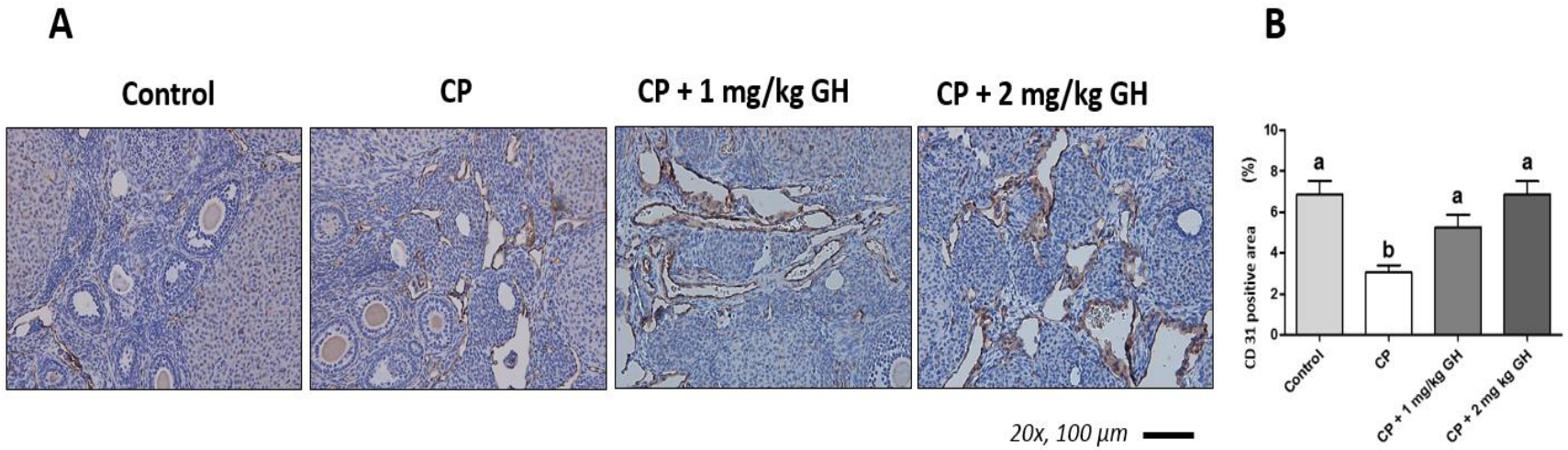
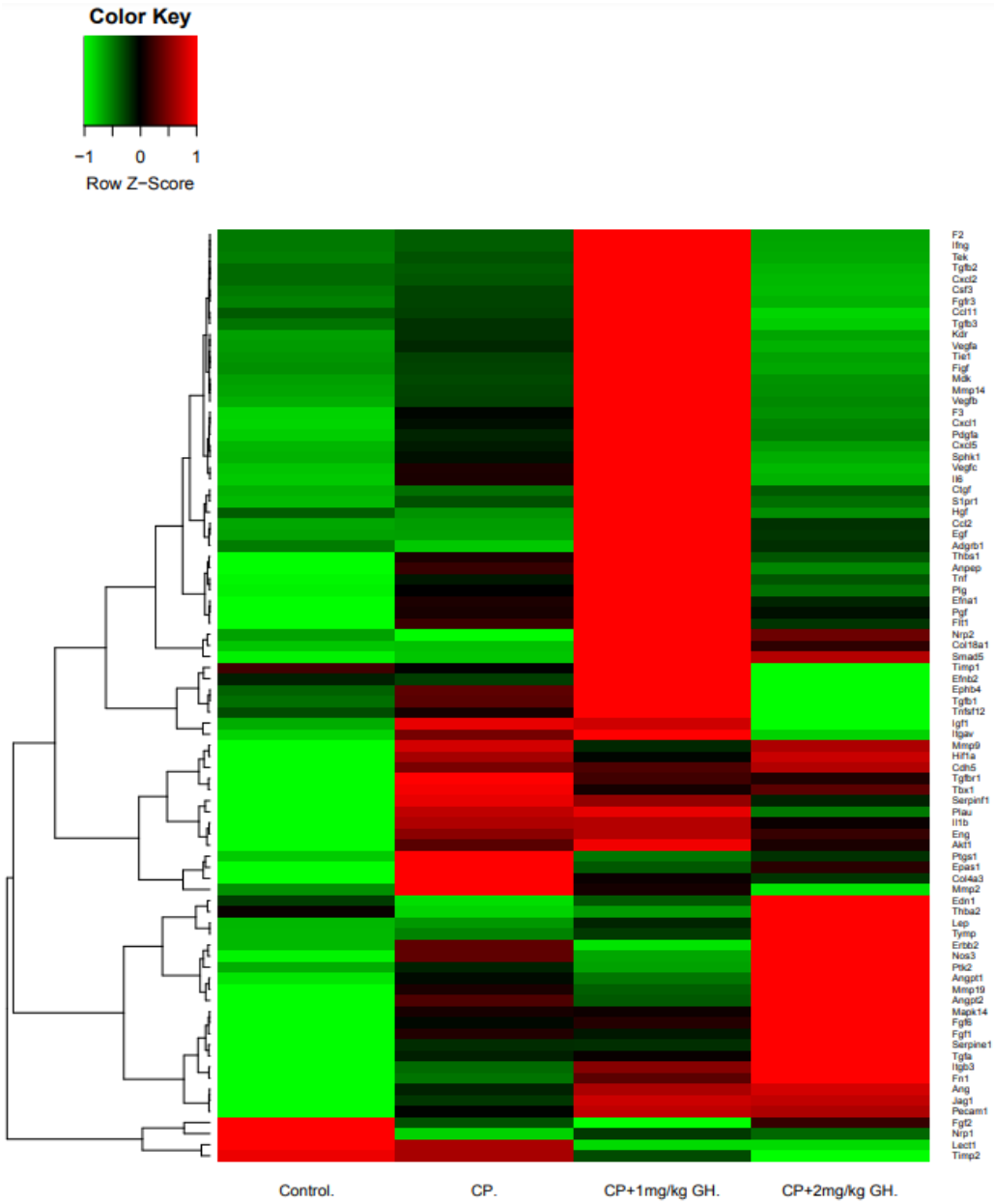



**Figure 7.** Immunohistochemical staining of ovarian tissue with CD 31. (A) Brown-stained cells are CD 31-positive cells. (B) Quantification of CD 31-positive area in ovarian tissues. Graphs are presented as mean ± SEM; n=5 and different superscript letters indicate statistically significant differences ($p < 0.05$). CP: cyclophosphamide; GH: growth hormone.

## 3.6 Angiogenesis-related gene expression

**Figure 8** shows the expression of 84 angiogenesis-related genes compared to the control group. **Table 3** demonstrates up-regulated genes with a > 4-fold change. In the GH-treated groups, three genes were up-regulated with a > 4-fold change, as shown in **Table 4** and **Figure 9** [leptin (*Lep*) (**Figure 9A**), platelet endothelial cell adhesion molecule 1 (*Pecam-1*) (**Figure 9B**), and angiogenin (*Ang*) (**Figure 9C**)].

**A**


Color Key
−1
0
1
Row Z−Score
F2
Ifng
Tek
Tgfb2
Cxcl2
Csf3
Fgfr3
Ccl11
Tgfb3
Kdr
Vegfa
Tie1
Figf
Mdk
Mmp14
Vegfb
F3
Cxcl1
Pdgfa
Cxcl5
Sphk1
Vegfc
Il6
Ctgf
S1pr1
Hgf
Ccl2
Egf
Adgrb1
Thbs1
Anpep
Tnf
Ptg
Efna1
Pgf
Flt1
Nrp2
Col18a1
Smad5
Timp1
Efnb2
Ephb4
Tgfb1
Tnfsf12
Igf1
Itgav
Mmp9
Hif1a
Cdh5
Tgfbr1
Tbx1
Serpinf1
Ptau
Il1b
Eng
Akt1
Ptgs1
Epas1
Col4a3
Mmp2
Edn1
Thba2
Lep
Tymp
Erbb2
Nos3
Ptk2
Angpt1
Mmp19
Angpt2
Mapk14
Fgf6
Fgf1
Serpine1
Tgfa
Itgb3
Fn1
Ang
Jag1
Pecam1
Fgf2
Nrp1
Lect1
Timp2
Control.
CP.
CP+1mg/kg GH.
CP+2mg/kg GH.

**B**

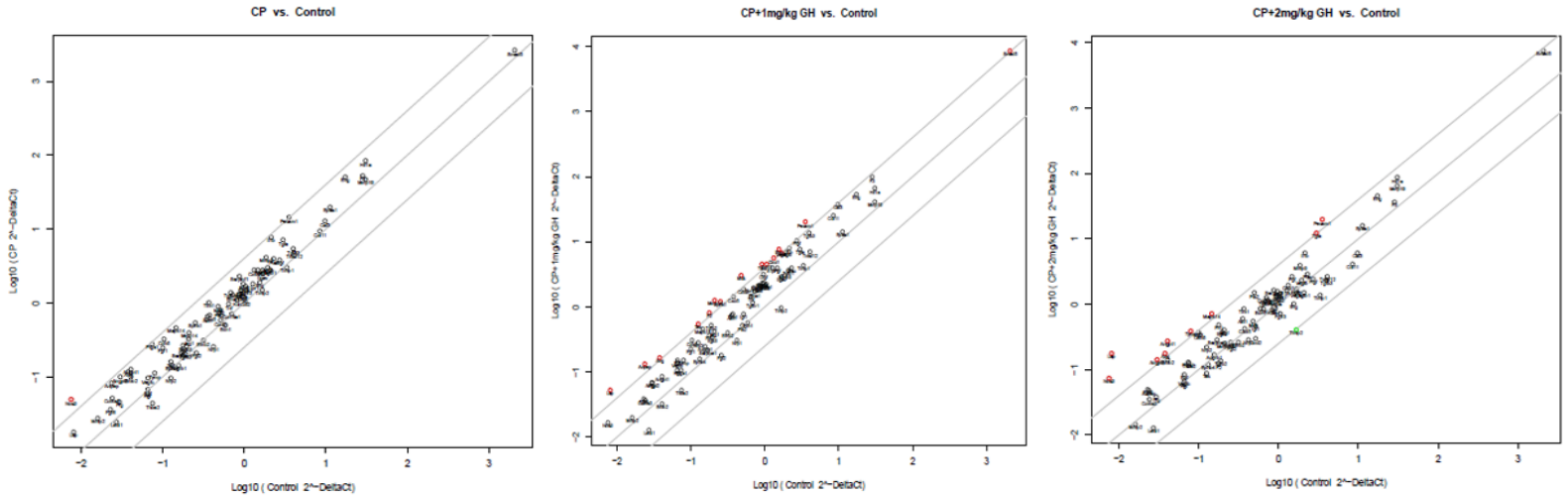



**Figure 8.** Angiogenesis-related gene expression by quantitative reverse transcription polymerase chain reaction (RT-qPCR). (A) Heatmap image, in which up-regulated and down-regulated genes are depicted in green and red colors, respectively. (B) Scatter plot. The red dots indicate up-regulation, and the green dots indicate down-regulation. The scatter graph depicts a log transformation plot of the relative expression level of each gene (2-Delta Ct) between control (x-axis) and CP (y-axis) groups. CP: cyclophosphamide; GH: growth hormone.

**Table 3**. Up-regulated genes with a fold change value > 4.0

| Gene | Description | Fold Change |
|---|---|---|
| | **CP vs. Control** | |
| Nos3 | Nitric oxide synthase 3 | 6.42 |
| | **CP+1mg/kg GH vs. Control** | |
| Lep | Leptin | 6.52 |
| Mdk | Midkine | 6.39 |
| Mmp14 | Matrix metalloproteinase 14 | 5.98 |
| Anpep | Alanyl (membrane) aminopeptidase | 5.64 |
| Pecam1 | Platelet endothelial cell adhesion molecule 1 | 5.61 |
| Ifng | Interferon Gamma | 4.91 |
| Sphk1 | Sphingosine kinase 1 | 4.81 |
| Tie1 | Tyrosine kinase with immunoglobulin-like and EGF-like domain 1 | 4.81 |
| Ang | Angiogenin | 4.35 |
| F2 | Coagulation factor ii (thrombin) | 4.56 |
| Tek | Tyrosin-protein kinase | 4.29 |
| Fgfr3 | Fibroblast growth factor receptor 3 | 4.21 |
| Cxcl1 | Chemokine (c-x-c motif) ligand 1 | 4.18 |
| Vegfa | Vascular endothelial growth factor-A | 4.08 |
| | **CP+2mg/kg GH vs. Control** | |
| Lep | Leptin | 21.78 |
| Nos3 | Nitric oxide synthase 3 | 9.93 |
| Angpt1 | Angiopoietin-1 | 6.71 |
| Pecam1 | Platelet endothelial cell adhesion molecule 1 | 5.44 |
| Tymp | Thymidine phosphorylase | 4.94 |
| Mapk14 | Mitogen-activated protein kinase 14 | 4.82 |
| Angpt2 | Angiopoietin-2 | 4.63 |
| Ang | Angiogenin | 4.61 |
| Tgfa | Transforming growth factor-alpha | 4.12 |

CP: cyclophosphamide; GH: Growth hormone.

**Table 4.** Up-regulated genes with a fold change value > 4.0 in the GH-treated group

| | Control | CP | CP+1mg/kg GH | CP+2mg/kg GH |
|---|---|---|---|---|
| Lep | 1.00 | 2.24 | 6.52 | 21.78 |
| Pecam1 | 1.00 | 3.99 | 5.61 | 5.44 |
| Ang | 1.00 | 3.03 | 4.35 | 4.61 |

CP: cyclophosphamide; GH: Growth hormone; Lep: Leptin;

Pecam1: Platelet endothelial cell adhesion molecule; Ang: Angiogenin

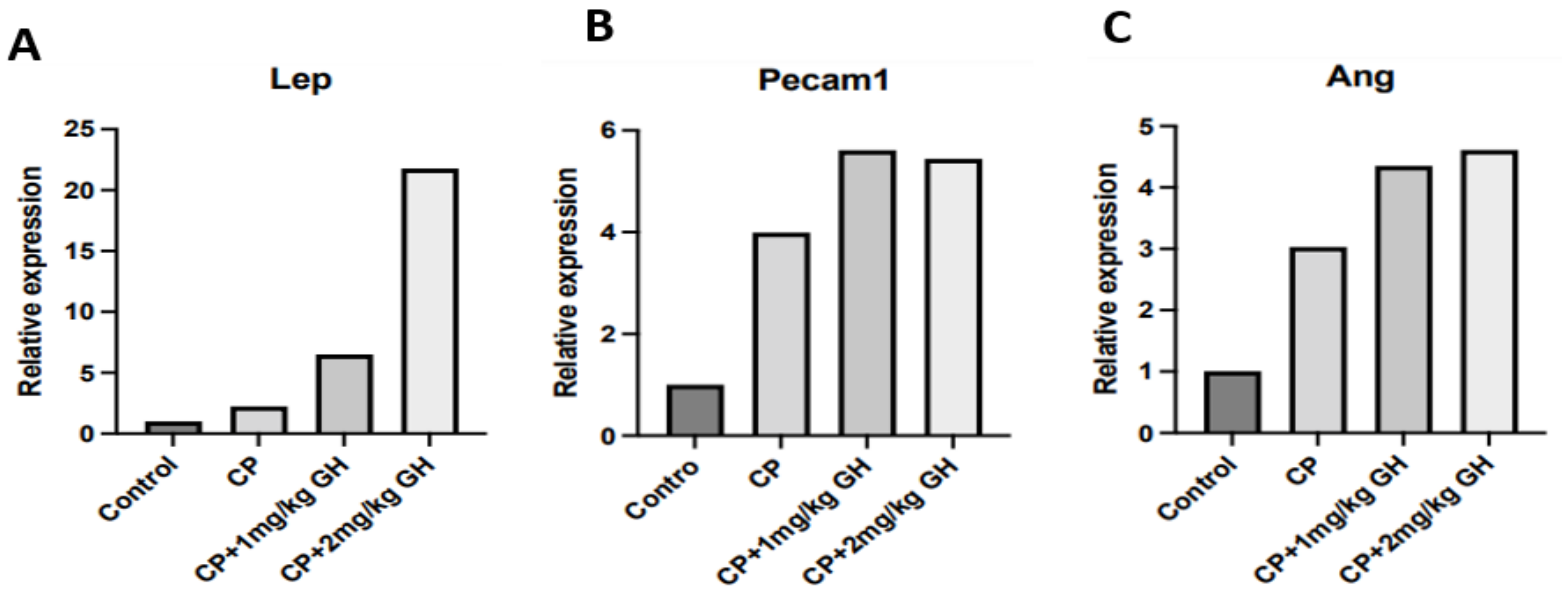



**Figure 9.** Bar graphs of up-regulated genes with a >4-fold change in the GH-treated group. The fold changes represent gene expressions compared to those of the control group. (A) Leptin (*Lep*) (B) Platelet endothelial cell adhesion molecule 1 (*Pecam1*) (C) Angiogenin (*Ang*) CP: cyclophosphamide; GH: growth hormone.

## 3.7 Western blot of CD 31

CD 31, previously a marker for determining vessel density, is a protein encoded by the *Pecam-1* gene. Western blot revealed that CD 31 was significantly increased in the GH 2 mg/kg treated group compared to the CP group (**Figure 10A, 10B**).

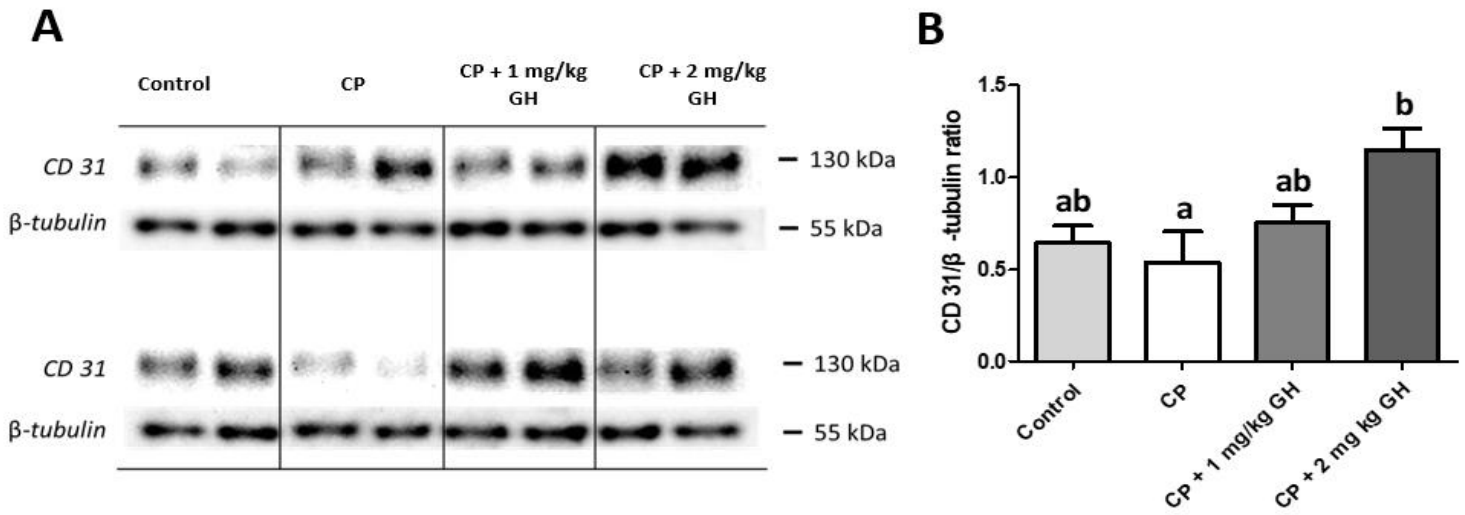



**Figure 10.** Evaluation of CD31 protein expression by western blot (A) Representative western blot image of CD31 (B) Relative quantification of CD31. Graphs are presented as mean ± SEM; n=5 and different superscript letters indicate statistically significant differences ($p < 0.05$). CP: cyclophosphamide; GH: growth hormone.

# 4. Discussion

This study showed the effects of GH on ovarian function recovery in ovarian insufficiency induced by the administration of CP. **Figure 11** shows the schematic results of the present study. CP damaged the non-growing follicles, such as the primordial and primary follicles. GH recovered ovarian function by decreasing apoptosis and fibrosis. GH also enhanced angiogenesis by up-regulation of Leptin (*Lep*), Platelet endothelial cell adhesion molecule 1 (*Pecam-1*), and Angiogenin (*Ang*). As a result, primordial and grade I follicles were increased. Furthermore, the fertilization rate and blastocyst formation rate were increased in IVF.

Anticancer treatment can impact ovarian reserve so that it could induce ovarian insufficiency. Ovarian insufficiency increases the risk of infertility, bone loss, and cardiovascular disease. Beyond these medical comorbidities, ovarian insufficiency impairs self-esteem and causes emotional distress (28). The highest risk of ovarian insufficiency in breast cancer is associated with the use of CP and its dose (29). CP-based chemotherapy significantly decreases the anti-mullerian hormone (AMH) level and causes treatment-related amenorrhea (3). Various strategies are being developed to enhance ovarian function to overcome these medical hurdles. Thus, this present study investigated the potential of GH as one of the therapeutic agents capable of ovarian function regeneration.

GH is a 191 amino acid protein that binds to the growth hormone receptor (GHR). Human oocytes, granulosa, and stromal cells express GHR and can be directly influenced by GH (30).

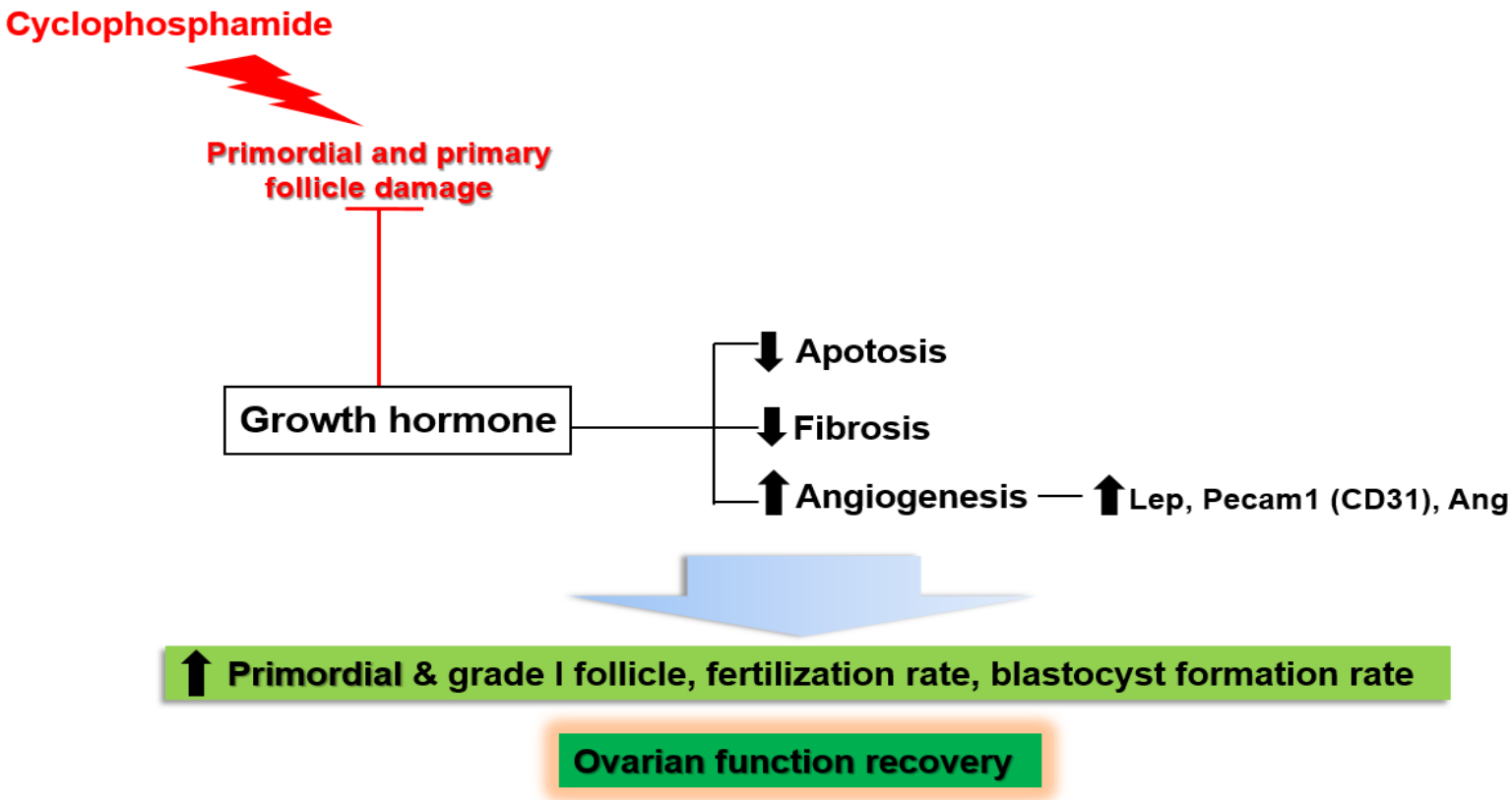



**Figure 11.** Schematic results of the current study.

GH indirectly affects ovarian function through autocrine or paracrine signaling mediated by the local production of secondary factors, particularly IGF-I (14). Through these direct and indirect pathways, GH influences folliculogenesis, oocyte maturation, and steroid synthesis (13). GH plays an essential role in the proliferation of ovarian follicles and is necessary for follicular maturation and survival (12). Locally released GH has a major role affecting ovarian function, but systemic GH produced by the anterior pituitary gland or exogenous administration also has an important role in female reproduction (13). GH replacement treatment recovered ovarian function with successful pregnancies in infertile patients with GH deficiency (31). This present study demonstrated that GH administration inhibited primordial follicle atresia and increased grade I follicles. The IVF outcome was also improved. Although statistical significance was not observed, the total number of follicles and grade 1 follicles were not increased as we expected in the GH 1 mg/kg treated group compared to the CP group. We speculated that these results could be explained by the small sample size with variation between objects. Moreover, these results suggest that high-dose GH might be needed to restore ovarian function.

The mechanism by which GH restores ovarian function, suggested by this study, is that GH reduces apoptosis and stromal fibrosis and enhances angiogenesis. Mahran et al. evaluated the radioprotective effects of GH. They injected GH 1 mg/kg in rats for seven days, starting three days before irradiation and lasting three days post-irradiation. GH significantly enhanced follicular development and ameliorated oxidative stress-medicated apoptosis by lowering cytochrome c and caspase-3 (18). Liu et al. injected GH 1 mg/kg in aged mice (8 months old) every two days for two months. GH reversed the age-associated depletion of ovarian reserve and the decline of oocyte quality. GH also reduced apoptosis by inhibiting the activation of Fos and

Jun signaling (32). Although those experiments were not in the same setting as the present study, GH may ameliorate apoptosis through various pathways.

To our knowledge, no study has explored the anti-fibrosis effects of growth hormones against ovarian fibrosis. Fibrosis is characterized by excessive proliferation of fibroblasts and deposition of extracellular matrix (ECM). Fibrosis develops in response to repeated tissue injury and inflammation (33). Ovarian fibrosis could be a reason for diminished ovarian function (34). Meirow et al. conducted a case-control study to evaluate the ovarian tissue harvested for cryopreservation (35). The case group (n=17) comprised young cancer patients previously administered chemotherapy, including CP. The control group (n=18) was not exposed to chemotherapy. Over 75% of study group patients showed ovarian cortical fibrosis with the disappearance of primordial follicles. Meirow et al. also found vascular damage showing severe narrowing and obliteration due to intimal fibrosis and hyalinization. These findings were consistent with this present study, which markedly increased fibrosis and decreased vessel density in the CP group. Meirow et al. suggested a mechanism of ovarian damage by chemotherapy as follows: exposure to chemotherapy caused injury and obstruction of blood vessels and ischemia of ovarian tissue. Finally, intact ovarian tissue was replaced with collagenous fibrosis lacking primordial follicles. In this study, fibrosis was significantly reduced in the GH-treated groups, hence we could speculate that ovarian fibrosis is reversible by GH and it would be a therapeutic strategy to enhance ovarian function.

As previously mentioned, we identified that GH promoted angiogenesis and decreased fibrosis in the damaged ovary. We focused on the angiogenesis effects of GH because, in any way, the supply of GH to the targeted organ needs adequate

vascular flow. Angiogenesis, the formation of new blood vessels, plays a pivotal role in maintaining normal ovarian function (36). Adequate blood supply to the ovaries is essential for receiving nutrients and delivering hormones and many factors. The female reproductive organs, specifically ovarian follicles, must experience a regulated angiogenesis process emerging dominant follicles and early corpus luteum in each menstrual cycle until menopause.

To understand the mechanism of GH on angiogenesis, we analyzed gene expression by RT- qPCR. We confirmed that GH increased in the expression of angiogenesis-related genes, including *Lep*, *Pecam-1*, and *Ang*.

*Lep* is an adipocyte-derived peptide hormone that regulates food intake, body mass, and reproductive functions, such as puberty (37). Capillary endothelial cells crosstalk with adipocytes and extracellular components via paracrine pathways and direct cell-to-cell interactions (38). Surprisingly, *Lep* is also regarded as a potent angiogenic factor. Endothelial cells express the *Lep* receptor and *Lep* promotes capillary-like tube formation in vitro. Furthermore, *Lep* increases corneal neovascularization in rats (39). In addition, ovarian follicular cells express *Lep* receptors. *Lep* promotes follicular development and oocyte maturation in synergistic effects with GH and IGF-1 (40).

*Pecam-1* (CD31) is a member of the Ig superfamily and is a single-chain transmembrane glycoprotein expressed on endothelial cells, platelets, and leukocytes (41). Angiogenesis refers to the formation of new blood vessels from pre-existing vasculatures by intussusception or sprouting (42). Angiogenesis requires cascade events such as enzymatic degradation of the basal membrane of the pre-existing vessels, migration of endothelial cells, and endothelial cell proliferation (43). *Pecam-1* has been implicated in the adhesive and signaling cascades required for endothelial

cell migration, which plays an essential role in angiogenesis (44, 45). *Pecam-1* is also involved in the endothelial cell-to-cell associations required for the organization of endothelial cells into tubular networks (46).

*Ang*, a member of the ribonuclease A superfamily, is a potent angiogenic molecule (47). For inducing angiogenesis, *Ang* is taken up by endothelial cells through receptor-mediated endocytosis and rapidly translocates to the nucleus, promoting ribonuclease activity, basement membrane degradation, signaling transduction, and nuclear translocation (48). In addition, the secreted *Ang* binds to the cell surface actin to induce basement membrane and extracellular matrix degradation, promoting endothelial cell invasion and migration into the surrounding tissue (49). Angiogenesis occurs as an orderly cascade of molecular events and starts with angiogenic factor secretion. In this study, GH increased angiogenesis by promoting the secretion of those important angiogenic factors including *Lep, Pecam-1,* and *Ang.*

The strength of the present study was that experiments were conducted in various aspects on the mechanism by which the GH restored ovarian function, including evaluation of apoptosis, fibrosis, and angiogenesis. In addition, RT-qPCR was also conducted to explore the mechanisms of GH on angiogenesis. Our findings will provide the basis for further studies on the pharmacological recovery of ovarian function. This present study has several limitations. First, this mouse model only provided relative short-term effects of GH on recovery of ovarian function. Second, it is not clear whether GH delayed declining ovarian function or restored it. It is necessary to compare the ovarian function before GH administration and on the day of ovarian sampling to determine if there is a difference in ovarian function decline. Third, although the GH concentration was determined after reviewing previous studies, it is a very high dose compared to the actual GH concentration administered

to humans. Fourth, GH could interfere with the activity of chemotherapy. Therefore, further studies on the long-term effects of GH on ovarian function and the elaboration of the protocol applied in human trials are recommended. In addition, GnRHa might be offered to patients when cryopreservation methods are not feasible in actual clinical practice. It would be helpful in clinical practice if the effects and mechanisms of GnRHa and GH are compared in further investigation.

In conclusion, this present study demonstrated the effect of GH on ovarian function recovery in ovarian insufficiency induced by the administration of CP. The mechanisms by which GH achieved these effects might be through decreasing apoptosis, stromal fibrosis, and increasing angiogenesis by up-regulating *Lep*, *Pecam-1*, and *Ang*.

# 5. References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: a cancer journal for clinicians. 2021;71(3):209-49.

2. Marklund A, Lundberg FE, Eloranta S, Hedayati E, Pettersson K, Rodriguez-Wallberg KA. Reproductive Outcomes After Breast Cancer in Women With vs Without Fertility Preservation. JAMA oncology. 2021;7(1):86-91.

3. Lambertini M, Peccatori FA, Demeestere I, Amant F, Wyns C, Stukenborg JB, et al. Fertility preservation and post-treatment pregnancies in post-pubertal cancer patients: ESMO Clinical Practice Guidelines(†). Annals of oncology : official journal of the European Society for Medical Oncology. 2020;31(12):1664-78.

4. Yu KD, Ge JY, Liu XY, Mo M, He M, Shao ZM. Cyclophosphamide-Free Adjuvant Chemotherapy for Ovarian Protection in Young Women With Breast Cancer: A Randomized Phase 3 Trial. Journal of the National Cancer Institute. 2021;113(10):1352-9.

5. Xiong J, Xue L, Li Y, Tang W, Chen D, Zhang J, et al. THERAPY OF ENDOCRINE DISEASE: Novel protection and treatment strategies for chemotherapy-associated ovarian damage. European journal of endocrinology. 2021;184(5):R177-r92.

6. Podfigurna-Stopa A, Czyzyk A, Grymowicz M, Smolarczyk R, Katulski K, Czajkowski K, et al. Premature ovarian insufficiency: the context of long-term effects. Journal of endocrinological investigation. 2016;39(9):983-90.

7. Oktay K, Harvey BE, Partridge AH, Quinn GP, Reinecke J, Taylor HS, et al.

Fertility Preservation in Patients With Cancer: ASCO Clinical Practice Guideline Update. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2018;36(19):1994-2001.

8. Cheng J, Ruan X, Du J, Jin F, Li Y, Liu X, et al. Ovarian tissue cryopreservation in a patient with breast cancer during pregnancy: a case report. Journal of ovarian research. 2021;14(1):176.

9. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion. Fertility and sterility. 2019;112(6):1022-33.

10. Paluch-Shimon S, Cardoso F, Partridge AH, Abulkhair O, Azim HA, Jr., Bianchi-Micheli G, et al. ESO-ESMO 4th International Consensus Guidelines for Breast Cancer in Young Women (BCY4). Annals of oncology : official journal of the European Society for Medical Oncology. 2020;31(6):674-96.

11. Mousaei Ghasroldasht M, Seok J, Park HS, Liakath Ali FB, Al-Hendy A. Stem Cell Therapy: From Idea to Clinical Practice. International journal of molecular sciences. 2022;23(5).

12. Xu YM, Hao GM, Gao BL. Application of Growth Hormone in in vitro Fertilization. Frontiers in endocrinology. 2019;10:502.

13. Hull KL, Harvey S. Growth hormone and reproduction: a review of endocrine and autocrine/paracrine interactions. International journal of endocrinology. 2014;2014:234014.

14. Ipsa E, Cruzat VF, Kagize JN, Yovich JL, Keane KN. Growth Hormone and Insulin-Like Growth Factor Action in Reproductive Tissues. Frontiers in endocrinology. 2019;10:777.

15. Cai MH, Gao LZ, Liang XY, Fang C, Wu YQ, Yang X. The Effect of Growth Hormone on the Clinical Outcomes of Poor Ovarian Reserve Patients Undergoing in

vitro Fertilization/Intracytoplasmic Sperm Injection Treatment: A Retrospective Study Based on POSEIDON Criteria. Frontiers in endocrinology. 2019;10:775.

16. Li XL, Wang L, Lv F, Huang XM, Wang LP, Pan Y, et al. The influence of different growth hormone addition protocols to poor ovarian responders on clinical outcomes in controlled ovary stimulation cycles: A systematic review and meta-analysis. Medicine. 2017;96(12):e6443.

17. Cozzolino M, Cecchino GN, Troiano G, Romanelli C. Growth hormone cotreatment for poor responders undergoing in vitro fertilization cycles: a systematic review and meta-analysis. Fertility and sterility. 2020;114(1):97-109.

18. Mahran YF, El-Demerdash E, Nada AS, El-Naga RN, Ali AA, Abdel-Naim AB. Growth Hormone Ameliorates the Radiotherapy-Induced Ovarian Follicular Loss in Rats: Impact on Oxidative Stress, Apoptosis and IGF-1/IGF-1R Axis. PloS one. 2015;10(10):e0140055.

19. Liu TE, Wang S, Zhang L, Guo L, Yu Z, Chen C, et al. Growth hormone treatment of premature ovarian failure in a mouse model via stimulation of the Notch-1 signaling pathway. Experimental and therapeutic medicine. 2016;12(1):215-21.

20. Semiz O, Evirgen O. The effect of growth hormone on ovarian follicular response and oocyte nuclear maturation in young and aged mice. Acta Histochem. 2009;111(2):104-11.

21. Yigiter M, Halici Z, Odabasoglu F, Keles ON, Atalay F, Unal B, et al. Growth hormone reduces tissue damage in rat ovaries subjected to torsion and detorsion: biochemical and histopathologic evaluation. European journal of obstetrics, gynecology, and reproductive biology. 2011;157(1):94-100.

22. Lundy T, Smith P, O'Connell A, Hudson NL, McNatty KP. Populations of granulosa cells in small follicles of the sheep ovary. Journal of reproduction and

fertility. 1999;115(2):251-62.

23. Gandolfi F, Paffoni A, Papasso Brambilla E, Bonetti S, Brevini TA, Ragni G. Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. Fertility and sterility. 2006;85 Suppl 1:1150-6.

24. Lee J, Kim EJ, Kong HS, Youm HW, Lee JR, Suh CS, et al. A combination of simvastatin and methylprednisolone improves the quality of vitrified-warmed ovarian tissue after auto-transplantation. Human reproduction (Oxford, England). 2015;30(11):2627-38.

25. Youm HW, Lee J, Kim EJ, Kong HS, Lee JR, Suh CS, et al. Effects of Angiopoietin-2 on Transplanted Mouse Ovarian Tissue. PloS one. 2016;11(11):e0166782.

26. Street JM, Souza AC, Alvarez-Prats A, Horino T, Hu X, Yuen PS, et al. Automated quantification of renal fibrosis with Sirius Red and polarization contrast microscopy. Physiological reports. 2014;2(7).

27. Schlüter A, Weller P, Kanaan O, Nel I, Heusgen L, Höing B, et al. CD31 and VEGF are prognostic biomarkers in early-stage, but not in late-stage, laryngeal squamous cell carcinoma. BMC cancer. 2018;18(1):272.

28. Committee opinion no. 605: primary ovarian insufficiency in adolescents and young women. Obstetrics and gynecology. 2014;124(1):193-7.

29. Razeti MG, Spinaci S, Spagnolo F, Massarotti C, Lambertini M. How I perform fertility preservation in breast cancer patients. ESMO open. 2021;6(3):100112.

30. Abir R, Garor R, Felz C, Nitke S, Krissi H, Fisch B. Growth hormone and its receptor in human ovaries from fetuses and adults. Fertility and sterility. 2008;90(4 Suppl):1333-9.

31. Giampietro A, Milardi D, Bianchi A, Fusco A, Cimino V, Valle D, et al. The effect of treatment with growth hormone on fertility outcome in eugonadal women with growth hormone deficiency: report of four cases and review of the literature. Fertility and sterility. 2009;91(3):930.e7-11.

32. Liu C, Li S, Li Y, Tian J, Sun X, Song T, et al. Growth hormone ameliorates the age-associated depletion of ovarian reserve and decline of oocyte quality via inhibiting the activation of Fos and Jun signaling. Aging. 2021;13(5):6765-81.

33. Umehara T, Winstanley YE, Andreas E, Morimoto A, Williams EJ, Smith KM, et al. Female reproductive life span is extended by targeted removal of fibrotic collagen from the mouse ovary. Science advances. 2022;8(24):eabn4564.

34. Zhou F, Shi LB, Zhang SY. Ovarian Fibrosis: A Phenomenon of Concern. Chinese medical journal. 2017;130(3):365-71.

35. Meirow D, Dor J, Kaufman B, Shrim A, Rabinovici J, Schiff E, et al. Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. Human reproduction (Oxford, England). 2007;22(6):1626-33.

36. Devesa J, Caicedo D. The Role of Growth Hormone on Ovarian Functioning and Ovarian Angiogenesis. Frontiers in endocrinology. 2019;10:450.

37. Obradovic M, Sudar-Milovanovic E, Soskic S, Essack M, Arya S, Stewart AJ, et al. Leptin and Obesity: Role and Clinical Implication. Frontiers in endocrinology. 2021;12:585887.

38. Cao Y. Angiogenesis modulates adipogenesis and obesity. The Journal of clinical investigation. 2007;117(9):2362-8.

39. Sierra-Honigmann MR, Nath AK, Murakami C, García-Cardeña G, Papapetropoulos A, Sessa WC, et al. Biological action of leptin as an angiogenic

factor. Science (New York, NY). 1998;281(5383):1683-6.

40. Childs GV, Odle AK, MacNicol MC, MacNicol AM. The Importance of Leptin to Reproduction. Endocrinology. 2021;162(2).

41. Johnson-Léger C, Aurrand-Lions M, Imhof BA. The parting of the endothelium: miracle, or simply a junctional affair? Journal of cell science. 2000;113 ( Pt 6):921-33.

42. Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. Circulation research. 2007;100(6):782-94.

43. Bruno JB, M.H.T. MJAr. Angiogenic factors and ovarian follicle development. Anim Reprod. 2009;6:371-9.

44. Cao G, Fehrenbach ML, Williams JT, Finklestein JM, Zhu JX, Delisser HM. Angiogenesis in platelet endothelial cell adhesion molecule-1-null mice. The American journal of pathology. 2009;175(2):903-15.

45. O'Brien CD, Cao G, Makrigiannakis A, DeLisser HM. Role of immunoreceptor tyrosine-based inhibitory motifs of PECAM-1 in PECAM-1-dependent cell migration. American journal of physiology Cell physiology. 2004;287(4):C1103-13.

46. Cao G, O'Brien CD, Zhou Z, Sanders SM, Greenbaum JN, Makrigiannakis A, et al. Involvement of human PECAM-1 in angiogenesis and in vitro endothelial cell migration. American journal of physiology Cell physiology. 2002;282(5):C1181-90.

47. Miyake M, Goodison S, Lawton A, Gomes-Giacoia E, Rosser CJ. Angiogenin promotes tumoral growth and angiogenesis by regulating matrix metallopeptidase-2 expression via the ERK1/2 pathway. Oncogene. 2015;34(7):890-901.

48. Gao X, Xu Z. Mechanisms of action of angiogenin. Acta biochimica et biophysica Sinica. 2008;40(7):619-24.

49. Weng C, Dong H, Bai R, Sheng J, Chen G, Ding K, et al. Angiogenin promotes

angiogenesis via the endonucleolytic decay of miR-141 in colorectal cancer. Molecular therapy Nucleic acids. 2022;27:1010-22.

국문 초록

# 난소부전 마우스 모델에서 성장호르몬에 의한 난소 기능회복 효과

김 수 미

의학과 산부인과학 전공

서울대학교 의과대학

**목적**

이 연구는 항암제인 cyclophosphamide(CP)를 투여하여 난소부전 마우스 모델을 구축한 후 성장호르몬을 투여하여 난소 기능 회복에 대한 효과를 확인하고 그 기전을 탐색하고자 하였다.

**연구 방법**

6주령 ICR 마우스에 CP를 400 mg/kg를 복강 내로 투여하여 난소부전을 유도한 후 네 군 (대조군, CP 투여군, 성장호르몬 1 mg/kg 투여군, 성장호르몬 2 mg/kg 투여군) 으로 나누었고 각 군당 마우스 10마리를 배정하였다. CP를 투여한 후 7일 뒤 성장호르몬을 7일간 투여하였고 투여 완료한 다음 날 각 군당 5마리의 마우스의 난소를 채취하여 조직학적 검사를 진행하였다. 나머지 5마리는 체외수정 (in vitro fertilization, IVF)을 진행하였다. 조직학적 검사와 체외수정을 통해 성장호르몬의 효

과를 확인 한 뒤 난소 기능을 회복시키는 효과를 확인하기 위하여 다음의 실험을 진행하였다. 세포사멸 (apoptosis)의 정도를 비교하기 위하여 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay를 시행하였다. 난소 조직의 섬유화 정도를 알아보기 위하여 Masson's trichrome staining을 시행하였다. 혈관신생 (angiogenesis)을 정량화하기 위하여 CD31 면역조직화학염색 (CD31 immunohistochemistry)을 시행하였다. 역전사 중합효소 연쇄반응 (reverse transcription polymerase chain reaction, RT- qPCR)을 통하여 혈관신생에 관여하는 유전자의 발현을 알아보았다.

**결과**

CP 투여는 원시난포와 1차 난포의 소멸을 유발하였다. 성장호르몬의 투여는 원시난포의 소멸을 방지하였고 난포의 질을 향상시켰다. 체외수정 결과를 살펴보면, CP 투여군은 수정률 및 배반포 형성률 (blastocyst formation rate)이 저하되었다. 그 반면, 성장호르몬 투여군은 성장호르몬을 투여한 용량에 비례하여 체외수정 결과가 향상되었다. 그 밖에 성장호르몬은 세포사멸 및 난소조직의 섬유화를 감소시키고 혈관신생을 향상시켰다. 혈관신생과 연관된 여러 유전자의 발현이 증가하였는데 그 중에서 특히 Leptin (*Lep*), Platelet endothelial cell adhesion molecule 1 (*Pecam-1*), Angiogenin (*Ang*) 유전자가 성장호르몬 투여군에서 발현이 증가하였다.

**결론**

CP를 투여하여 난소 부전을 유도한 뒤 성장호르몬을 투여하면 세포자멸사 및 난소조직 섬유화가 감소되고 혈관신생과 연관된 *Lep*, *Pecam-1*, *Ang* 유전자 발현이 증가하여 난소 기능 회복이 촉진된다.

**주요어**: 성장호르몬, 난소 부전, 시클로포스파미드, 난소암, 난소 재생, 가임력 보존

학 번: 2018-37972

# Acknowledgments

I want to take this opportunity to thank Professor Jung Ryeol Lee, my mentor, who led my research to the end. He is my lifelong teacher and a wonderful medical doctor who guides me along the path of medical science. His gracious talent for conceptualization, constant encouragement, and practical advice has been a source of immense support for me. Furthermore, he generously provided all of the support needed for this experiment and for preparing the thesis.

Special thanks also go to Jeong Young Yoo. I am grateful for her technical help and for providing materials to obtain the experimental results. I was impressed with her sincerity in the experimentations, and when discussing the laboratory results with her, I always found new inspiration.

Let me extend my infinite gratitude to my family. Without the patience, commitment, and encouragement of my precious family, I would never have completed my thesis.