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난소적출 마우스에서 지백지황탕 열수 추출물의 갱년기 장애 개선 효과 평가

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ABSTRACT

Aqueous Extracts of *Jibaekjihwang-tang* Ameliorate Ovariectomy-induced Climacterium Symptoms in Mouse

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Objectives: The purpose of this study was to evaluate the anti-climacterium effects of *Jibaekjihwang-tang* (JBJHT), especially on estrogenic, anti-obesity, hypolipidemic, hepatoprotective against fatty liver and anti-osteoporotic effects by Ovariectomy (OVX) mice.

Methods: In order to evaluate anti-climacterium effects of JBJHT, we used bilateral OVX female ddY mice. In this study, six groups were used; sham control, OVX control, estradiol, JBJHT 500, 250 and 125 mg/kg treated groups. Since 28 days after OVX surgery, JBJHT extracts were orally treated, and 17β-estradiol 0.03 μg/head were subcutaneously injected for 84 days, once a day. And then, we observed anti-climacterium effects classified into five categories; estrogenic, anti-obesity, hypolipidemic, hepatoprotective against fatty liver and anti-osteoporotic effects. The results were compared with 17β-estradiol 0.03 μg/head/day subcutaneous treated OVX mice.

Results: OVX control mice showed noticeable hypertrophic changes of adipocytes in abdominal fat pads, fatty liver, uterine atrophic changes, decreases of bone strength were also observed in OVX control. However, these estrogen-deficient climacterium symptoms were significantly and dose-dependently inhibited by JBJHT 500, 250 and 125 mg/kg treatment. Moreover, JBJHT 500 mg/kg showed comparable inhibitory effects as compared to those of estradiol 0.03 μg/head/day subcutaneous treatment.

Conclusions: The results suggest that oral administration of JBJHT 500, 250 and 125 mg/kg has clear dose-dependent anti-climacterium effects in OVX mice.

Key Words: *Jibaekjihwang-tang*, Ovariectomy, Climacterium, Estradiol

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Ⅰ. Introduction

Climacteric disturbance is a complicated symptom of perimenopausal women those entering menopause. In this period, many women suffer from multi-abnormal conditions, such as hot flash, sweat, depression, fatigue, insomnia, vaginal dryness and dyspareunia[1]. Some women also experience cognitive impairment, irritability, psychological symptoms, and increased risk for osteoporosis and cardiovascular disease[2]. These symptoms are closely related to estrogen deficiency and may also occur as secondary effects of obesity arising out of estrogen deficiency[3].

In western medicine, they usually employ hormone therapy on climacteric symptoms. But it increases risk of developing phlebothrombosis and coronary artery disease. Moreover, it might cause a high incidence of breast cancer in the long-term treatment[4].

In Korean Medicine, the climacterium symptoms have been considered from various perspectives. The typical reason of climacterium is renal deficiency (腎虛). ≪Somun・Sanggocheonjinnon≫ said that women can't be pregnant anymore after age 49, because Conception Vessel (任脈), Thoroughfare Vessel (太衝脈) and blood (血) are infirm with age[5]. This means that women reach menopause around 49, and it corresponds with the fact that modern women go through menopause 45-55[1]. There are various studies on climacteric disturbance in Korean Medicine[6-9].

Jibaekjihwang-tang (JBJHT) was recorded in ≪Euijonggeumgam[10]≫ for the first time. It is based on *Yukmijihwang-tang* (YMJHT) that has been used usually for renal disorders, diabetic mellitus and neurosis[11]. There were several reports dealing with the effectiveness of JBJHT, a case report of pigmented purpuric dermatosis[12] and experiment report of anti-hypertensive effects[13].

The reports about climacteric disturbance caused by renal deficiency (腎虛) are insufficient up to date. And the experimental study of anti-climacterium potentials of JBJHT has not been reported yet upon my knowledge. So I've intended to observe JBJHT's anti-climacterium effects; estrogenic, anti-obesity, hypolipidemic, hepatoprotective against fatty liver and anti-osteoporotic effects. And compared with 17β-estradiol subcutaneous administration, which is widely used as anti-climacterium treatment. Upon investigation, I ascertained that JBJHT has significant effect on menopausal disorder.

Ⅱ. Materials and Methods

1. Preparation and administration of test materials

Eight types of herbs consists of JBJHT were purchased from local vouche (Jecheon Hanbang Yakcho, Jecheon, Korea) after confirm the morphology under microscopy. The composition of individual herbs and

product regions were listed below (Table 1).

Total 174 g of JBJHT were boiled in 2,000 ml of distilled water for 4 hrs and 3 times at 60℃, and evaporated using automated round flasked evaporator (Eyela N-1110, Tokyo, Japan), then completely lyophilized. Total 34.38 g (yield = 19.76%) of lyophilized aqueous JBJHT extracts were acquired. JBJHT extracts, the light brown colored powder, were stored at -20℃ in a refrigerator to protect from light and humidity until used. JBJHT extracts were well dissolved up to 50 mg/ml in distilled water, at least. 17β-estradiol (Sigma-Aldrich, St. Louise, MO, USA), white powder used as the reference drug, was stored in a refrigerator at 4℃.

Table 1. Composition of JBJHT

Herbs	Scientific name	Korean name	Amounts (g)
Rehmanniae Radix Preparata	Rehmannia glutinosa (Gaertner) Liboschitz ex Steudel	熟地黃	16.00
Dioscoreae Rhizoma	Dioscorea batatas Decaisne	山 藥	8.00
Corni Fructus	Cornus officinalis Siebold et Zuccarini	山茱萸	8.00
Poria (*Hoelen*)	Poria cocos (Schw.) Wolf	白茯苓	6.00
Moutan Cortex Radicis	Paeonia suffruticosa Andrews	牧丹皮	6.00
Alismatis Rhizoma	Alisma orientale Juzepzuk	澤 瀉	6.00
Anemarrhenae Rhizoma	Anemarrhena asphodeloides Bunge	知 母	4.00
Phellodendri Cortex	Phellodendron amurense Ruprecht	黃 柏	4.00
Total	8 types		58.00

2. Animals and husbandry

Total one hundred healthy virgin female specific pathogen-free (SPF) outbred-mice, Kwl:ddY mice (6-week old upon receipt; Kiwa Laboratory Animal, Wakayama, Japan; Body weight ranged in 24-26 g upon receipt), were used after acclimatization for 16 days. Animals were devided into four to five in each polycarbonate cage, in a temperature (20-25 ℃) and humidity (50-55%) controlled room. Light : dark cycle was 12 hr : 12 hr, and standard rodent chow (Samyang, Seoul, Korea) and water were supplied free to access. After 27 days of OVX operation, eight mice in each group (total six groups) were selected based on the body weight deviations (Average 31.40±1.31 g of OVX mice, ranged in 29.1-33.7 g; Average 28.21±1.26 g of sham-operated mice, ranged in 26.4-30.1 g, respectively) as follows. All the animals were treated according to the national regulations of the usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) prior to animal experiment [Approval No DHU2015-031, April 13, 2015 (ANNEX)].

3. Experimental groups

Eight mice in each group, total six groups, were selected based on the body weight deviations. They were devided into sham control group, OVX control group, Estradiol reference group, and three different dosage of JBJHT (500, 250 and 125 mg/kg) as a experimental group (Table 2). From 28 days after OVX, JBJHT 500, 250 and 125 mg/kg were orally administered, once a day for 84 days, respectively. In addition, 17β-estradiol (Sigma-Aldrich, St. Louise, MO, USA) 0.03 μg was suspended in 0.2 ml of sterilized saline, and subcutaneously injected on the dorsal back skins in a volume of 0.2 ml/mouse (0.03 μg/head/day) according to the previous established methods[14]. In OVX and sham control mice, only distilled water were orally administered by equal volumes and periods, instead of herbal formulas, in this experiment.

Table 2. Experimental Design

Groups	Operation	Group identification	Treatment
Control	Sham surgery	Sham control	Distilled water 10 ml/kg/day, oral gavage
Control	OVX	OVX control	Distilled water 10 ml/kg/day, oral gavage
Reference	OVX	Estradiol	17β-estradiol 0.03 μg/head/day, subcutaneous injection
Experimental	OVX	The highest dosages	JBJHT 500 mg/kg/day, oral gavage
Experimental	OVX	The middle dosages	JBJHT 250 mg/kg/day, oral gavage
Experimental	OVX	The lowest dosages	JBJHT 125 mg/kg/day, oral gavage

4. Menopause inducement-bilateral OVX

Mice were anesthetized with 2 to 3% isoflurane (Hana Pharm. Co., Hwasung, Korea) in the mixture of 70% N_2O and 28.5% O_2, and were maintained with 1 to 1.5% isoflurane in the mixture of 70% N_2O and 28.5% O_2. The surgical protocol was carried out according to established methods[15] as follows. The OVX treatment group underwent open surgery involving bilateral OVX by incision of *linea alba*. Following surgery, the incision was closed in two layers. The muscular layers were sutured independently from peripheral tissues using dissolvable 3-0 vicryl sutures, and the skin was closed by continuous sutures using silk 3-0. The second group underwent a sham operation that similar incision in the *linea alba* was made, but bilateral OVX was not performed.

5. Body weight measurement

Changes of body weight were measured once a week, at least from at OVX, 1 day before administration, to sacrifice using an automatic electronic balance (Precisa Instrument, Zuerich, Switzland), respectively. At OVX, and from initiation

of administration to termination, all experimental animals were overnight fasted (water was not; about 18 hrs) to reduce the differences from feeding. In addition, body weight gains were calculated as follow.

EQUATION [1]. Body weight gains (g)

OVX recovery/induced periods (28 days) = Body weight at initial test substance treatment - body weight at the day of OVX surgery

After administration (84 days) = Body weight at sacrifice - body weight at start of administration

6. Measurement of BMD and body fat density

The mean BMD of total body and right femur were detected by *in live* dual-energy x-ray absorptionmetry (DEXA; InAlyzer, Medikors, Seungnam, Korea) with mean fat densities on the body and abdominal cavity regions of each mouse, respectively.

7. Organ weight measurement

At sacrifice, the uterus including vagina was collected after eliminations of the surrounding connective tissues, muscles and then measured at g levels regarding absolute wet-weights. To reduce the individual body weight differences, the relative weights (% of body weight) were also calculated as follow.

EQUATION [2]. Relative organ weights (% of body weight)

= [(Absolute abdominal fat pad, uterus or liver weights/Body weight at sacrifice) ×100]

8. Bone weight measurement

At sacrifice, the right side of femur was collected after eliminate surrounding connective tissues, muscles and any debris. The bone weight was measured at g levels regarding absolute wet-weight, and they were dried at 120℃ for 8 hrs in high temperature dry oven (LDO-080N, Daihan Labtech Co., Seoul, Korea) for measurements of dry bone weight. After that, dried bones were carbonized at 800℃ for 6 hrs in furnace (LEF-1055-1, Daihan Labtech Co., Seoul, Korea) regarded as ash absolute weight. To reduce the individual body weight differences, the relative weight (%) was also calculated based on the body weight at sacrifice and absolute wet/dry/ash weight as follow.

EQUATION [3]. Relative bone weights (% of body weight)

= [(Absolute bone weight/Body weight at sacrifice)×100]

9. Measurement of bone strength

Bone strength was detected as failure load (FL). FL of mid-shaft regions of right femur was detected by a three-point bending test to failure using computerized testing machine (SV-H1000, Japan Instrumentation System Co., Japan) as *N* (Newton), respectively.

10. Serum biochemistry

For serum biochemistry, 1 ml of whole

blood was collected from *vena cava* at sacrifice, and separated the serum by centrifugation at 15,000 rpm for 10 minutes under 4℃, using clotting activated serum tube. All serum samples were frozen at -150℃ using ultra deep freezer (Model MDF-1156, Sanyo, Tokyo, Japan) until they were assayed.

Serum AST, ALT, TC, LDL and TG levels were detected using an automated blood analyzer (Hemagen Analyst; Hemagen Diagnostic, Columbia, MD, USA), and HDL levels were also measured by other type automated blood analyzer (AU400; Olympus, Tokyo, Japan). Serum AST and ALT levels were detected as U/L levels, and serum TC, LDL, TG and HDL levels were detected as mg/dl levels. In addition, serum osteocalcin levels were detected using Mouse Osteocalcin ELISA Kit (Immutopics, San Clemente, CA, USA) as ng/ml levels, serum bALP levels were detected by Mouse bALP ELISA kit(Quidel Corp., San Diego, CA, USA) as U/ml with ELISA Reader (Tecan, Männedorf, Switzerland), respectively. In addition, serum estradiol contents were also measured using the chemiluminescent immunoassay technique (ECLIA, Roche e411 immunoassay analyzer, Roche, Penzberg, Germany) as pg/ml from the separated serum at sacrifice in all individual mice, respectively.

11. Histological procedures for abdominal fat pads, uterus and liver

After measuring of organ weight, regular parts of the abdominal fat pads containing full thicknesses, left uterus horn and left lateral lobes of liver were sampled and crossly trimmed. Sampled tissues were fixed in 10% neutral buffered formalin (NBF). After paraffin embedding, 3-4 μm serial sections were prepared. Representative sections were stained with hematoxylin and eosin (H&E) for light microscopical examination. In addition, portions of liver that had been dehydrated in 30% sucrose solutions were sectioned by cryostat for staining the lipids with oil red[16]. After that, the histological profiles of individual organs were observed. To observe more detail changes, total thickness of abdominal fat pads were measured using an automated image analysis process (*i* Solution FL ver9.1, IMT *i*-solution Inc., Quebec, Canada) as mm/mouse, and mean diameters of dorsal abdominal white adipocytes were calculated in restricted view fields on a computer monitor using an automated image analysis process as μm; at least 10 white adipocytes per each fat pads were considered as histomorphometrical analysis. In addition, total-full, mucosa and epithelial thicknesses of the uterus (μm/uterus) were also detected with percentages of uterine glands located in the mucosa (%/mucosa of uterus) using an automated image analyzer, respectively. To observe steatosis in the liver, percentage of fatty change regions in hepatic parenchyma was calculated as percentages in 1 field of liver (%/mm^2 of hepatic parenchyma) under oil red

staining, and mean diameters of hepatocytes were calculated in restricted view fields on a computer monitor under H&E staining using an automated image analysis process as μm; at least 10 hepatocytes per each liver were considered. The histopathologist was blinded to group distribution when this analysis was made.

12. Statistical analysis

All the data was expressed as mean± standard deviations (SD) of eight mice. Multiple comparison tests of different dose groups were conducted. Variance homogeneity was examined using the Levene test[17]. If the Levene test indicated no significant deviations from variance homogeneity, it was analyzed by one way ANOVA test followed by least-significant differences multi-comparison (LSD) test to determine which pairs of group comparison was significantly different. In case of significant deviations from variance homogeneity which was observed by Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. If a significant difference has observed in the Kruskal-Wallis H test, the Mann-Whitney U (MW) test was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, IBM SPSS Inc., Armonk, NY, USA)[18].

In addition, the percent changes between sham and OVX control mice were calculated to observe the severities of induced climacteric symptoms, estrogen depletion, obese, hyperlipidemia, hepatic steatosis and osteoporosis induced in this study, and the percent changes as compared with OVX control mice and test material treated mice were also calculated to help the understanding of the anti-climacterium effects of test substances as follow, according to previous method described by Kang et al.[16], respectively.

EQUATION [4]. Percent changes as compared with sham control (%)

=[((Data of OVX control - Data of sham control mice)/Data of sham control mice)×100]

EQUATION [5]. Percent changes as compared with OVX control (%)

=[((Data of test material treated mice - Data of OVX control)/Data of OVX control)×100]

Ⅲ. Results

1. Effects on body weight and gain

We selected eight mice per group showing more increases of body weight as compared with sham-operated mice, and regarded as good OVX animals at 27 days after OVX surgery (Average 31.40± 1.31 g of OVX mice, ranged in 29.1-33.7 g; Average 28.21±1.26 g of sham-operated mice, ranged in 26.4-30.1 g, respectively). And significant ($p<0.01$) increases of body weight were detected in all OVX mice as compared with sham control mice.

However, significant ($p<0.01$) decreases of body weight were detected in estradiol treated mice from 28 days after initial treatment, and from 35 days after initial treatment in JBJHT 500 mg/kg treated mice, from 42 and 56 days in JBJHT 250 and 125 mg/kg treated mice as compared with OVX control mice, respectively. In addition, all test substance treated mice showed significant ($p<0.01$) decreases of body weight gain during 84 days of treatment periods as compared with OVX control group, in this experiment (Table 3, Fig. 1).

Table 3. Changes of the Body Weight Gain in OVX Mice

Groups	Body weight (g)				Body weight gain (g)	
	At OVX [A]*	At 27 days after OVX [B]	At first treatment [C]*	At sacrifice [D]*	OVX recovery [B-A]	Treatment [D-C]
Control						
Sham	24.45±1.26	28.21±1.26	25.21±1.28	29.74±1.56	3.76±0.35	4.53±1.15
OVX	24.49±1.17	31.33±1.33[a]	28.36±1.35[a]	43.86±1.31[a]	6.84±0.38[a]	15.50±1.75[a]
17β-Estradiol						
0.03 μg/head	24.44±0.94	31.44±1.31[a]	28.38±1.15[a]	36.29±1.83[ab]	7.00±0.52[a]	7.91±1.76[ab]
JBJHT						
500 mg/kg	24.56±1.50	31.34±1.47[a]	28.28±1.48[a]	36.31±2.08[ab]	6.78±0.18[a]	8.04±1.89[ab]
250 mg/kg	24.39±1.27	31.50±1.45[a]	28.39±1.33[a]	38.29±2.23[ab]	7.11±0.38[a]	9.90±1.30[ab]
125 mg/kg	24.44±1.27	31.40±1.38[a]	28.46±1.10[a]	39.40±1.31[ab]	6.96±0.30[a]	10.94±0.87[ab]

*All animals were overnight fasted.

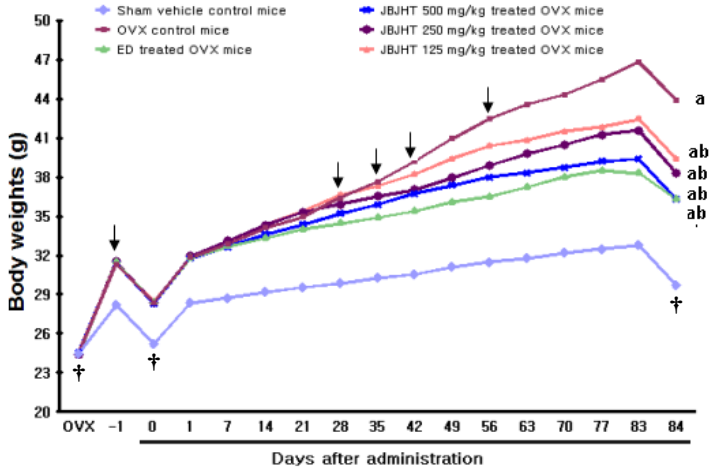

Fig. 1. Body weight changes in OVX mice.
[a] ($p<0.01$) as compared with sham control by LSD test
[b] ($p<0.01$) as compared with OVX control by LSD test
(†)All animals were overnight fasted.

2. Effects on the body fat densities

The total body and abdominal fat densities of OVX control mice were significantly ($p<0.01$) increased, as compared with sham control mice. However, significant ($p<0.01$) decreases of total body and abdominal fat densities were observed in all test substance administrated mice as compared with OVX control mice, respectively (Table 4).

Table 4. Changes of the Body Fat Density in OVX Mice

Groups	Fat density (%)	
	Total body	Abdominal
Control		
Sham	11.35±1.93	12.49±2.07
OVX	37.34±3.22[a]	40.09±4.24[c]
17β-Estradiol		
0.03 μg/head	21.11±3.85[ab]	22.53±4.23[cd]
JBJHT		
500 mg/kg	20.90±2.97[ab]	23.17±3.53[cd]
250 mg/kg	26.20±3.95[ab]	27.89±4.68[cd]
125 mg/kg	30.63±1.79[ab]	32.60±2.01[cd]

[a] ($p<0.01$) as compared with sham control by LSD test
[b] ($p<0.01$) as compared with OVX control by LSD test
[c] ($p<0.01$) as compared with sham control by MW test
[d] ($p<0.01$) as compared with OVX control by MW test

3. Effects on the serum Estradiol

Significant ($p<0.01$) decreases of the serum estradiol levels were observed in OVX control mice, as compared with sham control mice. However, significant ($p<0.01$) increases of the serum estradiol levels were observed in estradiol, JBJHT 500, 250 and 125 mg/kg treated mice as compared with OVX control mice, respectively, in this study (Fig. 2).

The serum estradiol levels in OVX control mice were changed as -78.81% as compared with sham control mice, and they were changed as 318.93, 216.05, 154.53 and 108.02% in estradiol, JBJHT 500, 250 and 125 mg/kg treated mice as compared with OVX control mice, respectively.

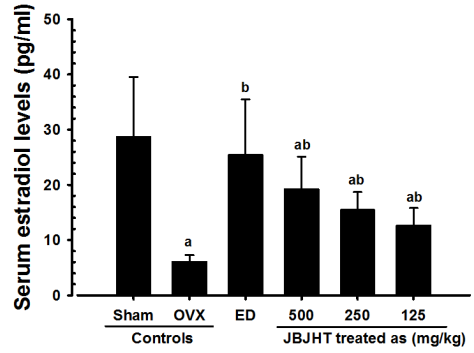

Fig. 2. Changes of the serum estradiol levels in OVX mice.
[a] ($p<0.01$) as compared with sham control mice by MW test
[b] ($p<0.01$) as compared with OVX control mice by MW test

4. Effects on the uterus weight

Significant ($p<0.01$) decreases of the uterus absolute and relative wet-weight were observed in OVX control mice as compared with sham control mice, respectively. However, significant ($p<0.01$) increases of the uterus weight were observed in all test substance treated mice, as compared with OVX control mice, respectively (Table 5).

Table 5. Changes of the Uterus Weight in OVX Mice

Groups	Absolute wet-weight (g)	Relative wet-weight (% of body weight)
Control		
Sham	0.270±0.095	0.906±0.319
OVX	0.026±0.010[a]	0.059±0.022[a]
17β-Estradiol		
0.03 μg/head	0.121±0.042[ac]	0.338±0.136[ac]
JBJHT		
500 mg/kg	0.117±0.046[ac]	0.325±0.136[ac]
250 mg/kg	0.075±0.016[ac]	0.197±0.042[ac]
125 mg/kg	0.063±0.017[ac]	0.160±0.047[ac]

[a] ($p<0.01$) and [b] ($p<0.05$) as compared with sham control by MW test
[c] ($p<0.01$) and [d] ($p<0.05$) as compared with OVX control by MW test

5. Effects on the serum AST, ALT, TC, LDL, HDL and TG

Significant ($p<0.01$) increases of the serum AST, ALT, TC, LDL and TG levels, and decreases of serum HDL levels were observed in OVX control mice as compared with sham control mice, respectively. However, significant ($p<0.01$ or $p<0.05$) decreases of the serum AST, ALT, TC, LDL and TG levels, and significant ($p<0.01$) increases of the serum HDL levels were observed in all test material treated mice as compared with OVX control mice, in this study (Table 6).

Table 6. Changes of the Serum AST, ALT, TC, LDL, HDL and TG Levels in OVX Mice

Groups	Serum biochemistry					
	AST (U/L)	ALT (U/L)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TG (mg/dl)
Control						
Sham	81.38±17.79	37.50±10.58	85.25±22.05	63.25±12.68	97.38±11.08	38.13±13.21
OVX	166.63±20.99[a]	83.63±16.94[a]	203.13±28.09[a]	178.75±19.65[a]	40.25±11.78[a]	168.00±26.27[c]
17β-Estradiol						
0.03 μg/head	107.00±13.18[ab]	54.38±10.24[ab]	150.38±21.54[ab]	118.75±18.87[ab]	75.75±12.84[ab]	94.75±13.93[cd]
JBJHT						
500 mg/kg	106.25±10.98[ab]	54.25±12.48[ab]	149.88±15.31[ab]	115.00±25.39[ab]	77.00±15.66[ab]	96.25±17.64[cd]
250 mg/kg	112.88±15.70[ab]	61.00±12.66[ab]	155.25±23.16[ab]	130.25±18.69[ab]	66.88±10.96[ab]	111.63±21.35[cd]
125 mg/kg	129.38±11.08[ab]	65.13±8.54[ab]	164.50±16.37[ab]	142.88±14.37[ab]	58.25±11.85[ab]	124.75±22.48[ce]

[a] ($p<0.01$) as compared with sham control by LSD test
[b] ($p<0.01$) as compared with OVX control by LSD test
[c] ($p<0.01$) as compared with sham control by MW test
[d] ($p<0.01$) and e ($p<0.05$) as compared with OVX control by MW test

6. Effects on the bone weight

Significant ($p<0.01$) decreases of the femur relative wet-weight, and absolute and relative dry and ash weight were observed in OVX control mice as compared with sham control mice, respectively. However, marked and significant ($p<0.01$ or $p<0.05$) increases of the femur relative wet-weight, and absolute and relative dry and ash weight were demonstrated

in all test substance treated mice, as compared with OVX control mice, in this study (Table 7).

Table 7. Changes of the Right Femur Weight in OVX Mice

Groups	Absolute weight (g)			Relative weight (% of body weight)		
	Wet	Dry	Ash	Wet	Dry	Ash
Control						
Sham	0.092±0.013	0.072±0.010	0.047±0.006	0.310±0.045	0.243±0.036	0.157±0.023
OVX	0.089±0.006	0.049±0.004[a]	0.025±0.004[a]	0.203±0.017[a]	0.111±0.009[d]	0.056±0.008[d]
17β-Estradiol						
0.03 μg/head	0.095±0.008	0.058±0.006[ab]	0.037±0.003[ab]	0.262±0.029[ab]	0.161±0.017[de]	0.102±0.014[de]
JBJHT						
500 mg/kg	0.094±0.010	0.058±0.005[ab]	0.036±0.004[ab]	0.261±0.034[ab]	0.161±0.019[de]	0.098±0.009[de]
250 mg/kg	0.093±0.008	0.057±0.005[ac]	0.033±0.003[ab]	0.244±0.027[ab]	0.149±0.010[de]	0.087±0.007[de]
125 mg/kg	0.092±0.007	0.055±0.004[ac]	0.032±0.004[ab]	0.234±0.021[ac]	0.140±0.009[de]	0.081±0.011[de]

[a] ($p<0.01$) as compared with sham control by LSD test
[b] ($p<0.01$) and [c] ($p<0.05$) as compared with OVX control by LSD test
[d] ($p<0.01$) as compared with sham control by MW test
[e] ($p<0.01$) as compared with OVX control by MW test

7. Effects on the serum Osteocalcin and bALP

Significant ($p<0.01$) increases of the serum osteocalcin levels, and decreases of serum bALP levels in OVX control mice were observed as compared with sham control mice. However, significant ($p<0.01$) decreases of the serum osteocalcin and increases of bALP levels were observed in estradiol and all test herbal formula treated mice as compared with OVX control mice, respectively, in this study (Fig. 3, 4).

The serum osteocalcin and bALP levels in OVX control mice were changed as 122.92 and -54.33% as compared with sham control mice, and they were changed as -36.55, -38.14, -31.96 and -25.13% of osteocalcin levels, and 59.28, 60.95, 43.27 and 31.82% of bALP levels in estradiol, JBJHT 500, 250 and 125 mg/kg treated mice as compared with OVX control mice, respectively.

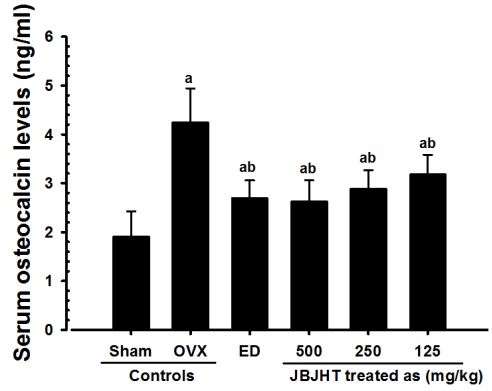

Fig. 3. Changes of the serum osteocalcin levels in OVX mice.
[a] ($p<0.01$) as compared with sham control by LSD test
[b] ($p<0.01$) as compared with OVX control by LSD test

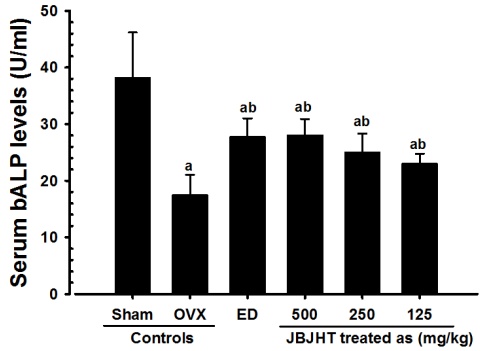

Fig. 4. Changes of the serum bALP levels in OVX mice.
[a] ($p<0.01$) as compared with sham control by MW test
[b] ($p<0.01$) as compared with OVX control by MW test

8. Effects on BMD and FL

The total body and femur mean BMD of OVX control mice were significantly ($p<0.01$) decreased as compared with sham control mice. However, significant ($p<0.01$) increases of total body and femur mean BMD were observed in estradiol and all three different dosages of JBJHT administrated mice as compared with OVX control mice, in this study (Table 8).

The strength (FL) of femur mid-shaft regions in OVX control mice was significantly ($p<0.01$) decreased as compared with sham control mice. However, significant ($p<0.01$ or $p<0.05$) increases of FL of femur were observed in all test substance administrated mice including estradiol and three different dosages of JBJHT, as compared with OVX control mice, in this study (Table 8).

Table 8. Changes of the Bone Mineral Density and Strength in OVX Mice

Groups	Bone mineral density (g/cm^2)		Bone strength (Newton)
	Total body	Femur (right)	Femur
Control			
Sham	0.0248±0.0017	0.0270±0.0011	21.84±4.76
OVX	0.0207±0.0010[d]	0.0226±0.0008[a]	9.53±2.65[a]
17β-Estradiol			
0.03 μg/head	0.0228±0.0007[ef]	0.0254±0.0008[ab]	16.79±3.06[ab]
JBJHT			
500 mg/kg	0.0228±0.0011[ef]	0.0254±0.0011[ab]	16.40±2.63[ab]
250 mg/kg	0.0224±0.0004[df]	0.0246±0.0008[ab]	15.14±3.40[ab]
125 mg/kg	0.0220±0.0005[dg]	0.0240±0.0007[ab]	13.81±2.55[ac]

[a] ($p<0.01$) as compared with sham control by LSD test
[b] ($p<0.01$) and [c] ($p<0.05$) as compared with OVX control by LSD test
[d] ($p<0.01$) and [e] ($p<0.05$) as compared with sham control by MW test
[f] ($p<0.01$) and [g] ($p<0.05$) as compared with OVX control by MW test

9. Histopathology: Abdominal fat pad, uterus and liver

Significant ($p<0.01$) increases of the thickness of abdominal fat pads, which is deposited into left abdominal muscles, and also increases of mean adipocyte diameters were observed in OVX control mice as compared with sham control mice. However, significant ($p<0.01$) decreases of the thickness of abdominal fat pads

and mean diameters of adipocytes were observed in all test substance administrated mice as compared with OVX control mice, in this study (Table 9).

Significant ($p<0.01$) decreases of the total, mucosa and epithelial thicknesses of the uterus, and percentages of uterine glands in the mucosa were observed in OVX control mice as compared with sham control mice. However, significant ($p<0.01$ or $p<0.05$) increases of the total, mucosa and epithelial thicknesses of the uterus, and percentages of uterine glands in the mucosa were observed in estradiol, JBJHT 500 and 250 mg/kg treated mice as compared with OVX control mice. In addition, JBJHT 125 mg/kg treated mice also showed significant ($p<0.01$) increases of the epithelial and mucosal thicknesses of the uterus, and non-significant but marked increases of the total uterus thicknesses and the percentages of uterine glands in the mucosa, as compared to those of OVX control mice, respectively (Table 9).

Significant ($p<0.01$) increases of the fatty change regions and mean diameters of hepatocytes were noticed in OVX control mice as compared with sham control mice. However, significant ($p<0.01$ or $p<0.05$) decreases of fatty change regions and mean diameters of hepatocytes were observed in all test substance administered mice as compared with OVX control mice, in this study (Table 9).

Table 9. Changes of the Histopathology-Histomorphometry for Abdominal Fat Pads, Uterus and Liver in OVX Mice

Groups	Fat pads		Uterus				Liver	
	Total Th (mm)	Adipocyte DM (μm)	Total Th (mm)	Epi Th (μm)	Mucosa Th (μm)	UG percentage (%)	FC region (%)	Hepatocyte DM (μm)
Control								
Sham	1.44 ±0.46	41.29 ±10.63	2.44 ±0.46	30.45 ±6.51	852.11 ±121.23	52.01 ±10.54	11.44 ±5.20	13.65 ±2.11
OVX	5.97 ±0.97[a]	128.21 ±24.80[a]	0.56 ±0.11[a]	5.73 ±1.66[a]	196.73 ±88.36[a]	14.36 ±3.82[a]	75.19 ±11.31[a]	29.98 ±5.43[a]
17β-Estradiol								
0.03 μg/head	2.58 ±0.55[ac]	68.03 ±16.38[ac]	1.27 ±0.34[ac]	21.59 ±4.06[ac]	537.19 ±103.61[ab]	34.99 ±11.13[ac]	37.49 ±10.45[ac]	19.18 ±1.96[ac]
JBJHT								
500 mg/kg	2.57 ±0.88[ac]	65.10 ±17.54[bc]	1.06 ±0.17[ac]	19.18 ±4.80[ac]	475.67 ±106.01[ac]	34.23 ±10.08[ac]	38.62 ±16.25[ac]	19.45 ±3.17[ac]
250 mg/kg	3.08 ±0.72[ac]	71.45 ±18.54[ac]	0.86 ±0.22[ad]	15.13 ±3.66[ac]	432.49 ±98.57[ac]	28.92 ±11.50[ac]	57.09 ±10.93[ac]	21.95 ±2.38[ac]
125 mg/kg	4.22 ±0.78[ac]	88.79 ±19.05[ac]	0.81 ±0.13[a]	14.10 ±4.39[ac]	379.02 ±78.62[ac]	23.41 ±6.77[a]	61.84 ±10.33[ad]	22.63 ±2.28[ac]

[a] ($p<0.01$) and [b] ($p<0.05$) as compared with sham control by LSD test
[c] ($p<0.01$) and [d] ($p<0.05$) as compared with OVX control by LSD test

Ⅳ. Discussion

Climacterium is a period of women's life those who are entering menopause caused by decline of reproductive capacity. It is related to degeneration of ovarian follicles, and this is the major cause of estrogen deficiency. In this period, various symptoms occur from physical to psychological problems and it is referred to climacteric disturbance[1]. According to a recent report, the symptoms appear in almost 70% of women in climacteric period, and 25% of them suffer considerable distress requiring a proper treatment[19].

Climacteric disturbance is classified into three categories: acute, subacute and chronic symptoms. The most common complaints of acute symptoms are vasomotor symptoms such as hot flash, sweat and palpitation and it comes with psychological problems such as depression, irritability and nervousness. Subacute symptoms are vaginal dryness, urinary urgency and dyspareunia caused by urogenital atrophy. Chronic symptoms are osteoporosis, cardiovascular disease and metabolic disease such as obesity, diabetes and hypertension[20,21].

In western medicine, hormone therapy is often employed on climacteric disturbance. However, recent report shows that estrogen increases risk of developing phlebothrombosis and coronary artery disease. Moreover, in case of more than 5 years' treatment, it can cause a high incidence of breast cancer[4]. Even though hormone therapy is the most effective treatment for hot flashes and urogenital atrophy, it leads to several side effects such as coronary artery disease, stroke, irregular bleeding, weight increase and anorexia[22].

In Korean Medicine, there are many case studies on climacteric disturbance. Since we consider that climacteric disturbance has various reasons, there are various treatments available with it; *Gamiguibi-tang*[6], *Danchisoyo-san*[7], *Shihogayonggolmoryo-tang*[8] *and Insamyangyeong-tang*[9]. There are also many experimental studies about effectiveness of herbal medicine and herbal acupunture on Estrogen-deficient animals induced by OVX[23-5]. However, since they are mainly inclined to effect for osteoporosis, it still leaves much to be desired.

Jibaekjihwang-tang (JBJHT) was recorded in ≪Euijonggeumgam[10]≫ for the first time. It is added *Anemarrhenae Rhizoma* (知母), *Phellodendri Cortex* (黃柏) to *Yukmijihwang-tang* (YMJHT) which has been used for several hundreds of years predominantly to treat renal disorders, diabetic mellitus and neurosis[11]. There were several reports dealing with the effectiveness of JBJHT, a case report of pigmented purpuric dermatosis[12] and experimental report of anti-hypertensive effects[13]. However, the study of anti-climacterium potentials of JBJHT has not been reported yet upon my knowledge.

Therefore, in this experiment, we've intended to observe the complex anti-

climacterium potentials of JBJHT aqueous extracts (yield=19.76%) with optimal dose ranges using bilateral OVX female ddY mice. Bilateral OVX female ddY mice are well-documented rodent model similar to women's climacterium symptoms, including cardiovascular diseases, obesity, hyperlipidemia, osteoporosis, organ steatosis and mental disorders[15]. In this study, we evaluated anti-climacteric effects in five categories; estrogenic effect, anti-obesity effect, hypolipidemic effect, hepatoprotective effect against fatty liver and anti-osteoporotic effect. After twenty-eight days of bilateral OVX surgery, three different dosages - 500, 250 and 125 mg/kg of JBJHT extracts were orally administered, once a day for 84 days. And then, we made observations of the changes of body weight and gain during experimental periods, serum TC, LDL, HDL, TG, AST, ALT, estradiol, osteocalcin and bALP levels, total body and abdominal fat densities, abdominal fat pad, liver and uterus weights, total body and femur BMD, femur weight and strength(FL).

In this study, compared with sham-operated control mice, OVX control mice showed the estrogen-deficient climacterium symptoms; increases of body weight and gains, food consumptions, accumulated body and abdominal fat mass densities, serum AST, ALT, TC, LDL, TG and osteocalcin levels, and decreases of uterus and femur weights, mean total body and femur BMD, femur strength, serum bALP and estradiol levels, respectively. In addition, marked hypertrophic changes of adipocytes in abdominal fat pads, fatty liver, uterine atrophic changes were observed by histopathological and histomorphometrical analysis. Consequently, obese, hyperlipidemia, fatty liver and osteoporosis were induced by bilateral OVX. However, these estrogen-deficient climacterium symptoms induced by OVX were significantly inhibited by 84 days of continuous treatment of estradiol, JBJHT 500, 250 and 125 mg/kg, respectively. Especially, JBJHT 500 mg/kg showed comparable inhibitory effects against estrogen-deficient climacterium symptoms as compared to those of estradiol 0.03 μg/head/day subcutaneous treatment.

Estrogen, which is mainly secreted by follicle and corpus luteum in ovary, is known as inhibiting leptin secrection, food consumption, lipogenesis in adipocyte[26]. In this study, OVX control group also showed significant increases of body weight and abdominal fat depositions with adipocyte hypertrophy. However, the obese related to these estrogen-deficiencies was inhibited by all of three different dosages treatment of JBJHT, dose-dependently.

Estrogen plays an important role in growing the structure and function of numerous female target organs such as uterus, vagina, and skeletal and cardiovascular systems[27]. Urogenital atrophy, caused by estrogen deficiency in climacteric women, brings about not only urinary urgency but also vaginal dryness, dyspareunia that may interfere with the sex life[28].

In this study, OVX control group induced significant decrease of uterine weights, serum estradiol levels and uterine atrophic changes as similar result to previous study[29]. However, these estrogen-deficient uterine atrophies induced by bilateral OVX were significantly inhibited by 84 days of continuous treatment of estradiol and three different dosages of JBJHT, dose-dependently.

Estrogen deficiency is one of the important risk factors for developing dyslipidemia. Previous studies reported significant increase of TC, LDL, TG, and decrease of HDL levels in postmenopausal women[30], and similar changes of serum lipids were also induced by OVX mice[31]. In this study, OVX induced significant increases of serum TC, LDL and TG levels, and decrease of serum HDL levels. However, OVX-induced hyperlipidemia were also significantly and dose-dependently inhibited by 84 days of continuous oral administration of all three different dosages of JBJHT and subcutaneous treatment of estradiol.

According to a recent report, fatty liver rates in postmenopausal women are higher than premenopausal women, and it is thought to be related to obese and hyperlipidemia caused by estrogen-deficiency[32]. And hypertrophy and fatty change of hepatocytes along with increased AST and ALT have observed in OVX rodents[33]. In this study, OVX control group induced significant increases of AST, ALT, and fatty liver. However, this fatty liver induced by bilateral OVX was significantly inhibited by 84 days of continuous treatment of estradiol and three different dosages of JBJHT, dose-dependently.

Osteoporosis is a metabolic bone disease that causes bone loss and increases frequency of fractures[34]. Since bone mineral density (BMD) and bone strengths are markedly decreased in postmenopausal women, it has been believed that osteoporosis is related to estrogen-deficiency[35]. According to recent studies, as a result of OVX induced osteoporosis, serum osteocalcin levels were increased but serum bALP levels were decreased[36]. Moreover, bone weight, BMD, bone strength were markedly decreased[37-9]. In this study, noticeable increases of osteocalcin levels were observed in OVX control mice with decreases of serum bALP levels, weight, and strength of the femur. However, this estrogen-deficient osteoporosis induced by bilateral OVX was significantly inhibited by 84 days of continuous treatment of estradiol, JBJHT 500, 250 and 125 mg/kg, respectively and JBJHT showed clear dose-dependent anti-osteoporotic activities.

The results suggest that oral administration of JBJHT has clear dose-dependent favorable anti-climacterium effects; estrogenic, anti-obesity, hypolipidemic, hepatoprotective against fatty liver and anti-osteoporotic activities in OVX mice. Therefore, it is expected that JBJHT will be promising as a novel alternative treatment for relieving the climacterium symptoms, especially on estrogen deficiency, obese,

hyperlipidemia, fatty liver and osteoporosis in menopausal women. The screening of the biological active compounds should be conducted in future with more detail mechanism studies.

Ⅴ. Conclusion

In this study, I reached the following results

1. Obese, related to Estrogen-deficiencies; increases of body weight, abdominal fat depositions with adipocyte hypertrophy, which is induced by bilateral OVX, was inhibited by all of three different dosages treatment of JBJHT, dose-dependently.
2. Estrogen-deficient uterine atrophies; decrease of uterine weights, total, mucosa and epithelial thicknesses, uterine glands in the mucosa, and serum estradiol levels, which is induced by bilateral OVX, was significantly inhibited by all three different dosages of JBJHT, dose-dependently.
3. Estrogen-deficient hyperlipidemia; increase of serum TC, LDL, TG, and decrease of HDL levels, which is induced by bilateral OVX, was also significantly and dose-dependently inhibited by all three different dosages of JBJHT.
4. Fatty liver, related to Estrogen-deficiencies; significant increases of AST, ALT, percentage of fatty change regions and mean diameters of hepatocytes, which is induced by bilateral OVX, was significantly inhibited by all three different dosages of JBJHT, dose-dependently.
5. Osteoporosis, related to Estrogen-deficiencies; significant increases of serum osteocalcin levels and decrease of serum bALP levels, Bone weights, BMD, bone strengths, which is induced by bilateral OVX, was significantly inhibited by all three different dosages of JBJHT, dose-dependently.

Therefore, oral administration of JBJHT improved ovariectomy-induced climacterium symptoms in mouse, and more researches should be done in future.

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국문초록

목 적: 본 연구에서는 지백지황탕 열수추출물(수율=19.76%)의 갱년기 장애 개선 효과를 확인하기 위해 난소적출(Ovariectomy, OVX) 마우스 모델을 이용하여 estrogen 유사 효과, 항비만 효과, 고지혈증 억제 효과, 지방간에 대한 보호 효과 및 골다공증 억제 효과의 5가지 생리활성 효과로 구분하여 평가하였다.

방 법: 본 연구에서는 지백지황탕의 갱년기 장애 개선 효과를 평가하기 위해 사람의 다양한 갱년기 장애와 유사한 증상을 나타내는 난소적출 마우스모델을 활용하였다. 총 6개의 실험군에서 각 8마리의 마우스를 사용하여 위수술 sham 대조군, OVX 대조군, Estradiol 대조 약물군, 지백지황탕 500, 250, 125 mg/kg 투약 실험군으로 나누어 실험하였다. OVX 수술 28일 후부터 지백지황탕 추출물을 각각 500, 250 and 125 mg/kg으로 매일 1 회씩 84일 동안 경구투여하고, 17β-estradiol 0.03 μg/head/day 피하 투여군과 비교 하여 estrogen 유사 효과, 항비만 효과, 고지혈증 억제 효과, 지방간에 대한 보호 효과 및 골다공증 억제효과로 구분하여 평가 하였다. 본 실험결과는 17β-estradiol 0.03 μg/head/day 피하 투여 마우스에서의 결과와 비교 평가 하였다.

결 과: 본 실험의 결과, OVX 대조군에서는 위수술 매체 대조군에 비해 현저한 체중 및 증체량, 체지방 및 복부 축적 지방량, 복부 축적 지방 중량, 혈청 중 AST, ALT, TC, LDL, TG 및 osteocalcin 함량의 증가가 자궁, 간 및 대퇴골 중량, 혈중 bALP 및 estradiol 함량, 평균 total body 골밀도 및 대퇴골 골밀도의 감소와 함께 인정되었으며, 현저한 복부 축적 지방 두께의 증가 및 지방세포의 비대, 간의 지방병증, 자궁의 불용성 위축, 대퇴골의 골량 감소 소견이 각각 인정되었다. 한편 이러한 OVX에 의해 유발된 estrogen 결핍성 폐경기 관련 갱년기 장애 소견이 지백지황탕 추출물 500, 250 및 125 mg/kg의 84일에 걸친 연속 투여에 의해 투여 용량 의존적으로 현저히 억제되었으며, 특히 지백지황탕 500 mg/kg은 estradiol 0.03 μg/head/day 피하투여군과 유사한 갱년기 장애 개선 효과를 OVX 마우스에서 나타내었다.

결 론: 이상의 결과에서, 지백지황탕 500, 250 및 125 mg/kg의 경구투여는 OVX 마우스에서 estrogen 결핍성 폐경기 관련 갱년기 장애(비만, 고지혈증, 간 지방병증 및 골다공증) 개선 효과를 투여 용량 의존적으로 나타내었다. 그러나 지백지황탕은 총 8종의 약제로 구성되어 있고, 각각 수많은 생리활성 물질을 함유하고 있어, 향후 생리활성을 나타내는 화학성분의 검색과 더불어 다양한 방면으로 기전적인 연구가 체계적으로 수행되어야 할 것으로 판단된다.

중심단어: 지백지황탕, 갱년기장애, 난소적출, 에스트라디올

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