

## 난소절제 흰쥐에서 엉겅퀴 추출물의 골다공증 보호 효과

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### Osteoprotective Effect of Extract from *Cirsium japonicum* var. *ussuriense* in Ovariectomized Rats

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**ABSTRACT :** This study was carried out to investigate the effects of the *Cirsium japonicum* var. *ussuriense* (*C. japonicum*) extract on serum level of hormones from induced osteoporosis by ovariectomized rats. Two month-old rats were ovariectomized (OVX), remained untreated for 8 weeks, and were subsequently administered *C. japonicum* (200 mg/kg) every day for 8 weeks. We examined the effects of treated *C. japonicum* on ovariectomy-related changes in Insulin-like Growth Factors (IGF), Insulin-like Growth Factor binding protein-3 (IGFBP-3), Estrogen, Calcium, and Phosphorus. After 8 weeks, the serum levels of IGF-I, -II, and IGFBP-3 were higher presented as compared to the other two groups ( $P < 0.05$ ), in the *C. japonicum* extract treatment on OVX rats. There were differences between OVX and *C. japonicum* extract treated OVX rats in serum levels of  $Ca^{2+}$ , but  $Ca^{2+}$  levels for the normal group was higher than for the other two groups. The *C. japonicum* extract increased both serum IGFs and IGFBP-3 levels on induced osteoporotic rat by ovariectomized. Thus, these results revealed that the *C. japonicum* extract is a possible role for improvement of osteoporosis induced-ovariectomized rats and has a great potential as an alternative tool for the treatment of osteoporosis.

**Key Words :** *Cirsium japonicum*, Osteoporosis, Ovariectomy, Serum

### INTRODUCTION

Osteoporosis is a disease characterized by low bone mass and deterioration of bone tissue. This leads to increased bone fragility and risk of fracture. Osteoporosis is often known as “the silent thief” because bone loss occurs without symptoms (Christiansen, 1993). A decrease in in women at the time of menopause and a decrease in testosterone in men is another major cause of bone loss. After menopause, the normal balance between bone formation and resorption is disrupted : osteoclasts (giant multi-nucleated cells) become more active, decreasing bone mass and increasing the chance of fracture. It is now

known that postmenopausal osteoporosis should be regarded as a product of inflammatory disease triggered by estrogen deficiency (Chung *et al.*, 2006). Ovariectomy (OVX) in the rat results in bone loss and micro-architecture deterioration (Ammann *et al.*, 1996) and is the most commonly used animal model of post-menopausal osteoporosis (Turner *et al.*, 2001). Although hormone replacement therapy has been proven to be efficacious in preventing bone loss, yet, it is not desirable to many women due to its side effects (Epstein, 2006). Estrogen deficiency results in a marked increase in proinflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and macrophage colony-

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stimulating factor (McLean, 2009). TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 stimulate osteoclast differentiation independently or in synergistic fashion (Jiro *et al.*, 2011). Because estrogen deficiency has been recognized as the key factor of osteoporosis, hormone replacement therapy (HRT) was used as an effective strategy for prevention and treatment of osteoporosis in postmenopausal women.

Recently, therapy for osteoporosis is aimed at the prevention of further bone loss, primarily by inhibiting bone resorption. Particularly, insulin-like growth factors (IGF) and their binding proteins (IGFBP), which could modulate IGF's actions, might play essential roles in bone formation (Nilsson *et al.*, 1994; Rosen *et al.*, 1994). In serum, most IGF-I (70-80%) forms a 150-kD complex by binding with IGFBP-3. The serum IGFBP-III level is considered to positively regulated by GH and/or IGF-I (Rosen and Pollak, 1999; Stewart and Rotwein, 1996). IGFBP-III acts as a regulator of growth-dependent IGF-I signaling through the enhancement of IGF-I stability in serum (Binoux and Hossenlopp, 1988). A major part of bound IGF-I is connected to IGFBP-III (Baxter, 1993). Low estrogen concentrations are known to increase the secretion of subsequent IGF-I synthesis during early puberty. Estrogens initiate the pubertal growth spurt and stimulate skeletal growth. Hence, sex steroid-related changes in GH and IGF-I secretion may impact on bone size and cross-sectional area (Juil, 2001). The popularity of herbal medicines is attributable to their easy take, therapeutic efficacy, relatively low cost, and assumed absence of toxic side effects (Park *et al.*, 2013). The use of natural products as an alternative treatment has been on the rise in the last few decades. *C. japonicum*, a wild perennial herb which belongs to *Asteraceae* family, has long been used as a natural medicine for treatment of hypertension, traumatic hemorrhage, and inflammation (Kim *et al.*, 2010). Recent studies have found that the water extracts of *C. japonicum* induce the activation of estrogen receptors and has estrogenic effects (Park *et al.*, 2008) and has been prescribed in the treatment of liver cancer (Lee *et al.*, 2003), uterine cancer, and leukemia (Yin *et al.*, 2008). But since now no studies have been carried out to evaluate whether *C. japonicum* has an antiosteoporosis activity in rats. The present research was conducted to investigate the effect of *C. japonicum* on postmenopausal osteoporosis in ovariectomized rats.

## MATERIALS AND METHODS

### 1. Experimental animals

The Sprague-Dawley rats were purchased from the Jungang Lab animal co, South Korea. A total of 60 female rats, 2-month-old, weighing 180-200 g were randomly assigned into 6 groups (10 animals/group). (i) sham, (ii) OVX\_control, (iii) 17 $\beta$ -estradiol, 17 $\beta$ -estradiol (E2) 30 mcg/kg/24 hour, (Sigma, St. Lois, MO, USA) *C. japonicum*. Except 17 $\beta$ -estradiol (positive control), which was administered every three days for 8 weeks, each treatment sample was administered once daily p.o. at a dose of (iv) 100, (v) 200 and (vi) 400 mg/kg for 8 weeks. The animals were housed in stainless steel cages having wire mesh bottom (3 animals per cage) in a light-controlled (12:12 hours light-dark cycle) room with a constant temperature (25 °C). All the rats had free access to food and water. The animals in group I were normal group. To induce menopause in rats, ovariectomies were performed in group I and group II. Ovariectomies were done under ketamine HCl (100 mg/kg) and xylazine (3 mg/kg) anesthesia. During 8 weeks, the animals of group I and II received solvent vehicle daily, whereas those of group III were administered *C. japonicum* orally (100, 200 and 400 mg/kg) daily for eight weeks. Body weight was determined weekly.

### 2. Preparation of *C. japonicum* extraction

The aerial parts of *C. japonicum* were collected on July 20, 2013 from Imsil herbal farming the Act at Imsil in Chonnam, Republic of Korea, and then specimens were taxonomically identified by an oriental doctor, S. W. Lee at National Institute of Horticultural & Herbal Science, RDA. We used water extraction because most traditional oriental herbal materials are decocted with boiling water. The crushed plant materials (100 g for each) were kept in a breaker at room temperature (25 ± 1). And it extracted under reflux with distilled water three times. After the extraction, the sample residues were dissolved in a known quantity of water and were repeatedly extracted until the extracts became clear. The obtained extract was then filtered through Whatman No. 1 filter paper and the filtrate was concentrated in a rotary evaporator (Buchi Rotavapor R-215, Flawil, Switzerland) under controlled vacuum. The concentrated extract was then dried in a

freeze dryer (Labconco, Free Zone 6 Liter, Kansas City, MO, USA), and yields were 16% (wt/wt) for *C. japonicum* in the dried state. They kept at 4°C in air tight container until further analysis.

### 3. Measurement of Femur/body weight

Body and femur weight was measured after *C. japonicum* seed treatment in osteoporosis induced-ovariectomized rats using chemical balance (Ohaus, Parsippany, NJ, USA).

### 4. Collection of blood and tissue samples

Serum was isolated from the blood samples by centrifugation at 3000 × g and 4°C for 5 min and stored at -70°C for biochemical measurements. Body and isolated femur weight was measured in osteoporosis induced-ovariectomized rats using chemical balance (Ohaus, Parsippany, NJ, USA)

### 5. Measurement of serum IGF-I, IGF-II and IGFBP-III levels

For the quantitative analysis of the serum IGF-I, IGF-II and IGFBP-III levels, free IGF-I and IGFBP-III enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA).

### 6. Estrogen, Ca<sup>2+</sup> and P assay

IGFBP-III and estrogen were assayed using an immunoradiometric assay kit (Diagnostic Systems Laboratories, Webster, TX, USA). The Ca<sup>2+</sup> and P assays used kits (Embiel Co., Gunpo, Korea).

### 7. Statistical analysis

All the data were presented as mean ± S.E.M. The effects of the different treatments were compared by Student's t test and were considered significant for values of  $p < 0.05$  and  $p < 0.01$ . Post hoc analysis was carried out using Duncan's Multiple Range Test (DMRT).

## RESULTS

### 1. Animal model

Body weight is shown in Fig.1. Ovariectomy increased the body weight (Normal: 266.60 ± 8.6; ovariectomized: 328.8 ± 7.4; *C. japonicum* (200 mg/kg) treated: 267.5 ± 11.4

( $p < 0.001$ ): *C. japonicum* (400 mg/kg) treated: 284.8 ± 16.5). There were no differences in body weight between normal, OVX and *C. japonicum*-treated until the end of the study, OVX group higher weights than the other groups. Body weight of OVX rats increased about 23.3%, after 8 week surgery. A relatively low dose of *C. japonicum*, 200 mg/kg, more inhibited the increase in body weight than did a high dose, 400 mg/kg.

### 2. Effect of ovariectomy and *C. japonicum* on serum IGF-I levels

Changes in serum level of IGF-I *C. japonicum* treatment in osteoporosis induced-OVX rat groups over time are shown in Fig. 2. On 8 weeks after ovariectomy, serum level of IGF-I was significantly higher in the 17-β estradiol and *C. japonicum* treated OVX group than in the OVX and sham groups ( $p < 0.01$ ) whereas slight changes were between the normal and OVX groups.

### 3. Effect of ovariectomy and *C. japonicum* on serum IGF-II levels

Serum level of IGF-II after *C. japonicum* treatment in osteoporosis induced-OVX rat groups was shown in Fig. 3. On 8 weeks after ovariectomy, serum level of IGF-II on the 17β-estradiol treated group were significantly higher than that of the OVX group (174.4 ng/mL). Serum level of IGF-II after *C. japonicum* (400 mg/kg) treatment was significantly higher than in the OVX groups ( $p < 0.05$ ) whereas slight changes were between the 100 mg/kg and 400 mg/kg of *C. japonicum* treated groups.

### 4. Effect of ovariectomy and *C. japonicum* on serum IGFBP-III and Estrogen levels

IGFBP-III levels after *C. japonicum* treatment in osteoporosis induced OVX rat groups are shown in Fig 4. On 8 weeks, IGFBP-III levels in the normal group decreased and *C. japonicum* treatment group significant differences compared with the OVX group ( $p < 0.05$ ). The serum levels of IGFBP-III in 17β-estradiol (600.1 ± 12.8 ng/mL) and the 200 mg/kg *C. japonicum* (579.7 ± 8.1 ng/mL) were higher than in OVX group (502.1 ± 30.1 ng/mL), but in *C. japonicum* (400 mg/kg) the serum IGFBP-III decreased to 563.4 ± 9.7 ng/mL. The difference was significant ( $p < 0.05$ ). Changes in estrogen levels of each group are shown in Fig. 5. On 8 weeks after ovariectomy, serum

estrogen levels of each group was difference as a consequence of OVX or the *C. japonicum* treated group, but there were no differences between *C. japonicum* treated group and OVX group.

### 5. Effect of ovariectomy and *C. japonicum* on serum Ca<sup>2+</sup> and P levels

Changes in serum level of Ca<sup>++</sup> and P after *C. japonicum* treated group are shown in Fig 6. 7. The serum levels of Ca<sup>++</sup> and P in all groups were differences compared with the OVX group ( $p < 0.05$ ). The serum levels of Ca<sup>++</sup> in OVX + *C. japonicum* (100 mg/kg) group ( $8.35 \pm 0.2$  ng/mL) was significantly lower than in OVX group ( $10.04 \pm 0.39$  ng/mL Fig. 6). On 8 weeks after ovariectomy, serum level of phosphorus on the 17 $\beta$ -estradiol and the 200 mg/kg *C. japonicum* treated group were significantly higher than that of the OVX group ( $6.96 \pm 0.96$  ng/mL), but in *C. japonicum* (400 mg/kg) the serum phosphorus decreased to  $8.29 \pm 0.80$  ng/mL.

### 6. Femur / body weight

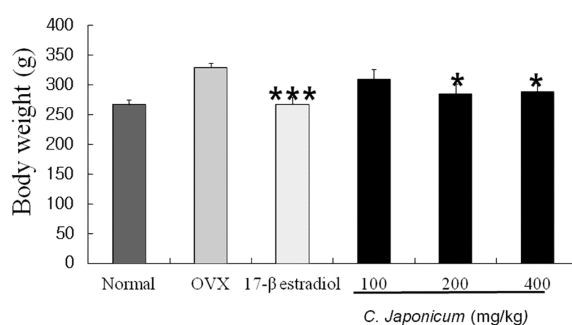
In Fig. 8, the ratio of femur/body weight on the 17 $\beta$ -estradiol and the *C. japonicum* (200, 400 mg/kg) treated group were significantly higher than that of the OVX group ( $0.38 \pm 0.06$  ng/mL).

## DISCUSSION

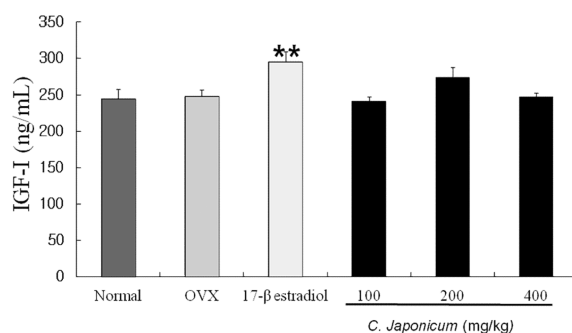
This study showed that the *C. japonicum* enlargements IGF/IGFBP on osteoporosis induced-ovariectomized rats. OVX dramatically increases body weights, while *C. japonicum* treatment presents almost normal levels (Fig. 1). Circulating levels of IGF-I, -II is low in men and women with osteoporosis (Frystyk *et al.*, 1994). The growth hormone/IGF-I system stimulates both the bone resorbing and bone-forming cells, but the superior effect is on bone formation, thus resulting in an increase in bone mass. Circulating level of IGF-I and IGFBP-III were also low in males and females with osteoporosis due to decreasing of bone formation or increasing of bone resorption (Nasu *et al.*, 1997). and increasing serum IGF-I levels of ovariectomized rats have been decreased (Rosen *et al.*, 1994). In serum IGF-II levels in *C. japonicum* treated OVX rats increased in 8 weeks just as osteoblast differentiation and activity aid. IGF-II appears to be due

to stimulatory effect of *C. japonicum* on osteoblast and osteoclast bone formation in this study (Fig. 2, 3).

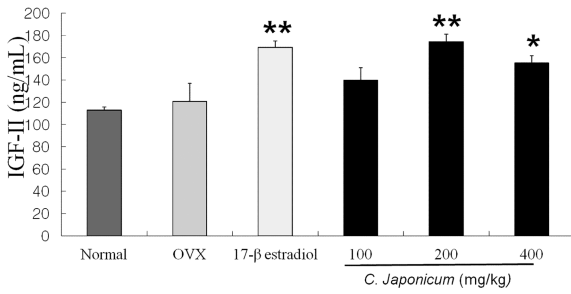
In this study we investigated IGFBP-III levels to determine the role of IGFBP-III in bone growth. The IGFBP-III levels were high in the *C. japonicum* group but not significantly different from those of the OVX group. The serum IGFBP-III levels did not exactly reflect the IGF-I level. However, the results indicate that the IGF-I and IGFBP-III levels had a directly proportional relationship. Most rats with high IGF-I levels had high IGFBP-III levels as well (Fig. 4). These findings suggest



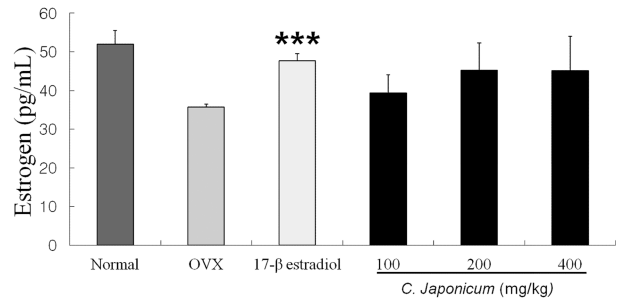
**Fig. 1. Changes of body weight on OVX and *C. japonicum* treatment.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. *P*-values denote significant difference from the OVX group and 17- $\beta$  estradiol treated group. \*\*\* $p < 0.001$  vs. the OVX group, \* $p < 0.05$  vs. the OVX group. Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group, *C. japonicum*; ovariectomized and *C. japonicum* treated group. 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol (30  $\mu$ g/kg) treated group.



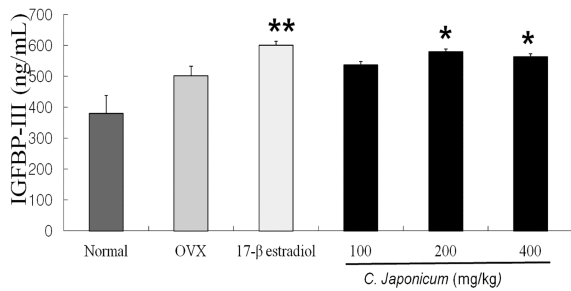
**Fig. 2. Effects of OVX and *C. japonicum* on serum IGF-I levels determined.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. *P*-values denote significant difference from the OVX group and *C. japonicum* treated group. Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group, *C. japonicum*; ovariectomized and *C. japonicum* treated group, 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol (30  $\mu$ g/kg) treated group. \*\* $p < 0.01$  vs. the OVX group.



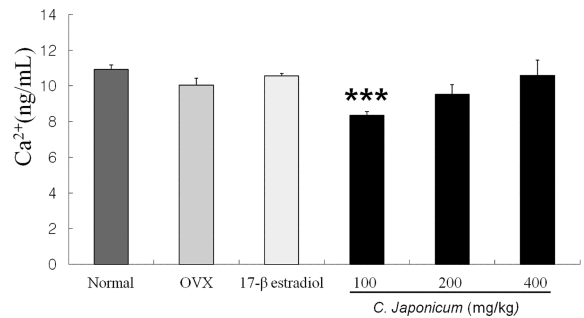
**Fig. 3. Effects of OVX and *C. japonicum* on serum IGF-II levels determined.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. *P*-values denote significant difference from the OVX group and *C. japonicum* treated group. \*\**p* < 0.01 vs. the OVX group, \**p* < 0.05 vs. the OVX group. Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group, *C. japonicum*; ovariectomized and *C. japonicum* treated group. 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol (30  $\mu$ g/kg) treated group. \*\**p* < 0.01 vs. the OVX group.



**Fig. 5. Effects of OVX and *C. japonicum* on serum estrogen levels determined.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group OVX+, *C. japonicum*; ovariectomized and *A. japonica* treated group. 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol treated group. \*\*\**p* < 0.001 vs. the OVX group.



**Fig. 4. Effects of OVX and *C. japonicum* on serum IGFBP-III levels determined.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. *P*-values denote significant difference from the OVX group and *C. japonicum* treated group (*p* < 0.05). Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group, *C. japonicum*; ovariectomized and *C. japonicum* treated group. \**p* < 0.05 vs. the OVX group. 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol (30  $\mu$ g/kg) treated group. \*\**p* < 0.01 vs. the OVX group.



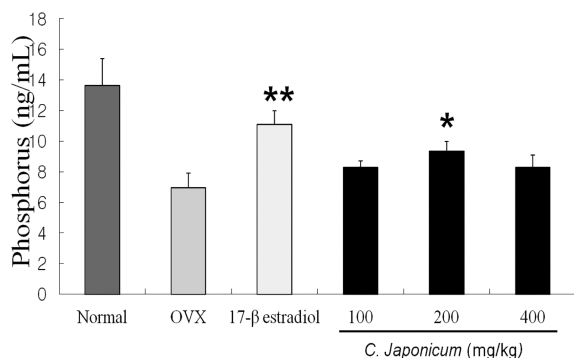
**Fig. 6. Effects of OVX and *C. japonicum* on serum Ca<sup>2+</sup> levels determined.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group OVX+, *C. japonicum*; ovariectomized and *C. japonicum* treated group. \*\*\**p* < 0.001 vs. the OVX group. 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol (30  $\mu$ g/kg) treated group.

that IGFBP-III also could be a biomarker of bone growth. We studied the increase of IGF-I during *C. japonicum* treated osteoporosis compared with the OVX group after ovariectomy. IGF-I induces early osteoblast gene expression in human mesenchymal stem cells (Koch *et al.*, 2005) and increased bone remodeling in transgenic mice with osteoblast targeted insulin-like growth factor-I (Jiang *et al.*, 2006).

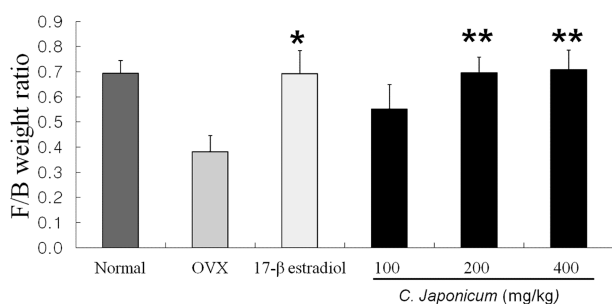
Estrogen play a major role acts in mineral homeostasis, 'the deficiency of estrogen is recognized as a major factor in loss of bone minerals in postmenopausal osteoporosis

(Danielsen and Flyvbjerg, 1996). IGF-I induces cell proliferation via the action in the granulose cells of the ovary and the Leydig cells of the testis and increases the synthesis of the female hormones (estradiol, progesterone) and the male hormone (testosterone) under the control of gonadotropic hormone (Kim *et al.*, 2003b) (Fig. 5). IGFBP-3, the quantitatively predominant IGFBP in circulation, is positively regulated by growth hormone and potentiates IGF action under most conditions. IGFBP-III has the unique property of being able to associate with an acid-labile subunit (ALS) after IGF binding, thus forming a 150 kd complex (Fig. 6, 7).

Longitudinal bone growth is concern with the expression of various growth hormones (GH). GH induces the IGF-I expression in the liver, and circulating IGF-I in serum



**Fig. 7. Effects of OVX and *C. japonicum* on serum phosphorus levels determined.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group, *C. japonicum*; ovariectomized and *C. japonicum* treated group. \* $p < 0.05$  vs. the OVX group. 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol treated group. \*\* $p < 0.01$  vs. the OVX group.



**Fig. 8. Effects of OVX and *C. japonicum* on F/B weight ratio levels determined.** Values are mean  $\pm$  S.E.M. The number of animals is 10/group. Normal; Normal and saline treated group, OVX; ovariectomized and saline treated group, *C. japonicum*; ovariectomized and *C. japonicum* treated group. \*\* $p < 0.01$  vs. the OVX group. 17- $\beta$  estradiol; ovariectomized and 17- $\beta$  estradiol (30  $\mu$ g/kg) treated group. \* $p < 0.05$  vs. the OVX group.

stimulates skeletal growth (Yakar *et al.*, 2002) and affects the expression of IGF-I in the bone (Roith *et al.*, 2001). In this study, OVX dramatically increases body weights, while *C. japonicum* treatment presents almost normal levels. Although the mechanisms by which OVX induces an increase in body weight are not clear, estrogen deficiency induced body fat accumulation and subsequently caused an increase in body weight (Dang *et al.*, 2002) (Fig. 8). Therefore, this study indicates that effects of serum IGFs expression by treated *C. japonicum* may be related to growth rate in OVX rats. In conclusion, *C. japonicum* is able to prevent OVX-induced in bone loss, suggesting that *C. japonicum* may be a reasonable

natural alternative for the prevention of postmenopausal osteoporosis.

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