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난소적출 마우스에서 固眞飮子 물 추출물의 골다공증 개선 효과

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ABSTRACT

Anti-osteoporotic Activity of *Gojineumja* Aqueous Extracts on the Ovariectomized Mice

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Objectives: The objective of this *in vivo* study is to observe the anti-osteoporotic activities of *Gojineumja* aqueous extracts (GJEJ) on the ovariectomized (OVX) mice as compared to those of risedronate sodium (RES).

Methods: Thirty five days after bilateral OVX, GJEJ was orally administered, for 35 days once a day and then the changes on the body weight and gain during experimental periods, femur weights, bone mineral density (BMD), bone strength (failure load), mineral contents - calcium (Ca) and inorganic phosphorus (IP), histological profiles and histomorphometrical analyses at sacrifice were conducted with serum biochemistry - osteocalcin contents and bone specific alkaline phosphatase (BALP) activities. And the results of GJEJ were compared with RES orally administered OVX mice.

Results: As a result of OVX, noticeable increase of body weight and gains and serum osteocalcin levels, decrease of serum BALP activities, femur weights, femur Ca and IP contents, BMD and strength were observed as compared to those of sham control mice, respectively. Also, the decrease of all histomorphometrical indices indicating the bone mass and structure, and the increase of indices about resorption were also detected in the femur of OVX control. However, these estrogen-deficient osteoporotic signs were significantly and dose-dependently inhibited by 35 days of continuous oral treatment of GJEJ, at dose levels of 500, 250 and 125 mg/kg, respectively. Especially, GJEJ 500 mg/kg showed favorable inhibitory activities against estrogen-deficient osteoporosis symptoms induced by OVX as comparable to those of RES 2.5 mg/kg.

Conclusions: The results in this study suggest that oral administrations of GJEJ have clear dose-dependent favorable anti-osteoporotic activities in OVX mice.

Key Words: Osteoporosis, *Gojineumja*, Ovariectomy (OVX)

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Ⅰ. Introduction

Osteoporosis is a systemic skeletal disorder that decreases bone strength and increases the risk of fracture[1]. In young adults, there is a balance between bone resorption and bone formation, but after 30 to 45 years of age, bone resorption exceeds bone formation. This phenomenon is further increased after women's menopause. This is because of estrogen deficiency, which results from the disruption of postmenopausal ovarian function and causes activation of new bone remodeling region and imbalance in the bone resorption and osteogenesis. Osteoporosis increases the frequency of hip, spine, and wrist fractures[2]. Hip fractures can cause serious problems in elderly patients which can reduce their quality of life[3].

In Korean Medicine literature, Gol-Wi (骨痿), Gol-Bi (骨痺), Gol-Go (骨枯), Gol-Geuk (骨極) that mean poor state of bone are most similar to osteoporosis, and the main reason of osteoporosis is renal deficiency (腎虛)[4,5].

Gojineumja (Guzhenyinzi in Chinese) is a polyherbal prescription that has been used as a tonic agent for long time, listed in ≪醫學入門≫[6] and ≪東醫寶鑑≫[7]. Until now, immunostimulatory[8], neuroprotective anti-dementia[9] and hepatoprotective activities[10] of *Gojineumja* aqueous extracts (GJEJ) have been revealed through animal experiments. The anti-osteoporotic activities of each herb in GJEJ has been well studied by other investigators[11,12]. However, there was no study on the effects of GJEJ, itself on osteoporosis induced by bilateral ovariectomy (OVX) in mice.

The aim of this study is to observe the anti-osteoporotic effects of GJEJ on the OVX-induced estrogen deficiency osteoporosis mice, a well documented animal model resemble to postmenopausal osteoporosis of women[13-5]. We investigated the changes on the body weight and gain, femur weights, serum biochemistry, bone mineral density (BMD), bone strength (failure load), mineral contents, histological profiles and histomorphometrical analyses. And the results of GJEJ were compared with Risedronate sodium (RES), a pyridyl bisphosphonate, binds to bone hydroxyapatites and inhibits osteoclast mediated bone resorption[16,17].

As a result of the experiment, it is thought that GJEJ showed significant anti-osteoporotic activity. So we report the experiment through this paper.

Ⅱ. Materials and methods

1. Preparations and administration of test materials

GJEJ (yield = 20.20%) as deep brown powders, were prepared by routine methods using rotary vacuum evaporator (N-1110, Eyela, Tokyo, Japan) and programmable freeze dryer (FDB-5503, Operon, Kimpo, Korea) from 2 folds (Total 96 g) of *Gojineumja*, which consists of 15 types of natural drugs (Total 48 g) (Table 1). Total 96 g of mixed herbs were boiled

in 1,000 ml of distilled water for 4 hrs at 60℃ 3 times, and evaporated by automated round flask evaporator (Eyela N-1110, Tokyo, Japan), and completely lyophilized. Total 19.39 g (yield = 20.20%) of lyophilized GJEJ were obtained, and stored in a refrigerator at -20℃ to protect it from light and humidity until used. The voucher specimens demonstrating this purchase and some specimens of GJEJ were kept in the Medical Research Center for Globalization of Herbal Formulation in Daegu Haany University (Code GJEJ2017KDC). RES was purchased from TEVA Tapi (Sheva, Israel), and also stored in a refrigerator at 4℃ to protect against light and degeneration. GJEJ was well dissolved upto 50 mg/ml concentration in distilled water, and upto 0.25 mg/ml in RES, at least.

Appropriated amounts of GJEJ were dissolved in distilled water in 50, 25 and 12.5 mg/ml concentrations, and administered by gastric gavages using a stainless steel zonde attached to 1 ml of syringe, in a volume of 10 ml/kg, equivalence to 500, 250 and 125 mg/kg, for 35 days once a day, from 5 weeks after OVX. RES was also dissolved in distilled water as 0.25 mg/ml concentrations, and orally administrated in a volume 10 ml/kg (as equivalence to 2.5 mg/kg), for 35 days once a day, from 5 weeks after OVX. In Sham-operated and OVX mice, equal volumes of vehicle (distilled water) were orally administered, instead of test substances to provide same restrain stresses from gastric gavage (Table 2). The dosages of GJEJ 500, 250 and 125 mg/kg were selected based on the previous *in vivo* efficacy studies of GJEJ[8-10], and the dosage of RES, 2.5 mg/kg was also selected based on the *in vivo* anti-osteoporosis efficacy studies[16,17], in the current experiment.

Table 1. Composition of GJEJ Used in This Study

Herbs	Scientific name	Korean name	Amounts (g)
Rehmanniae Radix Preparata	Rehmannia glutinosa Liboschitz ex Steudel	熟地黃	6
Dioscoreae Rhizoma	Dioscorea batatas Decaisne	山　藥	4
Ginseng Radix Alba	Panax ginseng C. A. Meyer	人　參	4
Angelicae Gigantis Radix	Angelica gigas Nakai	當　歸	4
Astragali Radix	Astragalus membranaceus Bunge	黃　芪	4
Phellodendri Cortex	Phellodendron amurense Ruprecht	黃　柏	4
Citri Unshii Pericarpium	Citrus unshiu Markovich	陳　皮	3.2
Poria (*Hoelen*)	Poria cocos (Schw.) Wolf	白茯苓	3.2
Eucommiae Cortex	Eucommia ulmoides Oliver	杜　仲	2.8
Glycyrrhizae Radix	Glycyrrhiza uralensis Fischer	甘　草	2.8
Atractylodis Rhizoma Alba	Atractylodes macrocephala Koidzumi	白　朮	2
Alismatis Rhizoma	Alisma orientale Juzepzuk	澤　瀉	2
Corni Fructus	Cornus officinalis Siebold et Zuccarini	山茱萸	2
Psoraleae Semen	Psoralea corylifolia Linne	破古紙	2
Schisandrae Fructus	Schisandra chinensis Baillon	五味子	2
Total	15 types		48.00

Table 2. Experimental Design Used in This Study

Groups	Surgery	Group identification	Treatment
Control	Sham	Sham control	Distilled water 10 ml/kg/day
	OVX	OVX control	Distilled water 10 ml/kg/day
Reference	OVX	RES	Risedronate sodium 2.5 mg/kg/day
Experimental	OVX	GJEJ 500	The highest dosages of GJEJ 500 mg/kg/day
	OVX	GJEJ 250	The middle dosages of GJEJ 250 mg/kg/day
	OVX	GJEJ 125	The lowest dosages of GJEJ 125 mg/kg/day

2. Animals and husbandry

Total 85 virgin female SPF/VAF outbred-mice, CrljOri:CD1[ICR] mice (7-week old on receipt; OrientBio; Seungnam, Korea) were used after 7 days acclimatization. Animals were assigned four per polycarbonate cage at temperature (20-25℃) and humidity (45-55%) controlled room. And Light : dark cycle of their cage was 12 hrs : 12 hrs. Water and standard rodent chow (Cat. No. 38057; Purinafeed, Seungnam, Korea) were supplied free to access. This experiments were conducted in accordance with 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, Korea) [Approval No DHU2017-032, April 11, 2017]. In addition, experiments on osteoporosis were based on US FDA Guideline "Guidelines for Preclinical Evaluation of Agents Used in The Prevention or Treatment Postmenopausal Osteoporosis (April, 1994)", Division of Metabolic and Endocrine Drug Products. After 7 days acclimatization, 75 mice were used as OVX-induced osteoporotic mice and remainder ten mice were used as sham operated control mice, in this experiment. At 34 days after OVX, 8 mice in each group were selected based on the body weight deviations (37.37±1.15 g of OVX mice, ranged in 35.1-40.0 g; 32.83±1.08 g of sham-operated mice, ranged in 31.5-34.7 g, respectively) as follows (Table 2). Forty mice were selected in seventy-five OVX-induced osteoporotic mice and eight mice were selected in ten sham operated control mice. All animals were overnight fasted (about 18 hrs, water was not limited) before OVX, initial administration and sacrifice, respectively.

3. OVX

Mice were anesthetized with 2-3% isoflurane (Hana Pharm. Co., Hwasung, Korea), in the mixture of 70% N_2O and 28.5% O_2, through rodent inhalation anesthesia apparatus (Surgivet, Waukesha, WI, USA) and rodent ventilator (Model 687, Harvard Apparatus, Cambridge, UK), and they were maintained with 1-1.5% isoflurane, in the mixture of 70% N_2O and 28.5% O_2. The surgical protocol was conducted according to established methods[13-5] as follows. The OVX treatment group received open surgery involving bilateral OVX with a midline incision of linea alba, and ligation with 1-0 silk

(Camel, Daebo Ind., Gwangju, Korea). Following surgery, the incision was closed in one layer using dissolvable 3-0 catgut sutures (4/0; F1154044, B. Braun Melsungen AG, Hessen, Germany). The second group of mice received a sham operation with similar incision of linea alba, except for bilateral OVX. Povidone Iodine (Betadine™, Korea Pharma, Hwasung, Korea) and Aluspray™ (Vétoquinol, Cedex, France) were used to wound protection.

4. Body weight measurements

Body weight changes were measured once a week, at least (1 day before OVX, at OVX, 1 day before administration, initiation of administration, 1, 7, 14, 21, 28 and 34 days after initial administration, and at sacrifice (24 hrs after last 35th administration), using an automatic electronic balance (XB320M, Precisa Instrument, Dietikon, Switzland), respectively. At OVX, initiation of administration and termination, all experimental mice were overnight fasted (about 18 hrs, water was not limited) to reduce the differences from feeding. Body weight gains were calculated as follow Equation [1]:

Equation [1]. Body weight gains (g)

OVX recovery/induced periods (35 days) = Body weight at initial test substance treatment-body weight at the day of OVX

After administration (35 days) = Body weight at sacrifice-body weight at initial test substance treatment

5. Measurement of BMD

The mean of BMD on total body and right femur were measured by in live dual-energy x-ray absorptionmetry (DEXA; InAlyzer, Medikors, Seungnam, Korea), once at 24 hrs after end of last 35th treatment of test substances.

6. Bone weight measurements

At end of 35 days continuous treatment from 35 days after bilateral OVX, the right sides of femur were collected after removal of the surrounding connective tissues, muscles and all debris. The weight of bones was measured at g levels regarding absolute wet-weights, and the bones were dried for 8 hrs at 200℃ in high temperature dry oven (LDO-080N, Daihan Labtech Co., Seoul, Korea) to measure dry bone weights. After that the dried bones were carbonized for 6 hrs at 800℃ in furnace (LEF-1055-1, Daihan Labtech Co., Seoul, Korea), it's regarded as ash absolute weights. The relative weight (%) was calculated based on the body weight at sacrifice and absolute wet/dry/ash weight as follow Equation [2], to reduce the individual body weight differences.

Equation [2]. Relative bone weights (% of body weight)

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

7. Measurement of bone strengths

Bone strength was detected as Failure load (FL). FL of mid-shaft regions of

dried right femur was measured by a three point bending test to failure by a computerized testing machine (SV-H1000, Japan Instrumentation System Co., Japan) as N (Newton).

8. Blood collection

For serum biochemistry, about 1 ml of whole blood was obtained from vena cava at sacrifice, and centrifuged to serum at 15,000 rpm for 10 minutes under 4℃, using clotting activated serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA). All serum samples were frozen at -150℃ using ultradeep freezer (MDF-1156, Sanyo, Tokyo, Japan) until they were used.

9. Serum biochemistry

Serum osteocalcin levels were detected using Osteocalcin mouse ELISA Kit (Cat. No., MBS495064, MyBioSource, San Diego, CA, USA) as ng/ml levels, and serum BALP activities were measured by Mouse BALP ELISA Kit (Cat. No. MBS2501503, MyBioSource, San Diego, CA, USA) as U/L levels with ELISA Reader (Sunrise, Tecan, Männedorf, Switzerland), respectively.

10. Measurement of femur mineral content

After measurement of ash bone weights of the right femur, they were grinded as a powder and dissolved in nitric acid. In diluted solution, Ca and IP contents were calculated as mg/g using orthocresolphthalein complexon and enzyme methods[13,14], respectively. In addition, Ca/IP ratio was calculated as follow Equation [3].

Equation [3]. Bone Ca/IP ratio = [(Bone Ca contents/Bone IP contents)]

11. Bone histological procedures

The left femur of each mouse was separated, fixed in 10% neutral buffered formalin (NBF), and decalcified in decalcifying solution [24.4% formic acid, and 0.5 N sodium hydroxide] for 3 days (mixed decalcifying solution was exchanges once a day). After that, trochlea head regions of the femur were longitudinally trimmed, embedded in paraffin using automated tissue processor (Shandon Citadel 2000, Thermo Scientific, Waltham, MA, USA) and embedding center (Shandon Histocentre 3, Thermo Scientific, Waltham, MA, USA), sectioned (3-4 μm) using automated microtome (RM2255, Leica Biosystems, Nussloch, Germany) and stained with Safranin O (SO) stain. The histological profiles were interpreted in each prepared histological samples. The histopathologist was blinded to the group distribution when this analysis was carried out. In addition, bone histomorphometry was conducted using a computer based automated image analyzer (iSolution FL ver 9.1, IMT i-solution Inc., Vancouver, Quebec, Canada) under microscopy (Eclipse 80i, Nikon, Tokyo, Japan) so as to examine bone mass and structure with bone resorption in a uniform area of epiphyseal or cortical bone regions of the femur (growth plate regions excluded). Cortical bone thickness was measured on the mid-shaft regions of the femur. Trabecular

bone volume (TBV, TV/BV; %), number of trabecular bone (Tbn), length (Tbl; μm) and thickness (Tbt; μm), and cortical bone thickness (Cbt; μm) were measured for bone mass and structure, and osteoclast cell number (Ocn) and ratio (OS/BS; %) were measured for bone resorption as our previous methods[13-5], respectively.

12. Statistical analyses

All values are expressed as mean± standard deviation (S.D.) of eight mice in this experiment. Multiple comparison tests were performed for different dose groups. Variance homogeneity was examined using the Levene test. If there is no significant deviations from variance homogeneity in the Levene test, the obtained data were analyzed by one way ANOVA test followed by least-significant differences multi-comparison (LSD) test to determine which pairs of group comparison were significantly different. If significant deviations of variance homogeneity were observed at Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. When there is a significant difference 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 14K, SPSS Inc., USA). Also, the percent changes between sham and OVX control were calculated to observe the severities of induced estrogen-deficient osteoporosis, and the percent changes between OVX control and test substance administered mice were calculated to investigate the efficacy of test materials as follow Equation [4] and [5], respectively[18,19].

Equation [4]. Percentage changes as compared to Sham control (%)

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

Equation [5]. Percentage changes as compared to OVX control (%)

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

Ⅲ. Results

1. Effects on body weights and gains

We selected eight mice in each group showed significant ($p<0.01$) increases of body weights as compared to sham-operated mice, and regarded as proper OVX animals at 34 days after OVX (37.37±1.15 g of OVX mice, ranged in 35.1-40.0 g; 32.83±1.08 g of sham-operated mice, ranged in 31.5-34.7 g respectively), consequently, significant ($p<0.01$) increases of body weights were observed in all OVX mice as compared to sham control mice with significant ($p<0.01$) increases of body weight gains during 5 weeks of OVX recovery/induce periods in this experiment. However, significant ($p<0.01$ or $p<0.05$) decreases of body weights were

observed from 28 days after initial oral administration of GJEJ 500 and 250 mg/kg, and also from 34 days after 1st administration of GJEJ 125 mg/kg as compared to OVX control mice, respectively. In addition, all GJEJ 500, 250 and 125 mg/kg administered mice showed significant ($p<0.01$) and dose-dependent decreases of body weight gains during 35 days of treatment as compared to OVX control, in this experiment. There is no significant changes on the body weights and gains in RES 2.5 mg/kg treated mice as compared to those of OVX control mice, throughout the whole experimental periods, in the present study (Fig. 1).

The body weight gains during 35 days of treatment in OVX control were changed as 496.34% as compared to sham control, but they were changed in RES 2.5 mg/kg, GJEJ 500, 250 and 125 mg/kg treated mice as -13.50, -52.76, -40.90 and -33.54% as compared to OVX control, respectively.

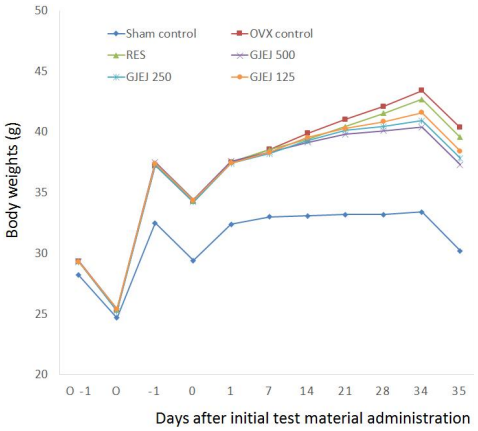

Fig. 1. Body weights observed in Sham-operated and OVX mice.
Values are expressed as Mean of eight mice.
The day O -1 means 1 day before OVX.
The day O means the day of OVX.
The day -1 means 1 day before start of administration at 34 days after OVX.
The day 0 means at start of administration, at 35 days after OVX.
The day 35 means 35 days after start of administration, at sacrifice.
All animals were overnight fasted before OVX, first administration and sacrifice, respectively.

2. Effects on the femur weights

Significant ($p<0.01$) decreases of the femur relative wet-weights, and absolute and relative dry and ash weights were noticed in OVX control mice as compared to sham control mice, respectively. However, significant ($p<0.01$ or $p<0.05$) increases of the femur wet/dry/ash weights were demonstrated in all test substance treated mice including RES 2.5 mg/kg orally treated mice as compared to OVX control mice, respectively. Especially, GJEJ 500, 250 and 125 mg/kg treated mice showed obvious dose-dependent increases of the femur relative wet-weights, and absolute and relative dry and ash weights as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, in the current study (Table 3, Table 4).

Table 3. Right Femur Absolute Weights in Sham-operated and OVX Mice

Groups	Absolute weights (g)		
	Wet	Dry	Ash
Controls			
Sham	0.097 ±0.008	0.075 ±0.006	0.049 ±0.004
OVX	0.093 ±0.005	0.053 ±0.004[a]	0.033 ±0.003[a]
Reference			
RES	0.093 ±0.009	0.072 ±0.007[c]	0.045 ±0.006[c]
GJEJ			
500	0.091 ±0.006	0.070 ±0.007[c]	0.043 ±0.005[ac]
250	0.090 ±0.005[b]	0.066 ±0.005[ac]	0.040 ±0.003[ac]
125	0.092 ±0.005	0.062 ±0.003[ac]	0.038 ±0.003[ac]

a : p<0.01 as compared to sham control by LSD test
b : p<0.05 as compared to sham control by LSD test
c : p<0.01 as compared to OVX control by LSD test

Table 4. Right Femur Relative Weights in Sham-operated and OVX Mice

Groups	Relative weights (% of body weight)		
	Wet	Dry	Ash
Controls			
Sham	0.315 ±0.035	0.245 ±0.027	0.159 ±0.016
OVX	0.230 ±0.015[a]	0.131 ±0.011[d]	0.082 ±0.008[a]
Reference			
RES	0.235 ±0.019[a]	0.182 ±0.015[de]	0.115 ±0.017[ab]
GJEJ			
500	0.245 ±0.020[a]	0.188 ±0.019[de]	0.116 ±0.013[ab]
250	0.237 ±0.015[a]	0.176 ±0.015[de]	0.107 ±0.008[ab]
125	0.239 ±0.015[a]	0.162 ±0.010[de]	0.099 ±0.010[ac]

a : p<0.01 as compared to sham control by LSD test
b : p<0.01 as compared to OVX control by LSD test
c : p<0.05 as compared to OVX control by LSD test
d : p<0.01 as compared to sham control by MW test
e : p<0.01 as compared to OVX control by MW test

3. Effects on the serum biochemistry: Osteocalcin contents and BALP activities

Significant ($p<0.01$) increases of the serum osteocalcin levels, and significant ($p<0.01$) decreases of serum BALP activities were detected in OVX control mice as compared to sham control mice, respectively. However, significant ($p<0.01$ or $p<0.05$) decreases of the serum osteocalcin levels and increases of BALP activities were detected in all three different dosages of GJEJ treated mice as compared to OVX control mice, dose-dependently. In this study, RES 2.5 mg/kg administered OVX mice also showed significant ($p<0.01$) decreased serum osteocalcin levels as compared to those of OVX control mice, but they showed quite similar serum BALP activities as compared to those of OVX control mice (Fig. 2, Fig. 3).

The serum osteocalcin levels and BALP activities in OVX control were changed as 124.22 and -54.60% as compared to sham control, but they were changed as -59.36, -41.31, -28.88 and -18.69% of serum osteocalcin levels, and 5.59, 78.36, 35.12 and 24.75% of serum BALP activities in RES 2.5 mg/kg, GJEJ 500, 250 and 125 mg/kg treated mice as compared to OVX control, respectively.

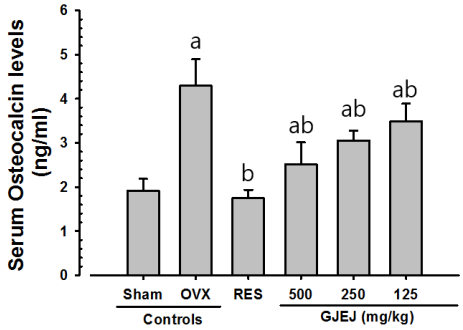

Fig. 2. Changes on the serum osteocalcin levels in Sham-operated and OVX mice.
a : p<0.01 as compared to sham control by LSD test
b : p<0.01 as compared to OVX control by LSD test

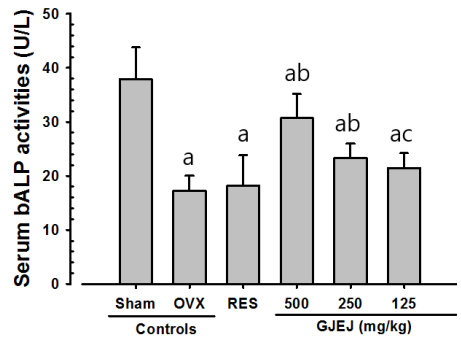

Fig. 3. Changes on the serum BALP levels in Sham-operated and OVX mice.
a : p<0.01 as compared to sham control by LSD test
b : p<0.01 as compared to OVX control by LSD test
c : p<0.05 as compared to OVX control by LSD test

4. Effects on the femur BMD

The total body and femur mean BMD of OVX mice were significantly (p<0.01) decreased as compared to sham control mice, respectively. However, significant (p<0.01) increases of total body and femur mean BMD were detected in all test substance administrated mice as compared to OVX control mice, respectively. Especially, all three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice also showed clear dose-dependent increases of the total body and femur mean BMD as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, in current study (Table 5).

5. Effects on bone strengths

The strengths (FL) of right dry femur mid-shaft regions in OVX control mice were significantly (p<0.01) decreased as compared to sham control mice. Significant (p<0.01 or p<0.05) increases of FL on the femur were detected in all test substance administrated mice including GJEJ 250 mg/kg as compared to OVX control mice, respectively. Especially, all three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice constantly showed definitive dose-dependent increases of the FL in the femur mid-shaft regions as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, in the current experiment (Table 5).

Table 5. Total Body and Right Femur BMD, Right Femur Strength in Sham-operated and OVX Mice

Groups	BMD (mg/cm^2)		Strength (Newton)
	Total body	Femur	Femur
Controls			
Sham	0.0249 ±0.0005	0.0272 ±0.0010	13.52 ±1.45
OVX	0.0213 ±0.0004[a]	0.0232 ±0.0003[d]	6.10 ±0.93[a]
Reference			
RES	0.0250 ±0.0007[b]	0.0263 ±0.0017[e]	10.45 ±1.72[ab]
GJEJ			
500	0.0246 ±0.0007[b]	0.0261 ±0.0015[e]	10.26 ±1.72[ab]
250	0.0231 ±0.0006[ab]	0.0251 ±0.0007[de]	8.43 ±1.17[ab]
125	0.0225 ±0.0004[ab]	0.0239 ±0.0004[de]	7.75 ±0.97[ac]

a : $p<0.01$ as compared to sham control by LSD test
b : $p<0.01$ as compared to OVX control by LSD test
c : $p<0.05$ as compared to OVX control by LSD test
d : $p<0.01$ as compared to sham control by MW test
e : $p<0.01$ as compared to OVX control by MW test

6. Effects on femur mineral contents - Ca and IP

Significant ($p<0.01$) decreases of femur Ca and IP contents were detected in OVX control as compared to sham control, respectively. However, significant ($p<0.01$ or $p<0.05$) increases of femur Ca and IP contents were detected in all test substance administrated mice including GJEJ 125 mg/kg as compared to OVX control mice, respectively. Especially, all three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed constant dose-dependent increases of the Ca and IP contents in the right femur as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, in our experiment (Table 6).

Table 6. Right Femur Mineral Contents in Sham-operated and OVX Mice

Groups	Mineral contents (mg/g)		Ratio
	Ca [A]	IP [B]	Ca/IP [A/B]
Controls			
Sham	60.86 ±10.35	48.19 ±7.82	1.26 ±0.07
OVX	32.26 ±3.83[a]	26.21 ±3.83[a]	1.25 ±0.23
Reference			
RES	55.72 ±10.63[b]	44.86 ±7.23[b]	1.27 ±0.34
GJEJ			
500	52.41 ±10.85[ab]	43.42 ±10.20[ab]	1.22 ±0.13
250	46.07 ±10.11[ab]	37.86 ±6.99[ab]	1.22 ±0.17
125	41.07 ±5.67[ac]	33.47 ±4.20[ac]	1.23 ±0.04

a : $p<0.01$ as compared to sham control by LSD test
b : $p<0.01$ as compared to OVX control by LSD test
c : $p<0.05$ as compared to OVX control by LSD test

7. Effects on the left femur histopathology

Relatively well-developed trabecular and cortical bone were observed in the left femur of sham control mice, whereas classical osteoporotic histological profiles were observed in OVX control mice as dramatic decreases of trabecular and cortical bone masses, increase of connective tissues in periosteum of cortical bone which is results from resorption of osteoid tissues associated with osteocalst activations in the current histopathological inspections. RES 2.5 mg/kg inhibited the trabecular

bone losses, but they did not influence on the cortical bone masses. However, dramatic increases of the bone mass and structures of the both trabecular and cortical bones were observed in all three different dosages of GJEJ 500, 250 and 125 mg/kg administered mice as compared to OVX control mice, related to their inhibitory activities on osteoclast cell activities, dose-dependently, in the current histopathological inspection (Table 7, 8).

Table 7. Left Femur Histomorphometry in Sham-operated and OVX Mice: Trabecular Bones

Groups	Trabecular histomorphometrical analysis			
	TV/BV (%)	Tbn (numbers/epiphyseal)	Tbl (μm)	Tbt (μm)
Controls				
Sham	40.79±5.68	19.88±2.90	1069.47±166.29	89.93±15.02
OVX	16.16±5.91[a]	8.38±1.60[e]	561.19±113.66[a]	35.61±5.77[e]
Reference				
RES	40.48±5.69[c]	30.25±7.29[ef]	1040.12±190.83[c]	39.09±11.77[e]
GJEJ				
500	39.85±5.50[c]	14.38±2.83[ef]	1018.42±128.85[c]	57.66±7.46[ef]
250	30.31±3.36[ac]	12.13±1.55[ef]	917.62±122.77[bc]	51.20±4.14[ef]
125	22.19±2.50[ad]	11.25±1.16[ef]	766.70±108.14[ac]	42.78±4.16[eg]

a : $p<0.01$ as compared to sham control by LSD test
b : $p<0.05$ as compared to sham control by LSD test
c : $p<0.01$ as compared to OVX control by LSD test
d : $p<0.05$ as compared to OVX control by LSD test
e : $p<0.01$ as compared to sham control by MW test
f : $p<0.01$ as compared to OVX control by MW test
g : $p<0.05$ as compared to OVX control by MW test

Table 8. Left Femur Histomorphometry in Sham-operated and OVX Mice: Cortical Bones and Osteoclast Cells

Groups	Cortical and osteoclast histomorphometrical analysis		
	Cbt (μm)	Ocn (number)	OS/BS (%)
Controls			
Sham	197.50±17.11	3.75±1.49	3.87±1.93
OVX	123.72±11.55[a]	16.63±2.00[d]	24.04±3.44[a]
Reference			
RES	125.66±10.13[a]	18.25±3.69[d]	7.39±2.17[ab]
GJEJ			
500	165.36±12.16[ab]	6.88±1.13[de]	11.35±1.36[ab]
250	155.12±11.54[ab]	8.25±1.28[de]	14.12±1.29[ab]
125	138.45±10.06[ac]	12.00±1.60[de]	17.32±1.70[ab]

a : $p<0.01$ as compared to sham control by LSD test
b : $p<0.01$ as compared to OVX control by LSD test
c : $p<0.05$ as compared to OVX control by LSD test
d : $p<0.01$ as compared to sham control by MW test
e : $p<0.01$ as compared to OVX control by MW test

1) Bone mass and structures

Significant ($p<0.01$) decrease of TV/BV, Tbn, Tbl, Tbt and Cbt were observed in OVX mice as compared to sham-operated mice in the femur, respectively. However, these decreases of bone mass and structures were inhibited significantly ($p<0.01$ or $p<0.05$) and dose-dependently by treatment of three dosages of GJEJ 500, 250 and 125 mg/kg as compared to OVX control mice, respectively. RES 2.5 mg/kg treated mice also showed significant ($p<0.01$) increases of the left femur TV/BV, Tbn and Tbl, but they did not critically influenced on the femur Cbt and Tbt as compared to those of OVX control mice, in our histopathological inspection (Table 7, 8).

2) Bone resorption

Significant ($p<0.01$) increases of Ocn and OS/BS were detected in OVX control mice as compared to sham control mice in the femur, respectively. However, these activation and increase of osteoclast cells were inhibited significantly ($p<0.01$) and dose-dependently by treatment of three dosages of GJEJ 500, 250 and 125 mg/kg as compared to OVX control mice, respectively. Although RES 2.5 mg/kg treated mice showed similar left femur Ocn as compared to those of OVX control mice, OS/BS in RES 2.5 mg/kg treated mice were significantly ($p<0.01$) decreased as compared to those of OVX control mice, in the current histopathological analysis (Table 7, 8).

Ⅳ. Discussion

Osteoporosis is a disease in which the risk of fracture increases because of the bone strength decrease[2]. It results from a disturbance in normal bone remodeling and imbalance between bone resorption and formation[2]. Bone remodeling of osteocytes is very important in determining and increasing bone mass in pathological condition such as osteoporosis[20]. Osteoporosis can cause serious problem because it significantly increases the frequency of fracture[2]. It has been estimated that a 50-year-old white woman in the USA has about an 11-18% risk of suffering a hip fracture in her lifetime[2,21].

There have been many attempts to develop new agents which is capable of preventing or treating bone diseases for many years[22]. Anti-resorptive agents are extensively used for osteoporosis treatment, but still there is a need for a highly efficacious antiresorptive agents with an excellent safety and tolerability profile[23]. Anabolic agents stimulating bone formation are less well known than anti-resorptive agents[23]. Continuous trials to find the anabolic agents have been accomplished[14].

Medicinal herbs are getting important in the pharmaceutical industry and they inspire the search for new potential sources of bioactive molecules[24,25]. Medicinal herbs and crude drug substances are considered a potential source of antioxidants in various diseases[26]. In this experimental study, we

examined whether *Gojineumja* is effective in the treatment of osteoporosis. *Gojineumja* is a Korean traditional polyherbal prescription has been used for tonic agent which is used when both yin (陰) and yang (陽) are insufficient[7]. ≪東醫寶鑑≫[7] said that people over middle age should always take *Gojineumja*. The main reason of osteoporosis is renal deficiency (腎虛)[5], and *Rehmanniae Radix, Dioscoreae Rhizoma, Eucommiae Cortex, Corni Fructus, Psoraleae Semen, Schisandrae Fructus* in *Gojineumja* have efficacy in renal deficiency (腎虛)[9,27]. That is why we decided to conduct this experiment.

There have been many studies on the efficacy of *Gojineumja*[8-10] and each herb in *Gojineumja*[11,12]. And there have been many studies on the efficacy of Korean Medicine prescription and each herb for osteoporosis induced by OVX[28,29]. However, There is no study to examine the effects of *Gojineumja* on osteoporosis.

Estrogen-deficient OVX osteoporosis model is useful for evaluation of osteoporotic drugs, because some parameters are clearly changed by ovariectomy in 4-6 weeks after operation. The effects of a osteoporotic drug would be based on bone weight, BMD, bone mineral contents, bone strength, serum biochemistry for bone turnover, resorption and formation, and histomorphometrical changes of bones in this model[13-5].

Risedronate sodium (RES), a pridinyl bisphosphonate, binds to bone hydroxyapatites and inhibits osteoclast[16]. It has been shown that RES prevents bone resorption by changing osteoclast cytoskeleton proteins[30] and induces osteoclast apoptosis through direct cytotoxic effects[31]. RES is effective in treatment and prevention of osteoporosis in postmenopausal women[32]. Therefore, RES was orally treated to mice as a positive control drug.

In this experiment, we intended to observe the dose-dependent anti-osteoporotic potentials of GJEJ on the bilateral OVX mice, as compared to those of RES at a dose level of 2.5 mg/kg. The dosages of GJEJ 500, 250 and 125 mg/kg were selected based on the previous *in vivo* efficacy studies of GJEJ[8-10], and the dosage of RES, 2.5 mg/kg was also selected based on the *in vivo* anti-osteoporosis efficacy studies[16,17].

Thirty five days after bilateral OVX, 500, 250 and 125 mg/kg of GJEJ were orally administered for 35 days once a day. Then the changes on the body weight and gain in experimental periods, femur weights, serum biochemistry(osteocalcin contents and BALP activities), BMD, bone strength, mineral contents - Ca and IP, histological profiles and histomorphometrical analyses at sacrifice were conducted.

As a result of OVX, noticeable increase of body weight and gains and serum osteocalcin levels, decrease of serum BALP activities, femur wet, dry and ash weights, femur Ca and IP contents, BMD and strength were observed as compared to those of sham vehicle control mice, respectively. In addition, the decrease of all histomorphometrical indices indicating

the bone mass and structure, and the increase of indices indicating the bone resorption were also detected in the femur of OVX control. It means the increase of bone turnover and decrease of bone formation, the estrogen-deficient osteoporosis.

However, these OVX-induced estrogen-deficient osteoporotic signs related to the increases of bone turnover and decreases of bone formation were significantly and dose-dependently inhibited by 35 days of continuous oral treatment of GJEJ, at doses of 500, 250 and 125 mg/kg, respectively. Especially, GJEJ 500 mg/kg showed favorable inhibitory activities against estrogen-deficient osteoporosis symptoms induced by OVX as comparable to those of RES 2.5 mg/kg. These findings are suggested that the GJEJ has potent anti-osteoporotic effects in OVX mice, as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, at least in this experiment condition. Therefore, It is expected that appropriate amounts of GJEJ will be a promising new protective agents for relieving the osteoporosis in menopausal women. Although, RES 2.5 mg/kg also favorably ameliorated the decreases of the femur BMD, strength and trabecular bone architectures induced by estrogen-deficient from OVX through the inhibited the increases of bone turnover, but they did not critically influenced on the bone formations - the serum BALP activities, cortical and trabecular bone thicknesses, well corresponded to the previous experiments by other investigators[17,33].

The increase of body weight and gains observed in all OVX mice as compared to those of sham control of this study were considered as general signs of estrogen-deficient status. Body weight changes have been used to predict bone density in OVX mice[34]. Existing data on the effect of OVX on the body weight in mice are inconsistent: one study has shown that OVX results in an increased body weight[35], whereas other reports have failed to do so[36,37]. The decreases of body weight or gains generally have been considered as a toxicological signs in normal states but it has been regarded as favorable signs in specific disease states such as obesity[14,15]. In the present result, significant increases of body weights and gains were also observed in all OVX mice as compared to sham control mice. However, all three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed significant and dose-dependent decreases of body weight gains during 35 days of treatment as compared to OVX control, in this experiment.

These findings on the body weights and gains were considered as indirect evidences that GJEJ has favorable inhibitory activities on OVX-induced metabolic disorders, enough to reduce body weight-loaded pathological changes - the osteoporosis, at least in a condition of the present study. No significant changes on the body weights and gains were observed in RES 2.5 mg/kg treated mice as compared to those of OVX control mice, throughout

the whole experimental periods, in the current result.

Although, it's generally known that the bone weight change was not critical index to evaluate the efficacy of anti-osteoporotic agents excepting ash bone weight[38], the increases trends of relative bone weights have been considered as a valuable markers of anti-osteoporotic activities[14,15]. Significant decreases of the femur relative wet-weights, and absolute and relative dry and ash weights were also noticed in OVX control mice as compared to sham control mice, in this study. However, marked increases of the femur wet/dry/ash weights were demonstrated in all test substance treated mice including RES 2.5 mg/kg orally treated mice as compared to OVX control mice, respectively. Especially, GJEJ 500, 250 and 125 mg/kg treated mice showed obvious dose-dependent increases of the femur relative wet-weights, and absolute and relative dry and ash weights as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, in the current study. It, therefore, is considered that GJEJ has favorable inhibitory activities on OVX-induced bone weight decreases as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, at least in a condition of the current study.

Although there was variable changes according to time of blood collection, types of study and kind of used animals, serum osteocalcin levels were generally known as a marker of "bone turnover" and BALP levels were generally known as a marker of "bone formation"[14,15,39,40]. In the present study, significant increases of the serum osteocalcin levels, and significant decreases of serum BALP activities were detected in OVX control mice as compared to sham control mice, respectively. However, significant decreases of the serum osteocalcin levels and increases of BALP activities were observed in all three different dosages of GJEJ 500, 250 and 125 mg/kg as compared to OVX control mice, dose-dependently. These findings on the serum biochemistrical analysis were considered as obvious evidences that GJEJ has bone formation and bone turnover inhibitory activities, enough to prevent osteoporosis, at least in a condition of this OVX mouse study. RES 2.5 mg/kg administered OVX mice also showed significantly decreased serum osteocalcin levels as compared to those of OVX control mice, but they showed quite similar serum BALP activities as compared to those of OVX control mice, in our study.

BMD has been regarded as a useful index for testing changes in bone quality, and they were significantly decreased in osteoporotic animal or human regardless of the cause[14,15,41,42]. BMD of bone provided predictable information on the efficacy of anti-osteoporotic agents[41]. It also provides diagnostic profiles of bone quality in human clinical studies[42]. BMD and bone strength were markedly decreased in osteoporosis regardless of causes[14,15,41,42]. In our experiment, the total body and femur BMD, femur strength of OVX control mice were also significantly decreased as compared to sham

control, respectively. However, significant increases of total body and femur BMD, femur FL were detected in all test substance administrated mice including GJEJ 250 mg/kg as compared to OVX control mice, respectively. Especially, GJEJ 500, 250 and 125 mg/kg treated mice showed noticeable dose-dependent increases of the total body and femur BMD, femur strength as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, constantly, in the present experiment. These findings were considered as direct evidences that GJEJ has increased the bone mass and bone strength preserve activities OVX mice, as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, at least in a condition of the this experiment.

Bone mineral contents were generally and significantly decreased as progress of osteoporosis[14,15]. Among bone mineral contents, Ca and IP contents were the most dramatically decreased mineral contents in animal's bone, but their ratio (Ca/IP) has not been significantly changed because of simultaneous decreases of the bone Ca and IP contents[14,15,43,44]. In the current analysis, significant decreases of femur Ca and IP contents were also detected in OVX control as compared to sham control, respectively. However, significant increases of femur Ca and IP contents were detected in all test substance administrated mice including GJEJ 500 mg/kg as compared to OVX control mice, respectively. Especially, all three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed constant dose-dependent increases of the Ca and IP contents in the right femur as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, in our experiment. These findings were considered as clear evidences that GJEJ has favorable inhibitory activities on OVX-induced bone mineral losses as comparable to those of RES 2.5 mg/kg in GJEJ 500 mg/kg, at least in a condition of the present study. There is no significant changes on the femur Ca/IP ratio which were demonstrated in OVX control mice as compared to those of sham control mice, and also no significant changes on the femur Ca/IP ratio which were observed in all test material administered mice including RES 2.5 mg/kg as compared to those of OVX control mice, in the present experiment.

A microscopic observation of bone provided great information about bone morphology[14,15,45]. The histological profiles in osteoporotic animals were significantly altered as compared to sham control regardless of the cause, especially on the trabecular and cortical bone, and the efficacy of various anti-osteoporosis agents have been assessed on the histology of bones[14,15,46]. In other words, some histomorphometrical indices of bone mass are clearly decreased but histomorphometrical indices of bone resorption are increased, and they have been provided reliable information to predict the efficacy of anti-osteoporotic agents[14,15,47,48]. In this study, classical osteoporotic histological

profiles were also observed in OVX control mice as dramatic decreases of trabecular and cortical bone masses, increase of connective tissues in cortical bone's periosteum results from resorption of osteoid tissues associated with osteoclast activations. Although RES 2.5 mg/kg inhibited the trabecular bone losses, it did not influence on the cortical bone masses. However, dramatic increases of the bone mass and structures in both trabecular and cortical bones were detected in three dosages of GJEJ 500, 250 and 125 mg/kg administered mice as compared to OVX control mice, related to inhibitory activities on osteoclast cell activities, respectively. These findings on the femur histopathological analysis were considered as obvious and reliable evidences that GJEJ has bone mass and structures preserve activities mediated by inhibition of the bone resorption and facilitated the bone formations in OVX mice, at least in a condition of this experiment.

The results in this study suggest that oral administrations of GJEJ, at dose levels of 500, 250 and 125 mg/kg have noticeable dose-dependent anti-osteoporotic activities in OVX mice, through bone mass and structures preserve activities mediated by inhibition of the bone resorption and facilitated the bone formations. Especially, GJEJ at dose level of 500 mg/kg shows anti-osteoporotic activities comparable to RES at dose level of 2.5 mg/kg. GJEJ at dose level of 500 mg/kg exhibits activities tendency superior to RES at dose level of 2.5 mg/kg in several indices (decreases of body weight gains, Ocn, increases of femur relative weights, BALP, Tbt, Cbt). Therefore, it is expected that appropriate amounts of GJEJ will be promising as a new potent protective agents for relieving the osteoporosis in menopausal women. Since GJEJ is composed of 15 herbs and each herb has various active ingredients, screening the biological active compounds should be carried out through more detailed mechanism studies in the future.

Ⅴ. Conclusion

In this study, we reached the following results:

1. Significant increases of body weights and gains were detected in bilateral OVX mice. Three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed significant and dose-dependent decreases of body weight gains.
2. Significant decreases of body femur wet, dry, and ash weights were detected in bilateral OVX mice. Three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed significant and mostly dose-dependent increases of femur wet, dry, and ash weights.
3. Significant increases of serum osteocalcin and decreases of serum BALP were detected in bilateral OVX mice. Three different dosages of GJEJ 500, 250

and 125 mg/kg treated mice showed significant and dose-dependent decreases of serum osteocalcin and increases of serum BALP.

4. Significant decreases of femur BMD were detected in bilateral OVX mice. Three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed significant and dose-dependent increases of femur BMD.
5. Significant decreases of bone strengths were detected in bilateral OVX mice. Three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed significant and dose-dependent increases of bone strengths.
6. Significant decreases of Femur mineral contents(Ca, IP) were detected in bilateral OVX mice. Three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed significant and dose-dependent increases of Femur mineral contents (Ca, IP).
7. Significant decreases of TV/BV, Tbn, Tbl, Tbt, Cbt and increases of Ocn, OS/BS were detected in bilateral OVX mice. Three different dosages of GJEJ 500, 250 and 125 mg/kg treated mice showed significant and dose-dependent increases of TV/BV, Tbn, Tbl, Tbt, Cbt and decreases of Ocn, OS/BS.

According to these results, GJEJ has significant anti-osteoporotic activity to osteoporosis. *Gojineumja* shows the potential for use as a treatment for osteoporosis. So more researches should be done in future.

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국문초록

목 적: 본 연구에서는 고진음자 물 추출물의 골다공증 개선 효과를 난소적출 마우스를 이용하여 risedronate sodium(RES) 2.5 mg/kg 투여군과 비교 평가하였다.

방 법: OVX 35일 후부터 고진음자 물 추출물을 매일 1회씩 35일간 연속 경구 투여하고, 체중, 대퇴골의 중량, 골밀도, 골강도, 무기질 - 칼슘(Ca) 및 무기인(inorganic phosphorus, IP) 함량, 골량 및 구조와 골흡수에 관한 조직병리학적 변화를 혈중 osteocalcin 함량 및 bone specific alkaline phosphatase(BALP) 활성의 변화와 함께 각각 측정하였다. 본 실험에서 고진음자 물 추출물에서의 결과는 RES 경구 투여 OVX 마우스에서의 결과와 비교 평가 하였다.

결 과: OVX대조군에서는 현저한 체중 증가와 함께 대퇴골의 중량, 골밀도, 골강도, 무기질 - Ca 및 IP 함량 감소 및 지주골과 피질골의 현저한 조직병리학적 감소가 함께 확인되었으며, 혈중 osteocalcin 함량의 증가와 함께 혈중 BALP 활성의 감소가 각각 확인되었다. 이에, 전형적인 estrogen 결핍성 골다공증 소견이 OVX 수술에 의해 유발되는 것으로 관찰되었다. 한편 이렇게 OVX에 의해 유발된 estrogen 결핍성 골다공증 소견이 모든 세 용량의 고진음자 물 추출물 500, 250 및 125 mg/kg의 35일에 걸친 연속 경구 투여에 의해 투여 용량 의존적으로 현저히 억제되었고, 특히 고진음자 물 추출물 500 mg/kg은 RES 2.5 mg/kg 투여군과 비교할 만한 골다공증 개선 효과를 나타내었다.

결 론: 이상의 결과에서, 고진음자 물 추출물은 난소적출 마우스에서 투여 용량 의존적으로 유효한 골다공증 개선 효과를 나타내는 것으로 관찰되었다. 따라서 적절한 용량의 고진음자 물 추출물은 새로운 보다 효과적인 estrogen 결핍성 골다공증 개선제로서의 개발 가능성이 매우 높을 것으로 기대된다. 한편 고진음자는 15종의 약제로 구성되어 있으며, 각각의 개별 약제는 수많은 생리활성 물질을 함유하고 있어, 앞으로 생리활성을 나타내는 화학성분의 검색과 함께 다양한 방면으로 기전 연구가 추가적으로 수행되어야 할 것으로 판단된다.

중심단어: 골다공증, 고진음자, 난소적출

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