

ORIGINAL ARTICLE

Effects of Hochuekkito, a traditional Japanese medicine (Kampo), on reproduction of aging female mice

Kim Cat Tuyen Vo¹  | Yorino Sato² | Yuta Kawagoe² | Kazuhiro Kawamura^{1,2}

¹Graduate School of Medicine, International University of Health and Welfare School of Medicine, Narita-shi, Japan

²Advanced Reproductive Medicine Research Center, International University of Health and Welfare School of Medicine, Narita-shi, Japan

Correspondence

Kazuhiro Kawamura, Advanced Reproductive Medicine Research Center, International University of Health and Welfare School of Medicine, 4-2 Kozunomori, Narita-shi, Chiba 286-0048, Japan.
Email: kazuhiroanami@gmail.com

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Abstract

Purpose: To determine the potentials of Hochuekkito (HET) treatment for aging infertility.

Methods: Mice at 36 weeks of age were fed without (control, $n = 40$) or with low (100 mg/kg/day, $n = 24$) and high (1000 mg/kg/day, $n = 38$) doses of HET for 12 weeks. Aging animals at 48 weeks of age were used for in vitro fertilization-embryo transfer (IVF-ET), and their ovaries were subjected to histological and quantitative inflammation analyses.

Results: HET administration decreased transcript levels of ovarian inflammatory markers, interleukin 6 (IL-6), IL-1 β , tumor necrosis factor (TNF)- α , and interferon-gamma (IFN- γ) but suppressed ovulation rates and the number of ovulated oocytes in aging mice. Furthermore, HET treatment decreased the rates of oocytes maturation and fertilization and the cumulus-cell expression of TNF- α -induced protein 6 and epidermal growth factor receptor. After IVF-ET, no improvement of declined live offspring rate by aging was achieved by HET administration, although there were no adverse effects on embryo development and implantation as well as gross morphology and bodyweight of pups.

Conclusion: Present study indicated HET treatment interfered with ovulation and fertilization in aging mice without affecting ovarian follicle development. No improvement on the age-associated decline of live offspring rate and follicle development was achieved after HET treatment.

KEYWORDS

Hochuekkito, infertility, Kampo, oocyte quality, ovulation

1 | INTRODUCTION

When chronological age increases, the female fertility potential decreases.¹ In advance-aged women, there is a natural depletion of ovarian follicles and oocyte quality, resulting in poor fecundity. The mechanisms of ovarian aging are multifactorial but not yet fully

elucidated.¹ One of the critical contributors of ovarian aging is age-related chronic inflammation known as inflammaging.² It is characterized by the enhancement of NOD-like receptor family pyrin domain containing 3 inflammasome NLRP3 which are known to accumulate during aging, leading to the increased expression of several pro-inflammatory cytokines including IL-6, IL-1 α , IL-1 β , IFN- γ , and

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TNF- α , age-related follicle depletion as well as reduction of oocyte quality.^{3–5} In addition, stroma fibrosis that is observed to increase during the aging process is one contributor to ovarian aging.^{6,7} In aging model mice with excessive fibrosis in the stroma, follicular dysgenesis occurred in bilayer secondary follicles.⁸

Kampo, one type of herbal medicine, has been gaining popularity and prescribed for diverse diseases. It is prepared from a single crude ingredient or a mixture of several crude ingredients in fixed formulae.⁹ Currently, Kampo is recognized as a well-integrated component of the Japanese medical system with 85% of prescriptions having the combination of Kampo and Western medicine.¹⁰ HET, one type of Kampo, also known as Bojungikki-tang in Korean and Bu-Zhong-Yi-Qi-Tang in Chinese, has become one of the most popular types of Kampo in several Asian countries¹¹ and used by 70% of women at reproductive age.¹⁰

HET showed anti-inflammatory effects on several chronic inflammatory conditions including aging.^{12–14} In infertility treatment, HET was demonstrated to improve the fertile competence in idiopathic male infertility and polycystic ovarian syndrome through immunomodulatory actions.^{15–17} However, there is no study on the anti-inflammatory effects of HET on aging females. Here, we sought to determine the potentials of HET treatment for infertility using an aging female animal model. We investigated the changes in ovarian inflammatory markers following HET treatment and the effects of HET on age-related fertility decline including ovarian follicle development, ovulation, and oocyte quality.

2 | MATERIALS AND METHOD

2.1 | Animals

Female ICR mice at 36 weeks of age ($n = 102$) were purchased from Japan SLC Inc. Additionally, female ICR mice at eight weeks of age ($n = 15$) were used to evaluate the fertility of young mice. These mice were housed five per cage under a controlled environment (12 h light/dark cycle, temperature 20–25°C and humidity 50%–60%). They were allowed free access to water via sipper bottle. All experimental procedures and animal housing conditions were approved by the Animal Care and Use Committee of the International University of Health and Welfare (Approval Number: 19005). Animal care was consistent with institutional and National Institutes of Health guidelines. To assess the effect of HET on the mice's body mass, all mice were weighed once a week by a digital scale to get the average body weight in each group. In addition, their behavior, fur color, drinking, and eating habit were also noted daily.

2.2 | Kampo materials

The animal food contained the HET extract supplied from Tsumura & Co. The dried HET powder extract was manufactured from a composition of 10 crude HET drugs in a fixed proportion. All medicinal plants were

purchased from Ibaraki Plant. Five gram of HET extract was produced from 4.0 g Astragali Radix (dried roots of *Astragalus membranaceus* Bunge), 4.0 g *Atractylodis lanceae* Rhizoma (rhizome of *Atractylodes lanceae* De Candolle, Compositae), 4.0 g Ginseng Radix (dried roots of *Panax ginseng* C.A. Meyer), 3 g *Angelica Radix* (dried roots of *Angelica acutiloba* Kitagawa, Umbelliferae), 2.0 g *Bupleuri Radix* (dried roots of *Bupleurum falcatum* L.), 2.0 g *Zizyphi Fructus* (dried fruits of *Zizyphus jujuba* Miller var. *inermis* Rehder), 2.0 g *Aurantii Bobilis Pericarpium* (dried pericarps of ripe fruits of *Citrus unshu* Markovich), 1.5 g *Glycyrrhizae radix* (dried roots of *Glycyrrhiza uralensis* Fisch et DC.), 1.0 g *Cimicifugae Rhizoma* (dried rhizome of rhizomes of *Cimicifuga simplex* Turczaninow), and 0.5 g *Zingiberis Rhizoma* (rhizomes of *Zingiber officinale* Roscoe). These crude drugs were standardized by Japanese Pharmacopoeia 17th Edition (Pharmaceutical and Medical Device Regulatory Science Society of Japan, 2016). The mixture of these crude drugs was added to purified water, extracted at 95°C for 1 h. Subsequently, the extraction solution was concentrated via the removal of water under reduced pressure. Spray-drying was used to yield the dried extract powder. The quality of HET extract was standardized based on the good manufacturing practice as defined by the Ministry of Health, Labour, and Welfare of Japan and the detailed chemical profile of HET determined by three-dimensional HPLC analysis can be obtained from a previous study.¹⁸

2.3 | Experimental design

Because the fertility of mice is known to decrease linearly after middle age (9–14 months),^{8,19,20} mice at 36 weeks of age were chosen as animals without an age-associated decline of fertility to start HET treatment. The mice were randomly divided into the following three groups: control group without HET ($n = 30$), low dose HET (100 mg/kg/day)-treated group ($n = 24$), and high dose HET (1000 mg/kg/day)-treated group ($n = 24$). To determine the effect of HET on oocyte maturation, 24 female ICR mice at 36 weeks of age were randomly divided into two groups: control group ($n = 10$) and high dose HET (1000 mg/kg/day)-treated group ($n = 14$). The doses of HET were determined according to the previous studies.^{21,22} These mice were housed and fed for 12 weeks until 48 weeks of age. Each mouse was fed with 6 g/day (30 g/cage) of food with or without HET. The food was changed daily, and almost all foods were ingested within a day by the animals.

2.4 | Histological examination

In every mouse, unilateral ovaries after ovulation were isolated for the histological examinations. The serial section process and the follicles counting methodology were performed as described previously.²³ In addition to dormant primordial and growing follicles, we also counted the number of ovulated follicles that have remaining granulosa cells (GCs) and no detected oocyte in all serial sections (Figure S1). The follicles were only counted and determined their types in the section where the nuclei of oocytes were clearly

observed. Because the nucleus is approximately 10–15 μm in size, which is larger than section thickness, repetitive counting of follicles was eliminated. Tunica albuginea (TA) thickness was measured as a sum of the basal membrane and tunica collagen layer as described previously.²⁴ The mean thickness of TA was measured in five randomly-chosen positions where basal and collagenous layers could be clearly observed (Figure S1).

2.5 | In vitro fertilization (IVF) and embryo transfer (ET)

To induce ovulation from preovulatory follicles, the mice were treated with an intraperitoneal injection of 20 IU human chorionic gonadotropin (hCG) (ASKA Pharmaceutical). Cumulus-oocyte complexes (COCs) were obtained from oviducts at 16 h after hCG administration. The number of ovulated mice and mature oocytes per mouse were noted. Collected COCs were placed in 50- μl drops of TYH medium (LSI Medience Corporation) and put into a CO_2 incubator before sperm introduction for fertilization. For IVF, ICR male mice (8–10 weeks of age) were sacrificed to remove epididymis. The subsequent sperm retrieval and the IVF procedure were conducted as described previously.²⁵ Then, the obtained zygotic stage embryos were cultured in 50- μl drops of KSOM medium (Merck Millipore) at 37°C and 5% CO_2 in the air for 3.5 days until blastocyst stage. For ET, 12–16 obtained blastocysts were transferred into the uterus of pseudopregnant ICR mouse (8 weeks of age) predated with vasectomized males of the same strain as a recipient ($n = 5\text{--}7$ animals). Pups retrieved from the uterus by cesarean section at 19.5 dpc were counted and weighted, together with determining the number of implantation sites and placentas. For evaluating the maturation status of oocytes, COCs obtained from mouse ovaries 14 h after hCG treatment were washed twice in L-15 medium (Thermo). COCs were subsequently transferred to L-15 medium containing 0.5 mM 3-Isobutyl 1-methylxanthine (Nakarai tesque) and 80 U/ml hyaluronidase (Sigma-Aldrich) at 37°C. The maturation stages of oocytes were examined after removing cumulus cells using a small-bore pipette under microscopy (Olympus). The mature oocytes were classified when having the extrusion image of the polar body, whereas the oocytes at the metaphase I or the germinal vesicle (GV) stage of meiosis or degenerated/fragmented oocytes were classified as the immature ones.

2.6 | Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate the anti-inflammatory effects of HET, one side of the ovary after ovulation in each group was obtained to quantify the transcript levels of inflammatory markers, IL-6, IL-1 β , TNF- α , and IFN- γ . To clarify the chronic effect of HET on the ovary, the expression levels of aging markers including p16, p21 p53, and plasminogen activator inhibitor 1 (Pai-1), a downstream target of p53 were quantified. We also measured the mRNA levels of TNF- α -induced protein

TABLE 1 Quantitative PCR primer sequences

Primer	Primer sequence (5'-3')
mGAPDH forward	GTGGCAAAGTGGAGATGGTTGCC
mGAPDH reverse	GATGATGACCCGTTTGGCTCC
mTNF- α forward	GCATGATCCGCGACGTGGAA
mTNF- α reverse	AGATCCATGCCGTTGGCCAG
mIFN- γ forward	AAAGAGATAATCTGGCTCTGC
mIFN- γ reverse	GCTCTGAGACAATGAACGCT
mIL-1 β forward	AAAAAAGCCTCGTGCTGTCG
mIL-1 β reverse	GTCGTTGCTTGGTTCTCCTTG
mIL-6 forward	TCCAGTTGCCTTCTTGGGAC
mIL-6 reverse	GTGTAATTAAGCCTCCGACTTG
mP16 forward	GCGTGCTAGCATGTGGCTTT
mP16 reverse	TCCTTCTGCTCCCTCCCTCT
mP21 forward	AATACCGTGGGTGTCAAAGCA
mP21 reverse	AGGGAGGGAGCCACAATACA
mP53 forward	GATGCCAAATGTACAGAGGAG
mP53 reverse	ACAGAAAAGGGGAGGGATGAA
mPai-1 forward	TTCCTCTCACTTCCACCCAAA
mPai-1 reverse	AGCAACAGCAACAGAAACAACAC
mTNF-AIP6 forward	ATACAAGCTCACCTACGCCGAA
mTNF-AIP6 reverse	ATCCATCCAGCAGCACAGACAT
mEGFR forward	CTGCCAAGGCACAAGTAACA
mEGFR reverse	ATTGGGACAGCTTGATCAC

6 (TNFAIP6) and EGFR (epidermal growth factor receptor) in the cumulus cells after fertilization to determine the impacts of HET on ovulation. Quantitative real-time RT-PCR was performed as described previously²⁵ using on a LightCycler[®] 96 System (Roche) as following protocol: 15 min at 95°C and followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. The used primer sequences are shown in Table 1.

2.7 | Statistical analyses

The results are shown as mean \pm standard error (SE). Statistical significance was determined using Dunnett's test and one side *T* test, with $p < 0.05$ being statistically significant.

3 | RESULTS

3.1 | The fertility difference between young and aging mice

We first confirmed the changes in fertility with aging in our animal model by comparing the outcomes of IVF-ET in young (8 weeks of age, $n = 15$) and aging control (48 weeks of age, $n = 29$) mouse groups. Because one mouse in the control group died during aging with no abnormal lesion by autopsy, there were total of 29 mice in the control group, 24 mice in low and high dose groups at the end of the study

period. The results indicated a significant decline in ovulation number (Figure 1A), and live pups rate (Figure 1E), together with an increase in abortion rate (Figure 1F) in the aging control mice. In contrast, no difference was found in rates of fertilization (Figure 1B), blastocyst formation (Figure 1C), and implantation (Figure 1D) in this study.

3.2 | HET treatment decreased inflammatory cytokines in ovaries

During the study period, the amount of daily remaining food was scarce in each cage and the mice developed normally with no

changes in drinking behavior, fur color, and other behaviors during the experiment. The bodyweight of mice in all groups showed no statistically significant difference (Figure 2). After 12 weeks of HET feeding, no changes in daily food intake were observed in the treated mice compared to the controls.

To check the anti-inflammatory effects of HET in murine ovaries, the levels of IL-6, IL-1 β , TNF- α , and IFN- γ transcripts were measured in ovaries. As shown in Figure 3, the levels of IL-6, IL-1 β , TNF- α , and IFN- γ transcripts were statistically lower in HET-treated mice than those in controls. In contrast, the transcript levels of cell senescence markers, p15, p21, p53, and Pai-1 were not changed by HET treatment (Figure 4).

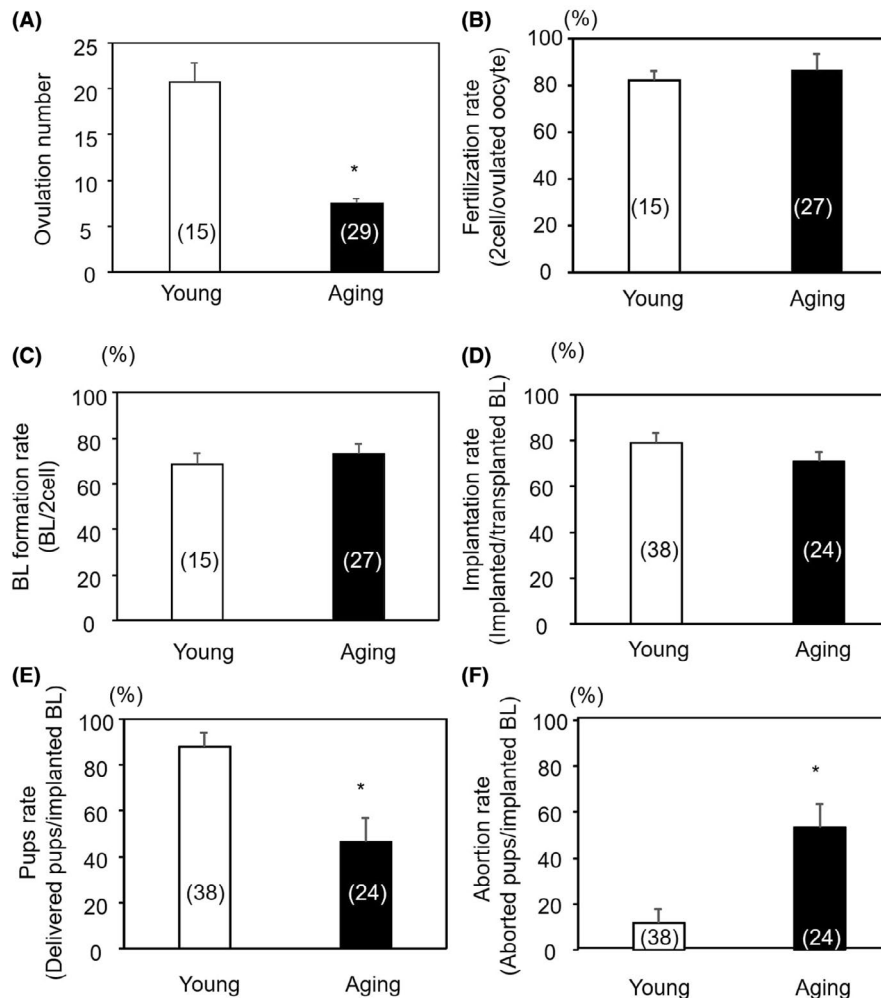


FIGURE 1 The effect of aging in fertility in this animal model. Changes in the fertility ability between young (8 weeks of age) and aging (48 weeks of age) mice were evaluated by IVF-ET. Ovulation was induced by hCG administration. (A) Ovulation number: The number of ovulated oocytes per animal. Numbers in parentheses represent animal numbers. The ovulated oocytes were inseminated with sperm and cultured with KSOM medium until blastocyst stage embryos. (B) Fertilization rate: Two-cell stage embryos (2 cells) per ovulated oocytes (a total of $n = 217$ – 311 ovulated oocytes in each group). Numbers in parentheses represent animal numbers. Among 29 animals, two aging mice did not ovulate. (C) Blastocyst (BL) formation rate: Blastocysts per two-cell stage embryos (a total of $n = 181$ – 256 two-cell stage embryos in each group). Numbers in parentheses represent animal numbers. For embryo transfer, blastocysts were transferred to the uterus of the foster mother ($n = 5$ – 10 animals). (D) Implantation rate: Implanted blastocysts per transplanted blastocysts (a total of $n = 38$ – 24 transplanted blastocysts in each group). Numbers in parentheses represent transplanted blastocyst numbers. (E) Pups and (F) abortion rates: delivered pups and aborted pups per implanted blastocysts (a total of $n = 38$ – 24 implanted blastocyst in each group). Numbers in parentheses represent implanted blastocyst numbers. Data were presented in mean \pm SE; * $p < 0.05$

3.3 | HET treatment suppressed ovulation in aging mice

After confirmation of decline in fertility in aging mice in our study and suppression of inflammatory cytokine expression following HET treatment, we evaluated the effects of HET on fertility in aging mice. As shown in Figure 5A, low and high doses of HET treatment decreased ovulation rates by 5.6% and 26.4%, respectively. Furthermore, in mice with ovulation, the number of ovulated

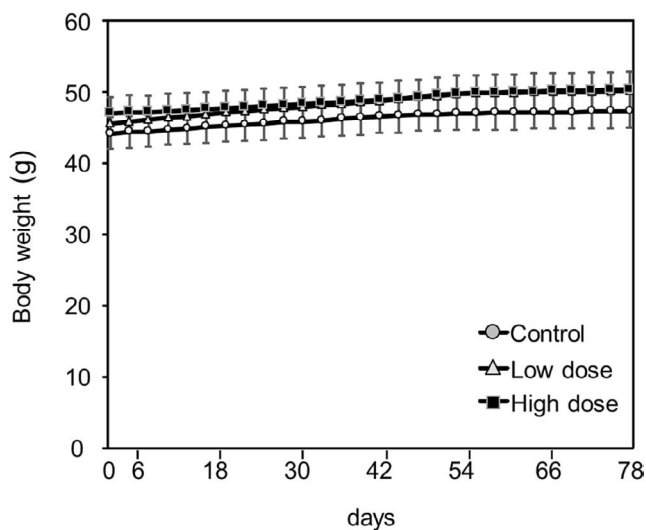


FIGURE 2 Changes in body weight of aging mice during HET treatment. Mice were treated with HET from 36 to 48 weeks of age. Animal body weights were measured every three days in each experimental group ($n = 24$ – 29). Data were presented in mean \pm SE

oocytes was significantly lower in the high-dose HET group as compared to that in the controls (Figure 5B). To assess the mechanism of ovulation suppression in HET-treated aging mice, follicular dynamics was evaluated by histological analyses in ovaries obtained from 10 randomly selected animals in each group. As shown in Figure 5C, the number of ovulated follicles in the HET-treated groups was lower than that in the controls. In contrast, the total number of follicles and proportions of growing follicles including primary, secondary, and large antral follicles were not different between groups, although the proportion of primordial follicles was negligible (Figure 5D) due to aging. To further address the effect of HET treatment on ovarian aging, ovarian fibrosis in stroma and cortex was evaluated by histological analysis. As shown in Figure 6A–C, similar fibrosis in the ovarian stroma was observed in all experimental groups, suggesting no obvious effect of HET treatment on stromal fibrosis. Regarding cortical fibrosis, TA thickness was not different between the control and HET-treated groups (Figure 6D).

3.4 | HET treatment suppressed fertilization but showed no effect on other IVF-ET outcomes in aging mice

We further evaluated the effects of HET treatment on different reproduction parameters during IVF-ET. As shown in Figure 7A, the fertilization rate declined in HET-treated aging mice. However, once fertilized, the embryos developed to the blastocyst stage without any significant differences as compared with the controls (Figure 7B). For further insight into the mechanism of low fertilization in HET groups, we assessed the changes of TNFAIP6 and EGFR

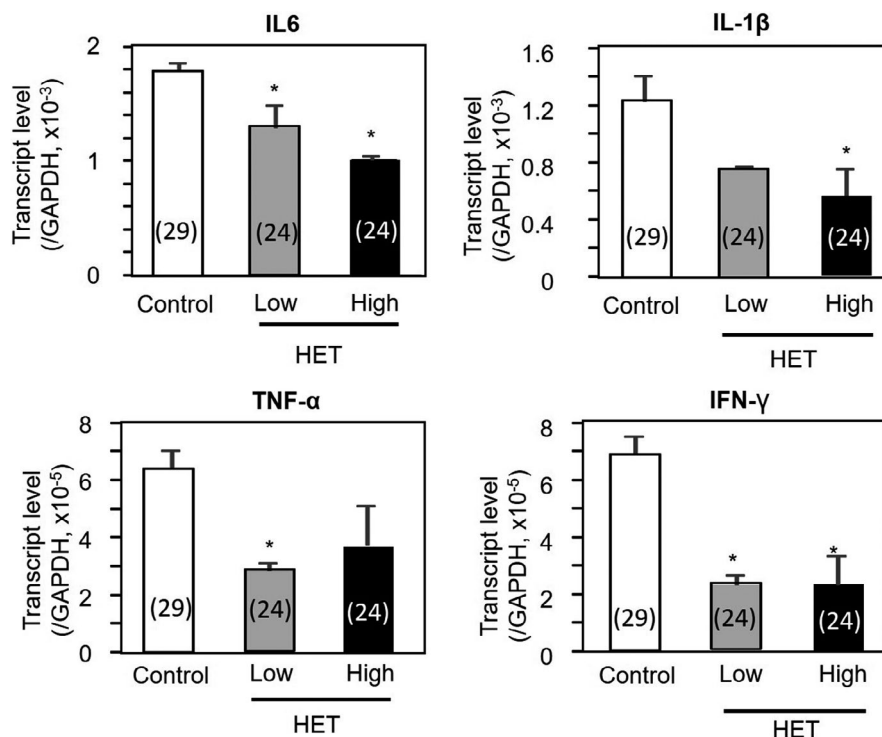


FIGURE 3 Effects of HET treatment on inflammatory cytokines on murine ovaries in aging animals. The unilateral whole ovary of each mouse was collected after ovulation induction by hCG injection to quantify the transcript levels of inflammatory markers, IL-6, IL-1 β , TNF- α , and IFN- γ . The expression of these markers was quantified using quantitative RT-PCR. Numbers in parentheses represent the number of ovaries examined. Data were presented in mean \pm SE; * $p < 0.05$

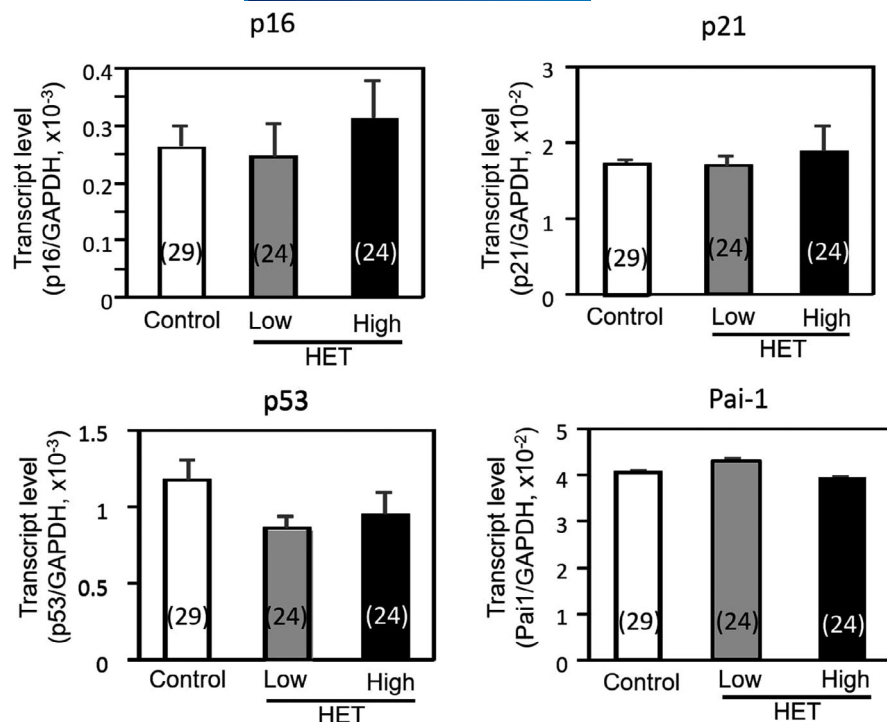


FIGURE 4 Effects of HET treatment on expression levels of cellular senescence markers in aging mice ovaries. The unilateral whole ovary of each mouse was collected after ovulation induction by hCG injection to quantify the transcript levels p16, p21, p53, and Pai-1. The expression of these markers was quantified using quantitative RT-PCR. Numbers in parentheses represent the number of ovaries examined. Data were presented in mean \pm SE; * $p < 0.05$

expressions known to play important roles in cumulus expansion and oocyte maturation. Although all ovulated COCs showed cumulus expansion in all groups and no apparent morphological difference in COCs between the two groups (Figure 7C), the expression of TNFAIP6 and EGFR transcripts in cumulus cells was extremely low in HET-fed mice (Figure 7D). Furthermore, the high doses HET group had a significantly lower maturation rate in oocytes ($p < 0.05$) (Figure 7E) as compared with the control group. In contrast to the low potential of fertilization in oocytes derived from HET-treated mice, no significant difference was detected in rates of implantation (Figure 8A), live pups (Figure 8B), and abortion (Figure 8C) after ET. Furthermore, HET treatment did not affect the pup's body weight and placenta weight (Figure 8D,E) as well as gross morphology of pups and placentas (Figure 8F).

4 | DISCUSSION

In this study, we determined whether HET treatment could improve the female fertility outcome of aging mice. Although HET administration suppressed the levels of ovarian inflammatory markers, the results of the IVF experiment indicated no improvement of aging-associated decline in fertility. Furthermore, HET treatment showed adverse effects on ovulation and fertilization without affecting ovarian follicle development.

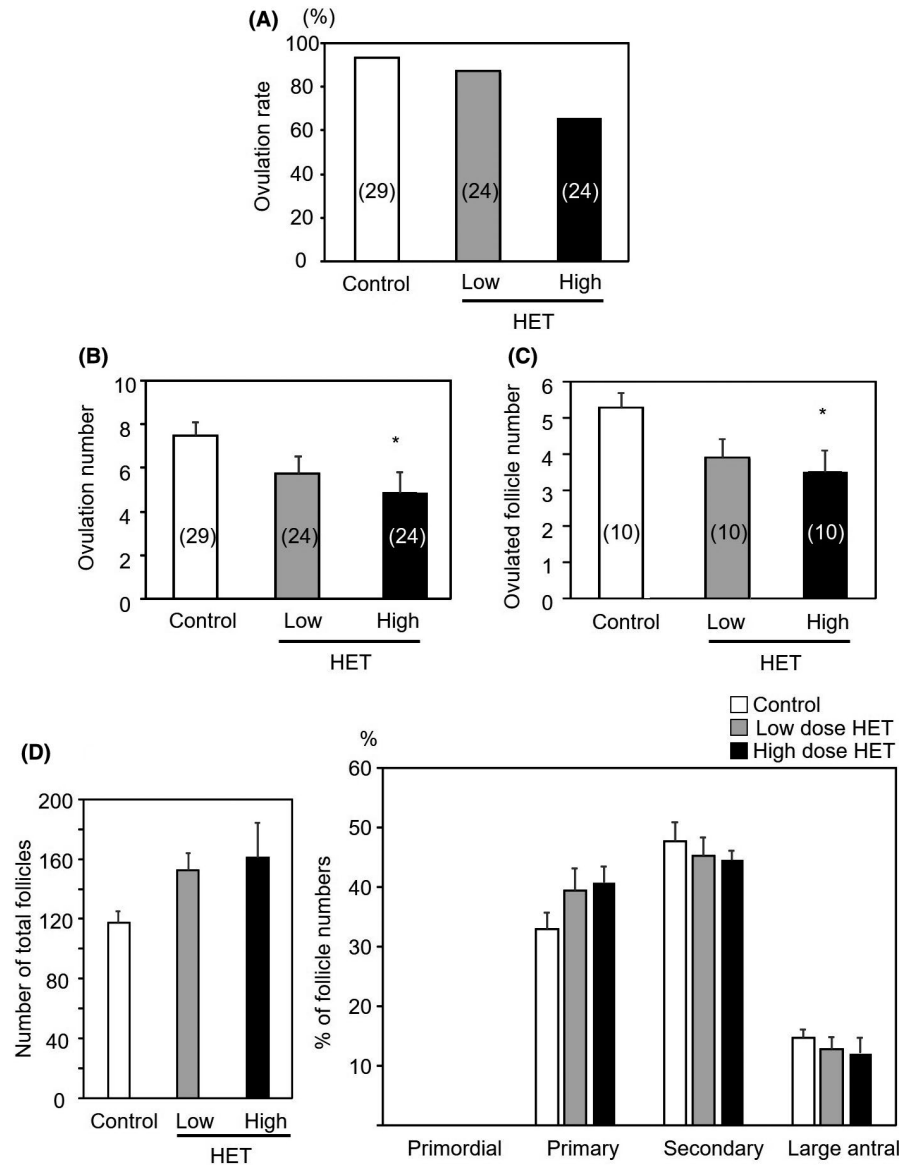
In our animal model, we confirmed the decline of fertility in aging mice. These aging-associated changes are consistent with previous studies.^{8,19,20} HET treatment did not change the body weight and gross morphology of animals, suggesting that HET exhibited its adverse effects on ovulation and fertilization via local actions, not by

systemic disorders. Although some anti-inflammatory substances such as chitosan oligosaccharide, anthocyanin, resveratrol, melatonin were reported to improve age-related infertility,²⁶ the in vivo HET treatment did not show any beneficial effects even at a high dose. Because HET is made from a mixture of several crude ingredients, the lack of beneficial effects of HET on reproduction might be caused by their complicated actions.

It is well documented that the ovulatory process is similar to the inflammatory responses. At the preovulatory stage, LH surge triggers dynamic changes in the ovulatory follicle leading to several intraovarian cytokine secretions with a rapid infiltration of leukocytes including the macrophages and T-lymphocytes, which secrete IL-6, IL-1 β , TNF- α , and INF- γ , resulting in an inflammation-like and complex biological process.²⁷⁻²⁹ Intraovarian IL-1 β stimulates the transition of GCs from proliferation to differentiation as well as their production of prostaglandins E, collagenases, hyaluronic acid, and proteoglycans.^{30,31} TNF- α upregulates matrixins and other collagenases, facilitating extracellular matrix remodeling in preparation for ovulation.^{31,32} Subsequently, IL-6 coordinates with IL-8 to promote cumulus expansion and follicle rupture.^{30,33} The IFN- γ participates in the corpus luteum formation.³⁴

To address the reason why the ovulated rate was lower in the HET-treated mice, we quantified the levels of several inflammatory markers in the ovary, including IL-6, IL-1 β , TNF- α , and INF- γ . In our study, the mRNA quantification showed decreases in IL-6, IL-1 β , TNF- α , and INF- γ levels following HET treatment in the ovary. These results suggest that HET treatment interfered with ovulation through suppression of the transcription of inflammatory cytokines which are essential for ovulation. Our results are

FIGURE 5 Effect of HET treatment on ovulation in aging mice. After 12 weeks of HET treatment, hCG was administered to aging animals at 48 weeks of age for ovulation induction. After ovulation, oocytes were obtained from oviducts and ovaries were collected for histological analyses. (A) Ovulation rate: The number of ovulated mice per total mice. (B) Ovulated oocyte number: The number of ovulated oocytes per animal. (C) Ovulated follicle number: The number of ovulated follicles were counted histologically in sections with HE staining. (D) Follicular dynamics. Numbers in parentheses represent animal numbers. Data were presented in mean \pm SE; * $p < 0.05$



consistent with other studies demonstrating the HET-mediated reduction of inflammatory cytokines' expression by modulating the immune system. HET administration reduced several inflammatory markers such as C-reactive protein, TNF- α , and IL-6 in elderly patients.¹² In mice having intestinal mucositis, the TNF- α , and IL-1 β also reduced after HET treatment.¹³ Furthermore, HET downregulated the expression of TNF- α in lung tissue,³⁵ the expression of IL-1 β , IL-6, and IFN- γ in the hippocampus in chronic fatigue syndrome mice,³⁶ and the expression of IFN- γ in bleomycin-induced lung injury mice.²² These earlier studies revealed that HET treatment suppressed the production of these inflammatory cytokines from the macrophages and T-lymphocyte in several inflammation conditions.^{22,35,37} In aging mouse ovaries, the NLRP3 inflammasome is activated in macrophages and lymphocytes, leading to an increase in pro-inflammatory cytokines including IL-6, TNF- α , IL-1 β , and IFN- γ regardless of the ovulation process.³⁻⁵ Thus, in addition to the suppression of inflammation in ovaries, similar actions of HET to other organs might decline

elevated transcript levels of inflammatory cytokines in aging ovaries. However, the molecular mechanisms underlying the suppression of inflammatory cytokine production by HET treatment are unknown. Including other possible mechanisms of HET, detailed molecular mechanisms will be assessed in future studies.

As a Kambo medicine, no adverse effect of HET on stimulation of oxidative stress accumulation and induction of cell cycle arrest was reported. However, HET treatment may accelerate ovarian aging rather than suppress it, resulting in a decreased number of ovulated oocytes, oocyte maturation, and fertilization rate. We analyzed the chronic action of HET on ovarian function by measuring the markers for cellular senescence. The data showed no difference of those makers as compared with controls, suggesting HET did not accelerate ovarian aging. We also assessed the effects of HET on stromal fibrosis as another important contributor to ovarian aging and found no obvious effect of HET on stromal fibrosis. This data further support the interpretation of lacking the acceleration of ovarian aging by HET treatment.

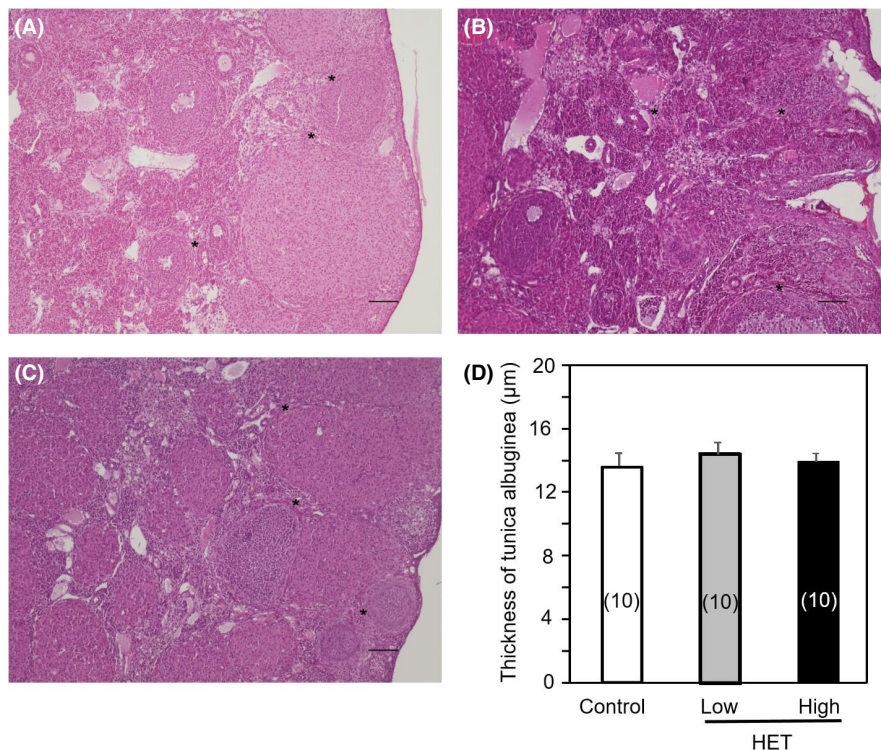


FIGURE 6 Effect of HET treatment on ovarian stromal and cortical fibrosis in aging mice. In each group, the unilateral whole ovaries of 10 mice were collected after ovulation induction and subjected to histology. (A–C), Representative images of stromal fibrosis in HE-stained ovarian tissues. *, fibrotic stroma in the control group (A), low HET treatment group (B), and high HET treatment group (C). Scale bars: 100 µm. (D) The measurement of TA thickness in the sections with HE staining. Numbers in parentheses represent the number of ovaries examined. Data were presented in mean \pm SE; * $p < 0.05$

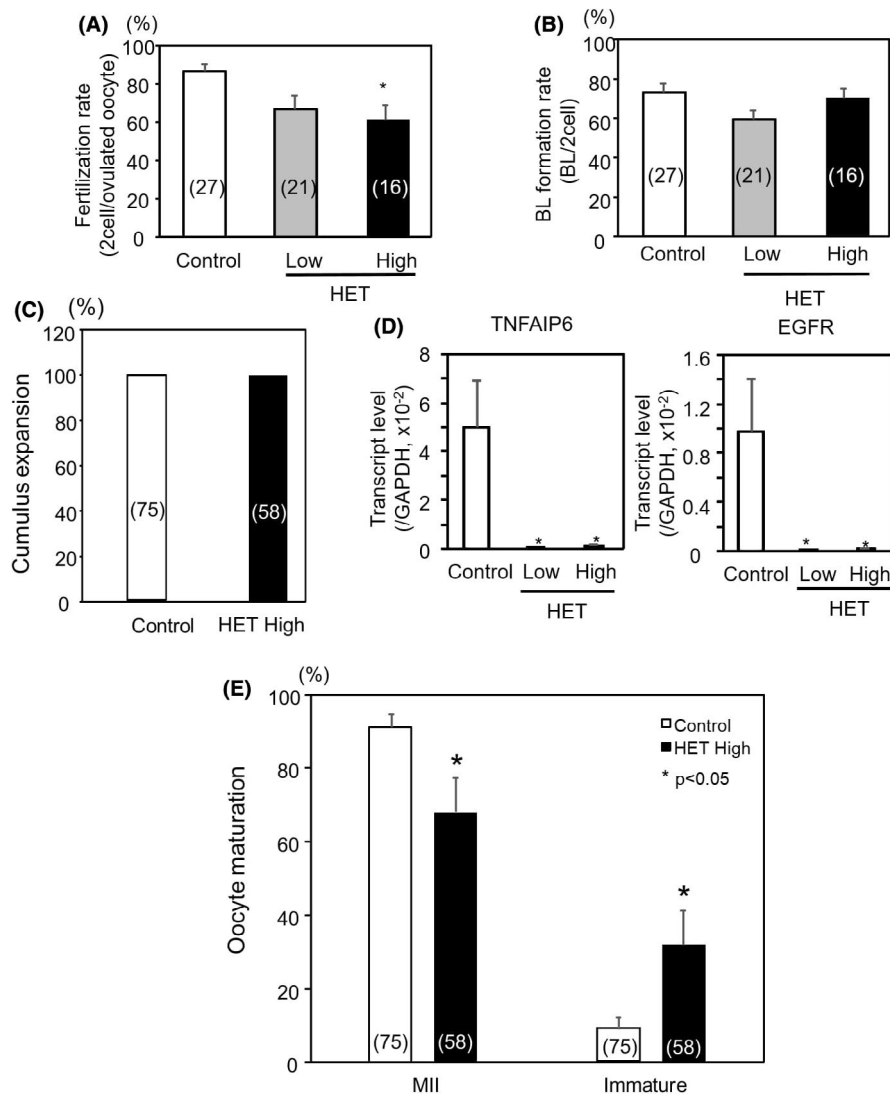
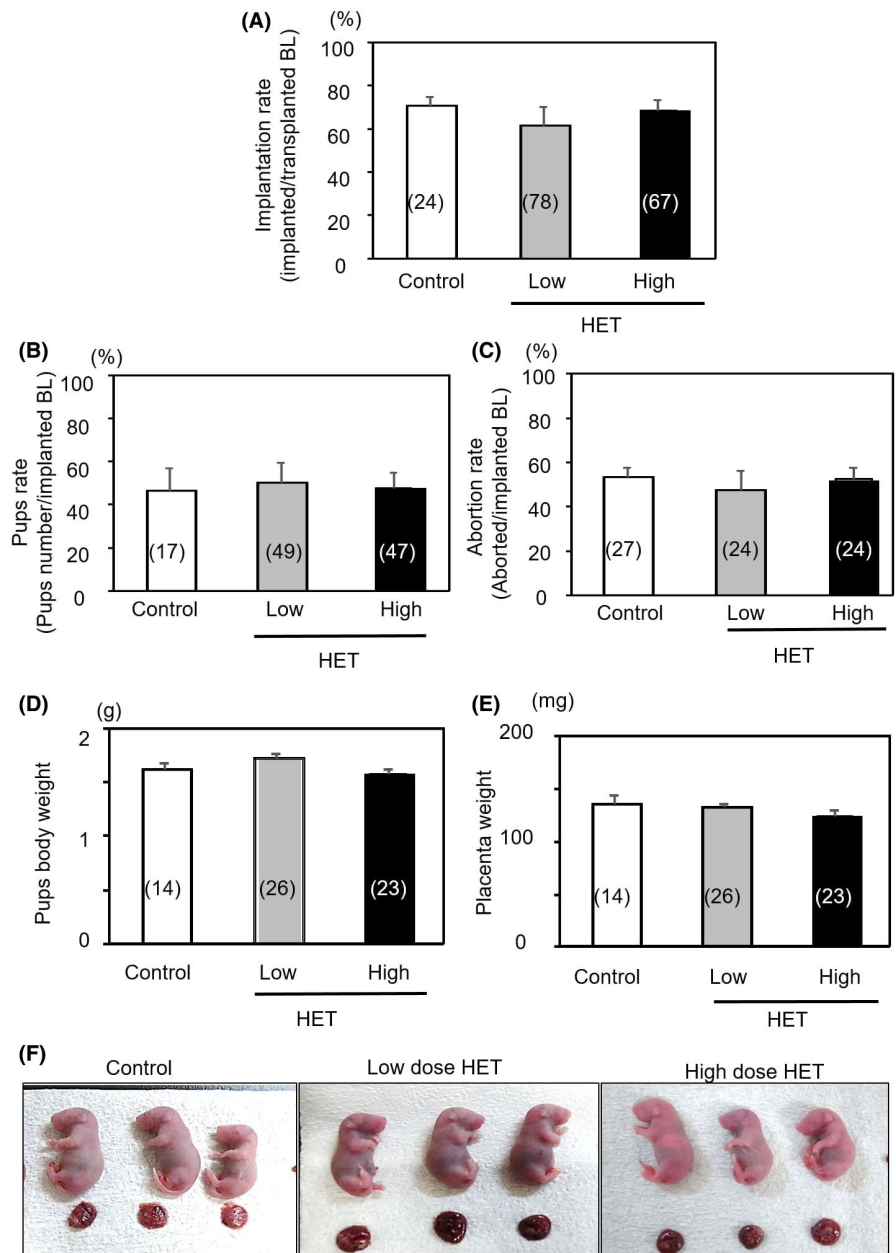


FIGURE 7 Effects of HET treatment on fertilization and cumulus cell expansion in aging mice. The ovulated oocytes were fertilized in vitro and allowed to develop into blastocysts. (A) Fertilization rate: Two-cell stage embryos (2 cell) per ovulated oocytes (a total of $n = 116$ –217 ovulated oocytes in each group). Numbers in parentheses represent animal numbers. (B) Blastocyst (BL) formation rate: blastocysts per two-cell stage embryos (a total of $n = 72$ –181, 2-cell stage embryos in each group). Numbers in parentheses represent animal numbers. (C) Cumulus expansion rate: Number of expanded cumulus per obtained COCs (a total of $n = 58$ or 75 COCs in each group). Numbers in parentheses represent COC numbers. (D) The transcript levels of TNFAIP6 and EGFR as makers for cumulus cell expansion in cumulus cells were obtained after fertilization. The mRNA levels were measured by quantitative real-time RT-PCR. (E) The effect of HET on oocyte maturation. Mature oocytes and immature oocytes rates: Number of MII oocytes and immature oocytes per obtained oocytes, respectively (a total of $n = 58$ or 75 obtained oocytes in each group). Numbers in parentheses represent COC numbers. Data were presented in mean \pm SE; * $p < 0.05$

FIGURE 8 Effect of HET treatment on implantation and fetal growth in aging mice. The fertilized embryos developed into blastocysts were transplanted into the uterus of recipient mice. On day 19.5 of pregnancy, the number of pups, implantation sites, and aborted fetuses were counted by caesarian sections. (A) Implantation rate: implanted blastocysts per transplanted blastocysts (a total of $n = 24$ – 78 transplanted blastocysts in each group). Numbers in parentheses represent transplanted blastocyst numbers. (B) Pups and (C) abortion rates: Delivered and aborted pups per implanted blastocysts (a total of $n = 24$ – 27 implanted blastocyst each group). Numbers in parentheses represent implanted blastocyst numbers. (D and E) The weights of pups and placentas on day 19.5 of pregnancy. Numbers in parentheses represent the numbers of fetuses and placentas. (F) Representative images of pups and placentas in each group on day 19.5 of pregnancy. Data were shown as mean \pm SE



An optimum expansion of COC and oocytes maturation is essential for the resumption of meiosis and subsequent fertilization.³⁸ Although we found the increases in non-ovulated large antral follicles in the HET-treated group, the cumulus expansion rates were not changed by HET treatment and the ovulated COCs showed morphologically normal expansion of cumulus cells in all groups. However, we demonstrated decreases in transcript levels of TNFAIP6 and EGFR in the cumulus cells obtained from COCs after insemination in HET-treated mice. In response to luteinizing hormone surge for ovulation, EGF-like peptides were produced from the mural GCs and then bound to their receptors (EGFR) expressing in the cumulus cells to induce cumulus expansion, oocyte cytoplasmic maturation, and meiotic resumption.³⁹ They also induce temporal mRNA expression of the cumulus expansion-related components including TNFAIP6 in cumulus cells, leading to cumulus expansion.³⁹ Furthermore, TNFAIP6 and EGFR are essential for proper gap junction communication between

cumulus cells and oocytes, the mitochondrial activity of oocytes, and first polar body extrusion at the resumption of meiosis.^{40–42} Indeed, we found the decline of maturation rate in ovulated oocytes by HET treatment. Therefore, the low fertilization in HET-treated animals is likely caused by functional changes in expanded cumulus cells and the maturation of oocytes induced by the anti-inflammation action of HET. This result suggested that HET might decline the expression of genes related to cumulus cell expansion directly even in young mice. Because our study originally aimed to determine the potentials of HET treatment for aging infertility, the effect of HET on young animals will be addressed in future studies.

Our study has some limitations. First, we could not clarify the temporal effects of HET on ovarian functions. Although we found the changes in reproductive events after 12 weeks of HET treatment, a shorter administration period of HET may also induce similar changes. Second, considering the potentials of HET for suppression

of both inflammaging and acute inflammatory changes during ovulation, we sampled ovaries after ovulation. Although a number of publications in other tissues support our finding on HET suppression of inflammatory cytokine expressions, we could not rule out the possibility that this result is simply reflecting the difference in the number of ovulations. Third, we have not addressed the response of follicles to FSH stimulation in HET-treated mice. To further clarify the effects of HET on ovarian functions, it is important to explore the follicle development under equine chorionic gonadotropin stimulation after cessation of HET treatment.

In conclusion, this is the first work identifying the impact of traditional Japanese herbal medicine, HET, on female aging infertility. Although we could not find the improvement of aging infertility by HET treatment, no adverse effect was found in the animals and their offspring except for ovulation and fertilization.

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CONFLICTS OF INTEREST

Kazuhiro Kawamura received the financial support of research funds from Tsumura & Co (Tokyo, Japan). Kazuhiro Kawamura declared that such financial supports have not influenced the outcome of the experimental data. Kim Cat Tuyen Vo, Yorino Sato, Yuta Kawagoe declare that they have no conflict of interest.

HUMAN RIGHTS STATEMENT AND INFORMED CONSENT

This article does not contain any study with human participants that have been performed by any of the authors.

ANIMAL STUDIES

All institutional and national guidelines for the care and use of laboratory animals were followed. All procedures and protocols were approved by the committee on animal research at the International University of Health and Welfare, Chiba, Japan (approval number: 19005).

ORCID

Kim Cat Tuyen Vo  <https://orcid.org/0000-0002-3771-1194>

REFERENCES

1. Tesarik J, Galán-Lázaro M, Mendoza-Tesarik R. Ovarian aging: molecular mechanisms and medical management. *Int J Mol Sci*. 2021;22(3):1371.
2. Foley KG, Pritchard MT, Duncan FE. Macrophage-derived multinucleated giant cells: hallmarks of the aging ovary. *Reproduction*. 2021;161(2):V5-V9.
3. Navarro-Pando JM, Alcocer-Gómez E, Castejón-Vega B, et al. Inhibition of the NLRP3 inflammasome prevents ovarian aging. *Sci Adv*. 2021;7(1):eabc7409. <https://doi.org/10.1126/sciadv.abc7409>
4. Lliberos C, Liew SH, Mansell A, Hutt KJ. The inflammasome contributes to depletion of the ovarian reserve during aging in mice. *Front Cell Dev Biol*. 2020;8:628473.
5. Lliberos C, Liew SH, Zareie P, La Gruta NL, Mansell A, Hutt K. Evaluation of inflammation and follicle depletion during ovarian ageing in mice. *Sci Rep*. 2021;11(1):278.
6. Briley SM, Jasti S, McCracken JM, et al. Reproductive age-associated fibrosis in the stroma of the mammalian ovary. *Reproduction*. 2016;152(3):245-260.
7. Umehara T, Richards JS, Shimada M. The stromal fibrosis in aging ovary. *Aging*. 2018;10(1):9-10.
8. Umehara T, Kawai T, Kawashima I, et al. The acceleration of reproductive aging in Nrg1(flox/flox); Cyp19-Cre female mice. *Aging Cell*. 2017;16(6):1288-1299.
9. Meresman GF, Götte M, Laschke MW. Plants as source of new therapies for endometriosis: a review of preclinical and clinical studies. *Hum Reprod Update*. 2021;27(2):367-392.
10. Yamana H, Ono S, Michihata N, Jo T, Yasunaga H. Outpatient prescriptions of Kampo formulations in Japan. *Intern Med*. 2020;59(22):2863-2869.
11. Hu L, Yao Z, Qin Z, et al. In vivo metabolic profiles of Bu-Zhong-Yi-Qi-Tang, a famous traditional Chinese medicine prescription, in rats by ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry. *J Pharm Biomed Anal*. 2019;171:81-98.
12. Tatsumi K, Shinozuka N, Nakayama K, Sekiya N, Kuriyama T, Fukuchi Y. Hochuekkito improves systemic inflammation and nutritional status in elderly patients with chronic obstructive pulmonary disease. *J Am Geriatr Soc*. 2009;57(1):169-170.
13. Gou H, Gu LY, Shang BZ, Xiong Y, Wang C. Protective effect of Bu-Zhong-Yi-Qi decoction, the water extract of Chinese traditional herbal medicine, on 5-fluorouracil-induced intestinal mucositis in mice. *Hum Exp Toxicol*. 2016;35(12):1243-1251.
14. Orita K, Yamate Y, Kobayashi H, Hiramoto K. Ameliorative Effect of Hochu-ekki-to on natural skin aging. *Pharmacology*. 2020;105(7-8):454-460.
15. Akashi T, Watanabe A, Morii A, Mizuno I, Fuse H. Effects of the herbal medicine hochuekkito on semen parameters and seminal plasma cytokine levels (TNF- α , IL-6, RANTES) in idiopathic male infertility. *Pharmacology*. 2013;91(5-6):314-321.
16. Park E, Choi C, Kim S, et al. Hochu-ekki-to treatment improves reproductive and immune modulation in the stress-induced rat model of polycystic ovarian syndrome. *Molecules*. 2017;22(6):978.
17. Furuya Y, Akashi T, Fuse H. Effect of Bu-zhong-yi-qi-tang on seminal plasma cytokine levels in patients with idiopathic male infertility. *Arch Androl*. 2004;50(1):11-14.
18. Dan K, Akiyoshi H, Munakata K, Hasegawa H, Watanabe K. A Kampo (traditional Japanese herbal) medicine, Hochuekkito, pre-treatment in mice prevented influenza virus replication accompanied with GM-CSF expression and increase in several defensin mRNA levels. *Pharmacology*. 2013;91(5-6):314-321.
19. Ozawa Y, Watanabe K, Toda T, et al. Heterosis extends the reproductive ability in aged female mice. *Biol Reprod*. 2019;100(4):1082-1089.
20. Chen L-J, Yang Z-X, Wang Y, et al. Single xenotransplant of rat brown adipose tissue prolonged the ovarian lifespan of aging mice by improving follicle survival. *Aging Cell*. 2019;18(6):e13024.
21. Kiyohara H, Nagai T, Munakata K, et al. Stimulating effect of Japanese herbal (kampo) medicine, hochuekkito on upper respiratory mucosal immune system. *Evid Based Complement Alternat Med*. 2006;3(4):459-467.
22. Tajima S, Bando M, Yamasawa H, et al. Preventive effect of hochuekkito, a Japanese herbal medicine, on bleomycin-induced lung injury in mice. *Respirology*. 2007;12(6):814-822.

23. Yorino S, Kawamura K. Rapamycin treatment maintains developmental potential of oocytes in mice and follicle reserve in human cortical fragments grafted into immune-deficient mice. *Mol Cell Endocrinol.* 2020;504:110694.
24. Grin L, Vo KCT, Sato Y, et al. Ageing and chronic disease-related changes in the morphometric characteristics of ovarian follicles in cynomolgus monkeys (*Macaca fascicularis*). *Hum Reprod.* 2021;36(10):2732-2742.
25. Kawagoe Y, Kawashima I, Sato Y, Okamoto N, Matsubara K, Kawamura K. CXCL5-CXCR2 signaling is a senescence-associated secretory phenotype in preimplantation embryos. *Aging Cell.* 2020;19(10):e13240.
26. Yang Z, Tang Z, Cao X, et al. Controlling chronic low-grade inflammation to improve follicle development and survival. *Am J Reprod Immunol.* 2020;84(2):e13265.
27. Omu AE, Al-Azemi MK, Makhseed M, et al. Differential expression of T-helper cytokines in the peritoneal fluid of women with normal ovarian cycle compared with women with chronic anovulation. *Acta Obstet Gynecol Scand.* 2003;82(7):603-609.
28. Duffy DM, Ko C, Jo M, Brannstrom M, Curry TE. Ovulation: parallels with inflammatory processes. *Endocr Rev.* 2019;40(2):369-416.
29. Yang Z, Kong B, Mosser DM, Zhang X. TLRs, macrophages, and NK cells: our understandings of their functions in uterus and ovary. *Int Immunopharmacol.* 2011;11(10):1442-1450.
30. Baskind NE, Orsi NM, Sharma V. Follicular-phase ovarian follicular fluid and plasma cytokine profiling of natural cycle in vitro fertilization patients. *Fertil Steril.* 2014;102(2):410-418.
31. Field SL, Dasgupta T, Cummings M, Orsi NM. Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation. *Mol Reprod Dev.* 2014;81(4):284-314.
32. Silva JRV, Lima FEO, Souza ALP, Silva AWB. Interleukin-1 β and TNF- α systems in ovarian follicles and their roles during follicular development, oocyte maturation and ovulation. *Zygote.* 2020;28(4):270-277.
33. Liu Z, de Matos DG, Fan HY, Shimada M, Palmer S, Richards JS. Interleukin-6: an autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. *Endocrinology.* 2009;150(7):3360-3368.
34. Abe H, Sakumoto R, Okuda K. Expression of matrix metalloproteinases in bovine luteal cells induced by prostaglandin F $_{2\alpha}$, interferon γ and tumor necrosis factor α . *J Reprod Dev.* 2015;61(4):277-286.
35. Isago H, Mitani A, Kohno S, et al. The Japanese Herbal (Kampo) medicine Hochuekkito attenuates lung inflammation in lung emphysema. *Biol Pharm Bull.* 2021;44(1):39-45.
36. He Q, Sawada M, Yamasaki N, et al. Neuroinflammation, oxidative stress, and neurogenesis in a mouse model of chronic fatigue syndrome, and the treatment with Kampo medicine. *Biol Pharm Bull.* 2020;43(1):110-115.
37. Sekiya M, Kiyohara H, Maruyama H, Yabe T, Yamada H. Modulation of chemokine expression on intestinal epithelial cells by Kampo (traditional Japanese herbal) medicine, Hochuekkito, and its active ingredients. *J Nat Med.* 2013;67(3):626-635.
38. Russell DL, Robker RL. Molecular mechanisms of ovulation: coordination through the cumulus complex. *Hum Reprod Update.* 2007;13(3):289-312.
39. Richani D, Gilchrist RB. The epidermal growth factor network: role in oocyte growth, maturation and developmental competence. *Hum Reprod Update.* 2018;24(1):1-14.
40. Zhang H, Lu S, Xu R, et al. Mechanisms of estradiol-induced EGF-like factor expression and oocyte maturation via G protein-coupled estrogen receptor. *Endocrinology.* 2020;161(12):bqaa190. <https://doi.org/10.1210/endo/bqaa190>
41. Kim J-W, Park H-J, Yang S-G, et al. Exogenous ganglioside GT1b enhances porcine oocyte maturation, including the cumulus cell expansion and activation of EGFR and ERK1/2 signaling. *Reprod Sci.* 2020;27(1):278-289.
42. Yamashita Y, Kawashima I, Yanai Y, Nishibori M, Richards JS, Shimada M. Hormone-induced expression of tumor necrosis factor alpha-converting enzyme/A disintegrin and metalloprotease-17 impacts porcine cumulus cell oocyte complex expansion and meiotic maturation via ligand activation of the epidermal growth factor receptor. *Endocrinology.* 2007;148(12):6164-6175.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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