Long-term supplementation with young coconut juice help prevent bone loss of orchidectomized rats by increasing connectivity density, percentage bone volume and osteoblast-/osteocyte-(ERα-/ERβ-) immunoreactive cells of the L5 vertebra and femur

Asma Asae[1], Albert M. Hutapea[2], Jirawat Saetan[1], Nisaudah Radenahmad[1]

[1] Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand

[2] Department of Pharmacy, Faculty of Science, Universitas Advent Indonesia Bandung, Indonesia

SUMMARY

Adult male rats were orchidectomized (ORX) and treated with 17β-estradiol or young coconut juice (YCJ) for ten weeks. Their femur and L5 vertebra were dissected, and the percentage of bone volume was measured using a Micro-CT scan. Immunohistochemistry was used to detect ERα and ERβ to determine if the osteoporotic protective effects of YCJ were due to the attachment of the YCJ active component(s) to estrogen receptors. It was found that both ERα and ERβ were found in osteocytes and osteoblasts, but not osteoclasts. Compared to normal rats, the percentage of bone volume and number of osteoblast- and osteocyte-reactive cells of both femur and L5 were significantly reduced in ORX rats. Those numbers in the ORX rats were restored to normal by injecting estradiol benzoate or by feeding YCJ to the rats, which was not dose-related. Significant correlations were detected between osteoblast-/osteocyte- (ERα-/ERβ)-reactive cells vs. serum E2 level. The optimal dose of this study was 10 mL/kg BW/day for ERα-of both osteoblasts and osteocytes and 40 mL/kg BW/day for ERβ-of both osteoblasts and osteocytes. The results suggest that YCJ may be as efficient as estradiol benzoate in attenuating osteoporosis, probably by being a selective estrogen receptor moderator.

Key words: *Cocos nucifera* L – *Arecaceae* – Micro-CT scan – Osteoblasts – Osteocytes – Osteoporosis

ABBREVIATIONS

CB	Calbindin
CIA	chemiluminescent immunoassay
E2	17β-estradiol

Corresponding author:
Dr. Nisaudah Radenahmad. Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. E-mail: nisaudah.r@psu.ac.th

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EB Estradiol benzoate
EDTA Ethylenediaminetetraacetic acid
ER Estrogen receptor
ERα Estrogen receptor-α
ERβ Estrogen receptor-β
GI Gastrointestinal
OPG Osteoprotegerin
ORX Orchidectomized
RANKL Receptor activator of nuclear factor κB ligand
SERM Selective estrogen receptor modulator
T Testosterone
VDR Vitamin D receptor
YCJ Young coconut juice

INTRODUCTION

Being one of the commonest human metabolic bone maladies, osteoporosis has been a growing health concern in both women and men (Sozen et al., 2017). Numerous studies reported that estrogens exert a vital role in human bone homeostasis (Noirrit-Esclassan et al., 2021). Bone loss in aging men is due to androgen deficiency (Shigehara et al., 2021). Several studies, however, reported the effect of estrogen in maintaining men's bone mass (Noirrit-Esclassan et al., 2021). Although testosterone, one of the androgenic hormones, can be converted to estrogen in peripheral tissue, its level is not as high as in women. Effective prevention of bone loss has been proven by estrogen replacement treatment. However, this therapy also causes gynecomastia (Chen et al., 2015), increasing the risk for benign prostate cancer and prostatic hyperplasia (Capogrosso et al., 2021). Therefore, a better choice than estrogens is the plant-derived nonsteroidal compounds phytoestrogens, with biological activities like estrogen, maybe. Various phytoestrogens inhibit bone loss with limited consequences on reproduction organ tissues that function as selective estrogen receptor modulators (SERMs) (Zhou et al., 2021). Recently, the discovery of SERMs has pointed to the fact that estrogenic substances that positively affect bone but do not affect the gonads and the mammary glands available. Our previous studies for the past ten years strongly indicated that YCJ could act as a kind of SERMs (Balit et al., 2018; Payanglee et al., 2021; Radenahmad et al., 2009, 2010, 2012, 2015; Suwanpal et al., 2011).

Our team has gradually accumulated preliminary studies to investigate the anti-osteoporotic effects of YCJ for many years. We first started with relevant cells involved with gastrointestinal (GI) functions in the GI tract (Radenahmad et al., 2014). Those studies indicated that the reduction of the GI argyrophil cells density might influence osteoporosis among orchidectomized (ORX) rats. In turn, this phenomenon would add to the motility, calcium absorption activities, hormone production, and secretion processes in the GI tract that is often evident in osteoporotic andropause and elderly patients. Diminishing absorption of calcium, and perhaps of other minerals in the GI tract in the ORX rats, may have caused osteoporosis that can be treated with exogenous estrogen, e.g., estradiol benzoate (EB) and phytoestrogen, or YCJ feeding (Yusuh et al., 2010). The study then moved to the next step, investigating cartilage and bone of temporomandibular joint of ORX rats. We found that YCJ with a high dose of YCJ at 100 mL/kg BW/day could increase the thickness of the cartilage and mandibular cancellous bone in cell layer ORX rats (Suwanpal et al., 2011; Yusuh et al., 2010). We found that ORX rats that were given a dose of 100 mL/kg BW/day YCJ produced an unfavorable glycogen deposition in the liver (Radenahmad et al., 2012, unpublished data). As an established model of osteoporosis, castrated rats (Blouin et al., 2008) develop substantial osteoporosis with a decrease to about 35% in bone mass (Mohamad et al., 2018; Chin and Ima-Nirwana, 2015). In addition, we have proven that YCJ at three lower doses (10, 20, and 40 mL/kg BW) had no feminizing nor adverse effects on male gonads (Balit et al., 2018). Therefore, in the present study, these three lower doses were used to investigate whether it effectively prevents osteoporosis without adverse effects in ORX rats.

Bone cell phytoestrogen therapy has been reported by many studies. Kim (2021) found that isoflavones are structurally similar to 17-β-estradiol and bind to estrogen α and β receptors (Kim, 2021). Domazetovic et al. (2020) reported that blueberry juice can prevent the inhibition of osteogenic differentiation and mineralization induced by oxidative stress and triggered by glutathione depletion using human osteoblast-like

SaOS-2 cells (Domazetovic et al., 2020). Icariside I and icariside II of *Epimedii Folium* were reported by Liu et al. (2017) as two active compounds that greatly enhanced cell proliferation and osteoblast development. Estrogen is crucial for male bone health, acting through ERα (Noirrit-Esclassan et al., 2021). Characteristically, phytoestrogens show affinity to α and β estrogen receptors, like 17β-estradiol (E2) (Domínguez-López et al., 2020; Rietjens et al., 2017), and these two estrogen receptors are found in both mice and human bones (Khalid and Krum, 2016). It was found that ERα of osteocytes has an essential function in male mice trabecular bone formation (Farman, 2019). Panche et al., (2016) reported that flavonoids found in most phytoestrogens possess wound-healing properties. Our previous studies showed that YCJ accelerated wound healing (Radenahmad et al., 2012; 2015). Therefore, YCJ was presumably considered as one of the flavonoid groups that could have anti-osteoporotic effects.

Altogether, the present study aimed to explore the functioning properties of YCJ (*Cocos nucifera*, L) in preventing bone loss in the femur and the L5 vertebra, a proper model of osteoporosis among ORX rats. This study used immunohistochemistry of estrogen receptor (ER) of both ERα and ERβ in osteoblasts and osteocytes to explore whether YCJ could act through these receptors. As an established method, the micro-CT is popularly used to quantify *in vivo* anti-osteoporotic agents (Effendy et al., 2013), and microradiographs of post-mortem undecalcified histological sections provide higher resolution. Thus, this study used a micro-CT scan to investigate microarchitectural changes of the bones in detail, i.e., number, length, width, and localization.

MATERIAL AND METHODS

YCJ preparation

Substantial volume of YCJ was obtained from Khlong Hoi Khong district, Hat Yai, Songkhla, Thailand, which was then processed to become powder. The YCJ powder was maintained at -30°C temperature until used. Solution of the powder was freshly prepared before being given to the rats. Our previous publication provides the complete preparation and administration of the YCJ (Radenahmad et al., 2006).

Animals

The study used 8-month-old, 250-300 g BW adult male Wistar rats obtained from Mahidol University (Salaya, Thailand). The animals were then maintained at our animal House laboratory facilities, given standard food pellets, housed in contamination-free room that was artificially lighted with a 12h dark/light cycle, with25 ± 1°C temperature, and 50 ± 5% humidity. All animals were given normal care complying with Animal Care and Use Committee of Prince of Songkla University and the National Institutes of Health guidelines (NIH publication 86-23 revised 1985). The research protocol was approved under license number 01/59.

Experimental design

At the end of a one-week acclimatization period, the animals were divided into seven groups, each consisting of ten rats, then treated for ten weeks. First group, the baseline control (NC) animals without any treatment, sacrificed on the first day of the experiment. This first group was used as an initial baseline value in determining skeletal tissue changes resulting from aging and surgery operations (Kalu, 1991). Second group, sham-operated rats (SC). Third group, orchidectomized rats (OC). Fourth group (OE), ORX rats injected estradiol benzoate intraperitoneally with a dose of (2.5 µg/kg BW/day) for three days per week, as in our previous studies (Radenahmad et al., 2009; 2012), Fifth, sixth and seventh groups each consisted of ORX rats with YCJ gavage dose of 10 (OJ10), 20 (OJ20), and 40 ml/kg BW/day (OJ40), respectively (Table 1a). The EB injection and the feeding with YCJ began a week after orchidectomy. SC and OC rats were forced-fed with reverse osmosis water, the injection vehicles. The feeding with YCJ was done once daily, a week after the orchidectomy. The rats were sacrificed at the end of the ten-week feeding and injection treatment. The L5 vertebral body and the femur were removed, fixated using neutral formalin (10%), then decalcified. This was then followed by paraffin sectioning and immunohistochemical staining processes (Radenahmad

Table 1. Animal grouping, antibodies, concentration, and manufacturer used for bone sections of each rat.

1a. Animal grouping (10 rats per group).

Groups	Treatments
NC	Normal (normal control)
SC	Sham-operated, received reverse-osmosis water (sham control)
OC	Orchidectomized, received reverse-osmosis water (orchidectomized control)
OE	Orchidectomized, and injected with estradiol benzoate (EB, 2.5 µg/kg BW) 3 days a week, for 10 wks
OJ10	Orchidectomized, received YCJ at 10 mL/kg BW/d for 10 wks
OJ20	Orchidectomized, received YCJ at 20mL/kg BW/d for 10 wks
OJ40	Orchidectomized, received YCJ at 40mL/kg BW/d for 10 wks

1b. Immunostaining for each rat bone sections.

Section No.	Staining
1-2	H & E staining, for histological orientation
3-4	Immunostaining for mouse anti-estrogen receptor α (aa-120-170) antibody (MAB447, Millipore, CA, USA)
5-6	Immunostaining for rabbit anti-estrogen receptor β antibody (PA1-310B, Thermo Fisher Scientific, IL, USA)
7-8	Immunostaining, omitting primary antibodies (negative control), one for each antibody

1c. Dilutions of each antibody.

	Antibodies	
Bones	ER-alpha	ER-beta
Femur	1:400	1:100
Lumbar (L5)	1:200	1:100

et al., 2009). Serum estradiol and testosterone was measured using the chemiluminescent immunoassay (CIA) technique (ECLIA, Modular E 170C, Estradiol II 03000079 122, Roche, Germany and ECLIA, Modular E 170C, testosterone 11776061 122, Roche, Germany, respectively). The CIA technique details have been explained in our previous publication (Radenahmad et al., 2009).

Bone decalcification

Muscles were removed as much as possible from the bones, then fixed in 10% neutral buffer formalin for seven days. All well-fixed specimens were decalcified with 10% EDTA (ethylenediaminetetraacetic acid) chelating agent twenty times their volume changed once a week for ten weeks. The endpoint of decalcification was adjusted by X-ray (the most accurate method).

Bone morphology assessment

The excised L5 and femur were thawed at room temperature for two hours before scanning process using the micro-computed tomography method (Scanco Medical, Bassersdorf, Switzerland), and evaluation using Scanco program (version 6.5-1, Scanco Medical). The optical microscope (Axioskop 40) was used to measure %bone volume with a digital video camera (NOPL-A662, Media Cybernetics, Silver Spring, MD, USA) and the imaging software (CellSense version 1.6, Germany). The Bone volume/total volume (BV/TV), bone mineral density (BMD), trabecular thickness (Tb.Th), trabecular separations (Tb.Sp), trabecular numbers (Tb.N.), and connectivity density (Conn.D) are the structural parameters measured. The collected data were compared between groups and different levels of vertebra or femur within the group.

1.1. Percentage prevention calculation

BMD values from micro-CT scan of the control and the experimental groups were calculated for % prevention using a formula as follows (Urasopon et al., 2007):

$$\% \text{ prevention} = \left(\frac{\text{average value of treatment groups - average value of the OC group x 100}}{\text{average value of the SC group - average value of the OC group}} \right)$$

1.2. Percentage change calculation

BMD values from micro-CT scan of the control and the experimental groups were calculated for % change using a formula as follows (Urasopon et al., 2007):

$$\% \text{ change} = \left(\frac{\text{average value of treatment groups- average value of the OC group x100}}{\text{average value of the OC group}} \right)$$

1.3. Percentage Bone volume (H&E staining)

Histomorphometric analysis was done on randomly five areas of three separate 60 µm trabecular bone areas. Analysis of the bone area (%) was done using the formula described by Parfitt et al. (1987) and Suwanpal et al. (2011):

$$\text{Bone volume (\%)} = \left(\frac{\text{area of trabeculae x100}}{\text{area of trabeculae+ marrow space}} \right)$$

Immunohistochemistry

Each block was sliced with microtome to eight 5 µm-thick sections, stained with Hematoxylin and Eosin (H&E), and prepared for ERα and ERβ antibody immunostaining (Table 1b and 1c). The glass slides were coated with a poly-L-lysine solution for immunostaining. The positive controls for ERα and ERβ immunostaining from normal female rats were done on the uterus and ovary sections, respectively. The whole preparation process was guided according to our method previously reported (Radenahmad et al., 2009; 2012). The details of all antibodies, concentrations, and manufacturers used for bone sections of each rat described in Table 1b and 1c.

Quantitative analysis of ERα and ERβ immunoreactive cells

The immunoreactive cells from the L5 vertebral body and the femur were counted using light microscopy (LM) at 40x magnification power. Using an image analysis system (Samba Technologies, Meylan, France), two double-blind observers analyzed ten random fields of each slide. The mean number of immunoreactive cells/mm^2 was obtained by averaging the three antibody readings in each section.

Statistical analysis

The calculations of the sample size used Altman's nomogram. The microscopic fields were randomized for selection using Excel version 5.0. Results were expressed as mean ± SEM. The confidence interval used was $p<0.05$. Shapiro-Wilk test was used to test the normal distribution, one-way ANOVA followed by the LSD test was performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Body weight

After the ORX rats were treated with EB or YCJ for ten weeks, the body weight of the OE group on the day of termination was significantly lower than that on the day of onset (Table 2). Com-

Table 2. Body weight (g) (mean ± SEM) of the 7 groups of rats examined, NC = baseline normal control group, SC = sham-operated group, OC = orchidectomized rats, OE = orchidectomized rats + estradiol benzoate (EB), OJ10 = orchidectomized rats receiving YCJ 10 mL/kgBW, OJ20 = orchidectomized rats receiving YCJ 20 mL/kgBW, OJ40 = orchidectomized rats receiving YCJ 40 mL/kgBW. *p<0.05 comparison between the body weight at the commencement of the study and the day that the rats were terminated.

Groups BW	NC (n=10)	SC (n=10)	OC (n=10)	OE (n=10)	OJ10 (n=10)	OJ20 (n=10)	OJ40 (n=10)
BW Started	552.60 ± 32.69	540.70 ± 28.82	530.00 ± 27.33	571.70 ± 32.50	520.50 ± 22.14	504.70 ± 21.58	485.30 ± 21.06
BW finished	552.60 ± 32.69	528.20 ± 17.53	493.80 ± 17.69	489.30 ± 19.94 *	511.70 ± 21.12	485.10 ± 16.95	473.40 ± 15.04

pared between the initiation day and the last day of the experiment after force feeding with YCJ for 10 weeks, the body weight of all the 3 OJ groups (OJ10, OJ20 and OJ40) was lower, even though statistically significant was not exhibited.

Bone Parameters

Bone volume percentage. The results presented in Figs. 1A and 1B demonstrates 3D images of micro-CT measurements of the L5 vertebra and femur, respectively. Following orchidectomy (OC group), the percentage of bone volume (Figs. 2A, a-b) was significantly lower in L5 (Fig. 2A, a) but not in the femur (Fig 2A, b) compared with the control groups (NC and SC groups). That percentage of bone volume increased when the ORX rats were treated with EB (OE group) or YCJ treatments. With YCJ treatments (OJ10, OJ20, and OJ40 groups), the bone volume percentage increased but was not dose-related. The OJ20 was the best dose for both the L5 vertebra (Fig. 2A, a) and femur (Fig. 2A, b). Nevertheless, the bone vol-

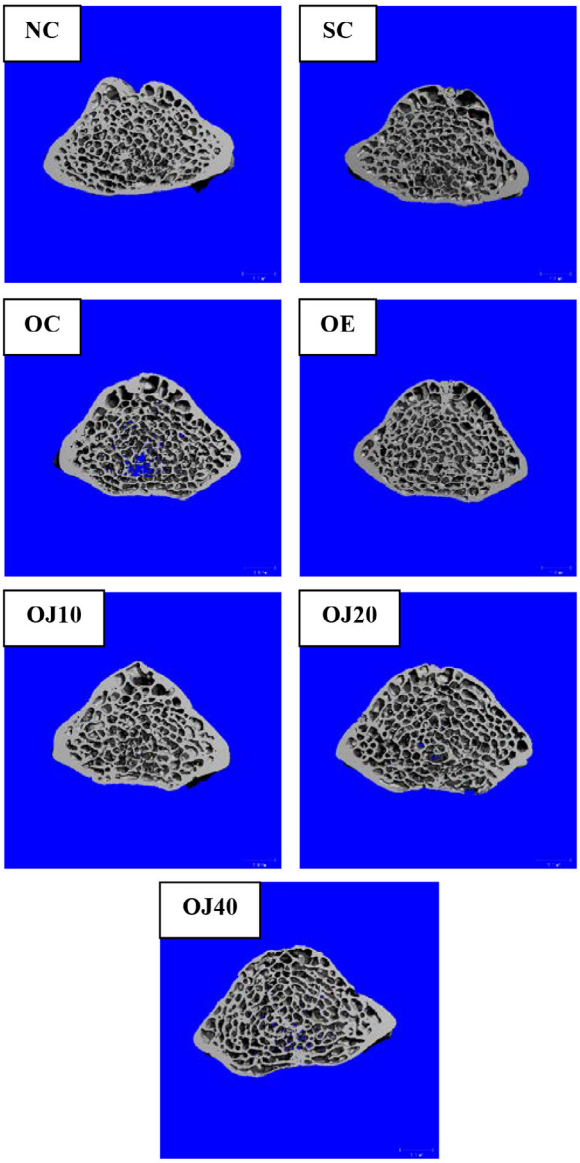


Fig. 1A.- The 3D image of micro-CT measurement of the vertebral body of 5th lumbar (L5). NC = baseline control group; SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; OJ40 = orchidectomized rat receiving YCJ 40 mL/kgBW/day.

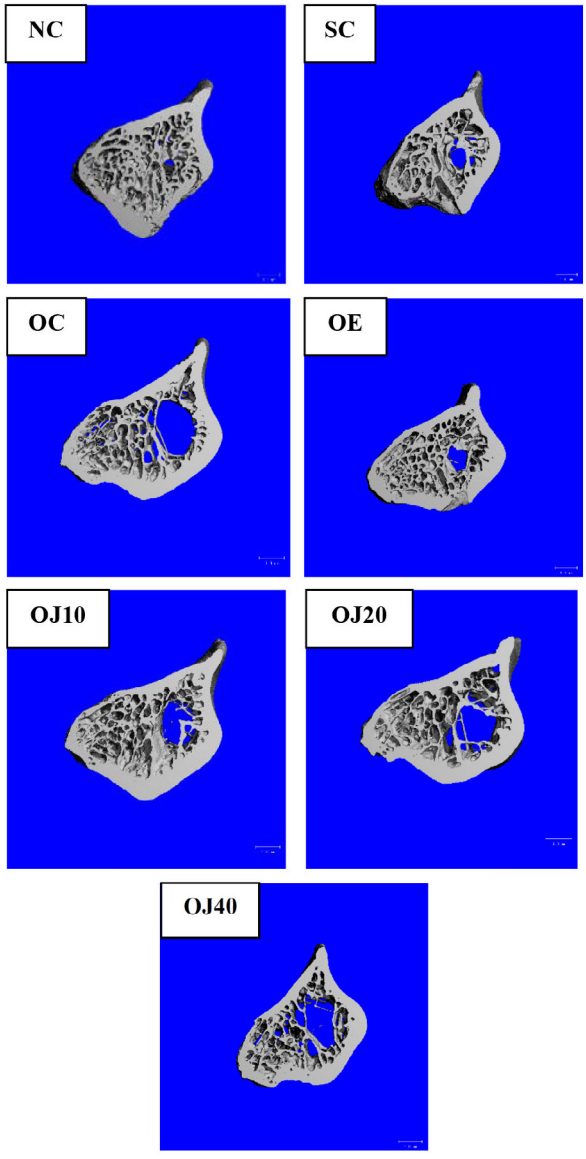


Fig. 1B.- The 3D image of micro-CT measurement of the left femur. NC = baseline control group; SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; OJ40 = orchidectomized rat receiving YCJ 40 mL/kgBW/day.

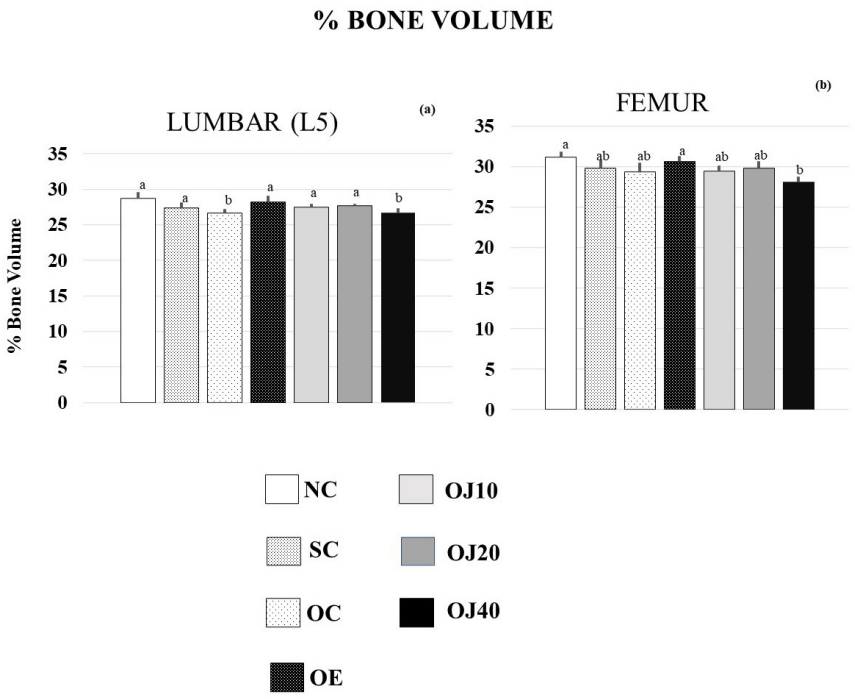


Fig. 2A.- The percentage of bone volume of the lumbar (L5) vertebra and the femur of 7 groups examined. Data expressed as mean ± SEM. Columns superscript with different letters are significantly different at $p < 0.05$. NC = baseline control group; SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; OJ40 = orchidectomized rat receiving YCJ 40 mL/kgBW/day.

ume percentage of OJ10 and OJ20 groups in both the L5 vertebra and femur were not significantly different from each other (Figs. 2A, a-b). Among three doses of YCJ treatments, that of the OJ40 group was the lowest (Figs. 2A, a-b), even though in the femur, the significant difference was not detected when compared with the OJ10 and OJ20 groups (Fig. 2A, b).

Bone density connectivity. The micro-CT scan image of the femur indicated that Conn.D of the ORX group (OC group) was significantly less than the baseline group (NC group), while the ORX groups fed with YCJ were not different significantly from either the sham (SC) or baseline (NC) groups. BV/TV of the ORX groups fed with YCJ (OJ groups) was significantly less than the baseline group, but the difference was not significant from the ORX group (Fig. 2C, e). Other bone parameters of the OJ groups, e.g., BMD% prevention, BMD% change, Tb.Th., Tb.N., Tb.Sp. was not significantly different from the sham or ORX groups (Figs. 2C, c, d, f, g, and h). In contrast, all bone parameters in the micro-CT scans of the L5 of the OJ groups were not significantly different when compared to the control groups (Figs. 2B, a-h). Unexpectedly, the Conn.D of the L5 of the OJ40 group was higher than that of the NC, OC, OE, OJ10, and OJ20 groups. Interestingly, the Conn.D. of both OJ40 and the SC groups was not significantly different (Fig. 2B, b).

Estrogen Receptors

Fig. 3 demonstrates ERα-ir and ERβ-ir reactive cells of both femur and the L5 vertebra compared between the ORX+YCJ (OJ) groups and the control (SC, OC, and OE) groups. Reactivity of both ERα and ERβ were detected in osteocytes and osteoblasts in femur bone and L5 vertebrae *(*Fig. 3*)*.

ERα-ir cells. Fig 4A depicts the number of ERα-ir cells of osteoblasts and osteocytes of the L5 vertebra (Figs. 4A, a, b) and the femur (Figs. 4A, c, d). In the NC and SC groups, the reactive cells were observed at high frequency. Following orchidectomy (OC group), the numbers of ERα -ir cells significantly dropped, particularly osteoblasts of L5 (Fig. 4A, a). The values of ERα-osteoblasts (Figs.

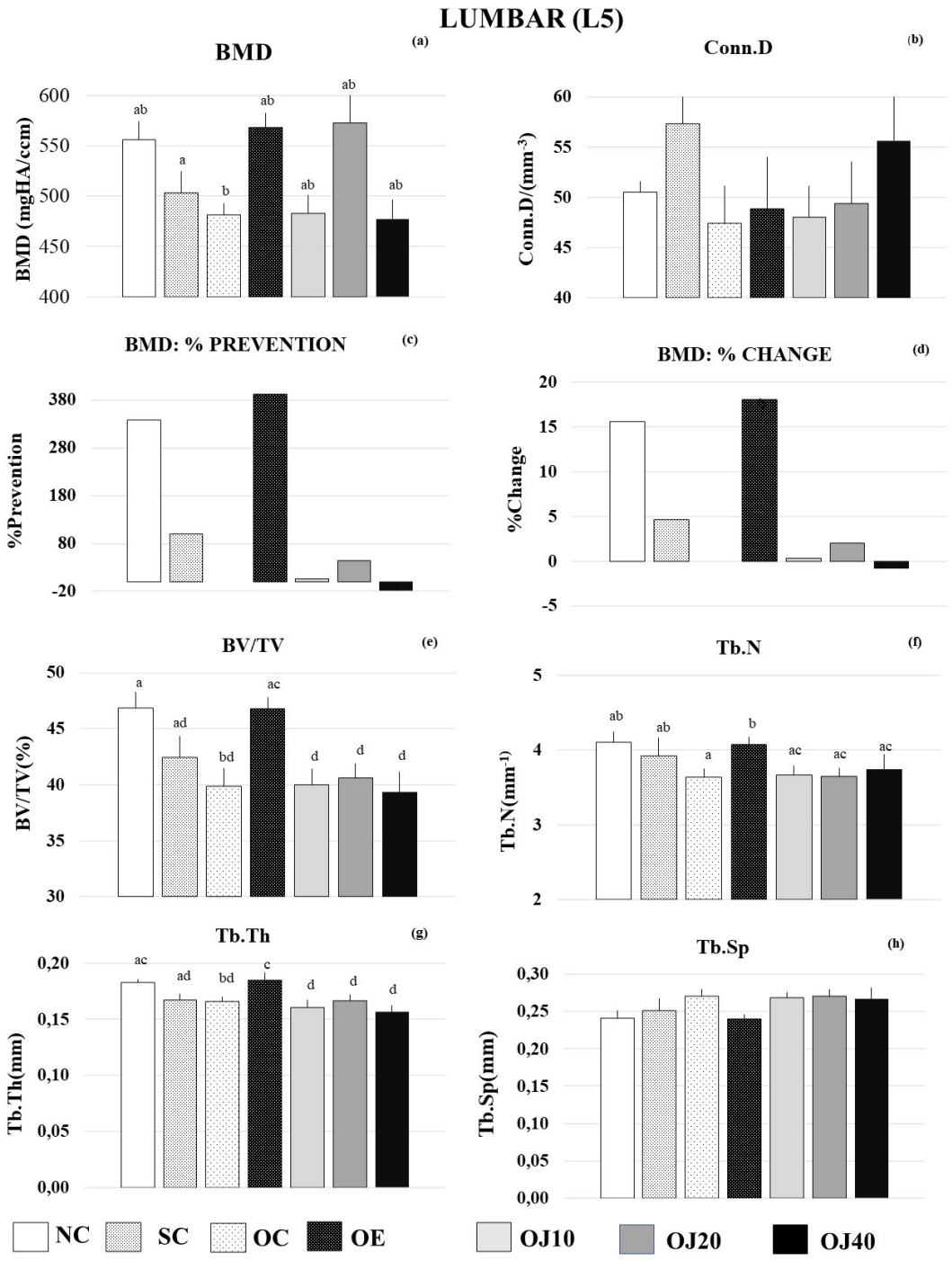


Fig. 2B.- The results of histomorphometric analyses of the 5th lumbar vertebra (L5). Data expressed as mean ± SEM. Columns superscript with different letters are significantly different at $p < 0.05$. BMD = bone mineral density; Conn.D = Connectivity density; BMD % Prevention = percentage prevention of the experimental/control groups; BMD % Change = percentage changes of the experimental/control groups; BV/TV = trabecular bone volume fraction; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation of lumbar (L5).Data expressed as mean ± SEM. NC = baseline control group; SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; OJ40 = orchidectomized rat receiving YCJ 40 mL/kgBW/day.

4A, a, c), but not of osteocytes (Figs. 4A, b, d), of both L5 and femur, significantly increased when the ORX rats were treated with EB (OE group). When ORX rats were treated with YCJ (OJ10, OJ20, and OJ40 groups), the numbers of the osteoblast- and osteocyte-reactive cells increased but were not dose-related (Figs. 4A, a-d). When compared to each other, the difference in the number of ERα–ir cells in all three OJ groups were not significant from each other. Similarly, the osteoblasts

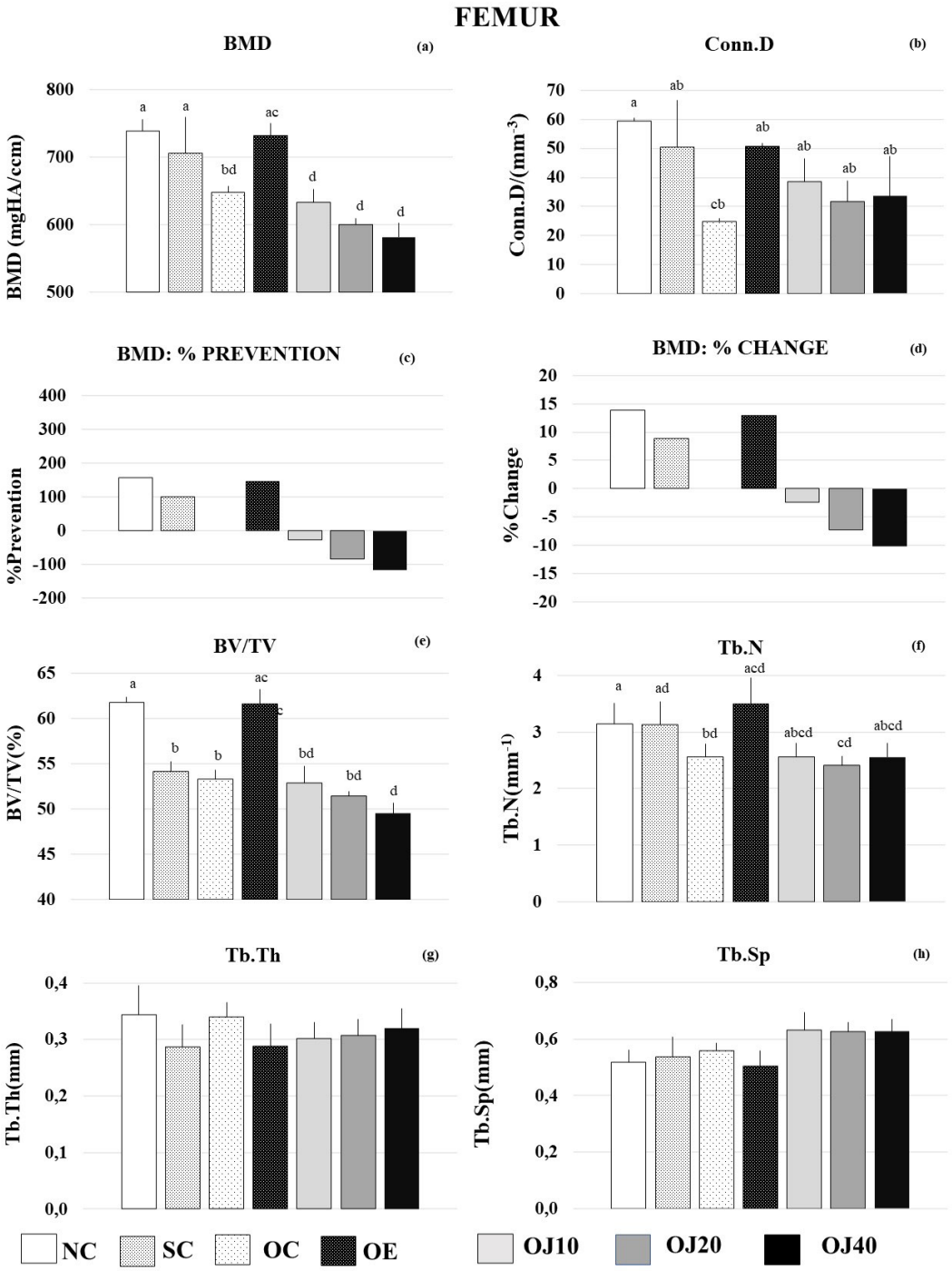


Fig. 2C.- The results of histomorphometric analyses of the femur. Data expressed as mean ± SEM. Columns superscript with different letters are significantly different at $p < 0.05$. BMD = bone mineral density; Conn.D = Connectivity density; BMD % Prevention = percentage prevention of the experimental/control groups; BMD % Change = percentage changes of the experimental/control groups; BV/TV = trabecular bone volume fraction; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation of femur. Data expressed as mean ± SEM. NC = baseline control group; SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; OJ40 = orchidectomized rat receiving YCJ 40 mL/kgBW/day.

and osteocytes in all these three OJ groups and the control (NC, SC, OC, and OE) groups were not significantly different (Figs. 4A, a-d).

ERβ-ir cells. Fig. 4B depicts the number of ERβ-ir cells of osteoblasts and osteocytes of the L5 vertebra and the femur. In the NC and SC groups, these reactive cells were observed at high frequency. Following orchidectomy (OC group), the number of ERβ–ir in osteoblasts of L5 (Fig. 4B, a) but not the femur (Fig. 4B, c) significantly decreased. That

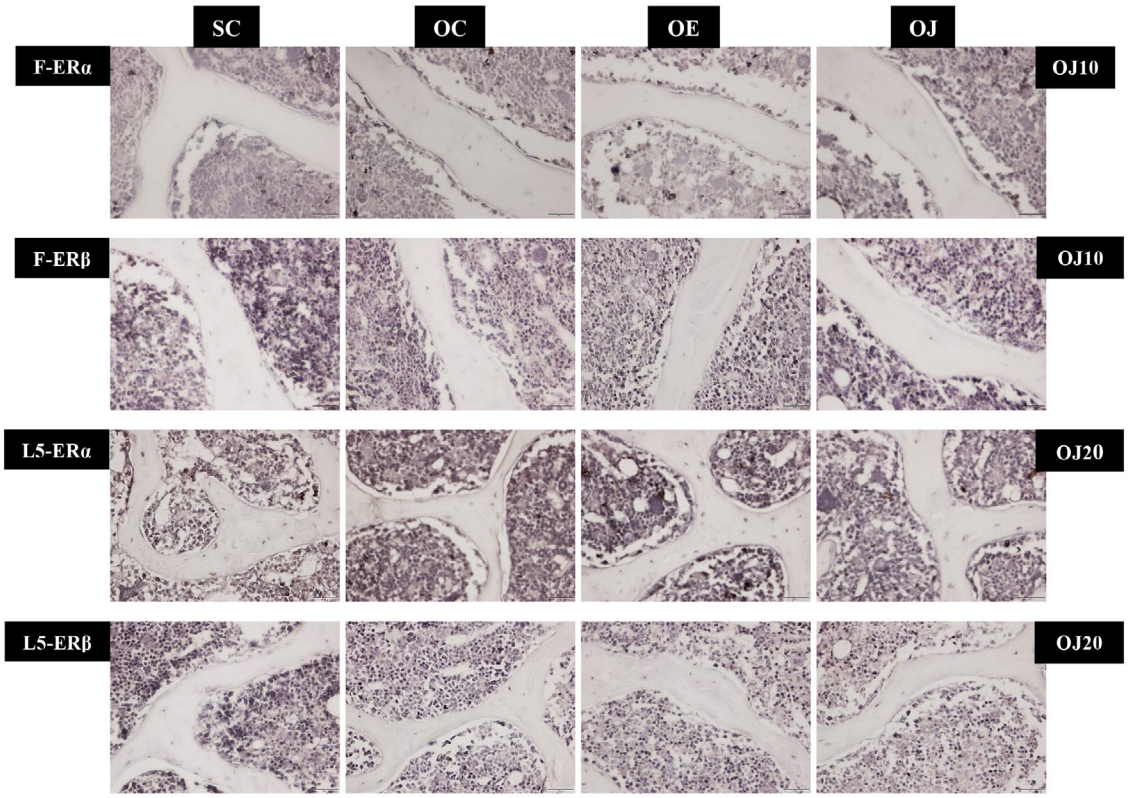


Fig. 3.- Immunohistochemical staining of ERα and ERβ cells of the femur (F-ERα and F-ERβ) and the lumbar vertebra (L5) (L-ERα and F-ERβ) at 40x magnification. Both ERα-ir and ERβ-ircells were detected on 2 kinds of bone cells: osteoblasts and osteocytes of both femur and lumbar (L5). SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; Scale bar = 20 µm.

values increased when the ORX rats were treated with EB (OE group) (Fig. 4B, a-d). With YCJ treatments (OJ10, OJ20, and OJ40 groups), the numbers of the osteoblast- and osteocyte-reactive cells increased but not in a dose-related manner (Figs. 4B, b, c, d), except in osteoblasts of L5 (Fig. 4B, a). There was no significant difference in the number of ERβ–ir cells of the three OJ groups when compared to each other nor when compared with the control (NC, SC, OC, and OE) groups (Fig. 4B, a-d).

To find out if the numbers of ERα-, ERβ- osteoblasts and osteocytes correlated with serum E2 or serum testosterone (T), the number of ERα-, ERβ-cells were plotted against those of these serum sex hormone levels in the same animals, regardless of groups (Fig. 5, a-h).

DISCUSSION

The 3D reconstructions of the femur and L5 are presented in Figs. 1A and 1B. The Micro-CT scan image of the femur showed that the BMD of the OC group was less than the NC and SC groups, while those of the ORX groups given YCJ were not significantly different from the OC group. Comparison of other parameters of the femur e.g., BMD: % prevention and % change, Tb.N, Tb.Th, Tb.Sp of the YCJ and the controls (NC, SC, OE) groups showed no significant difference, except for the Conn.D and BV/TV parameters. The OC group showed significantly less Conn.D than the NC group, while the Conn.D of all YCJ treated groups showed no significant difference from the SC, NC or OE groups (Fig. 2C, b). These results agree with the results of the previous studies (Bouxsein et al., 2010; Kleerekoper et al., 1985; Parfitt, 1987) that trabecular bone Conn.D value is a better indicator of osteoporosis than bone density. The femur's BV/TV parameter of the OE group was significantly higher than that of the three OJ and the SC groups (Fig. 2C, e). This also agrees with Lindberg (2002), who found that the proximal tibia of seven-month-old ORX mice given 17β-estradiol benzoate 0.7 µg/day five days/week for three weeks has a significantly higher BV/TV value than that of the vehicle group. In addition, except for the

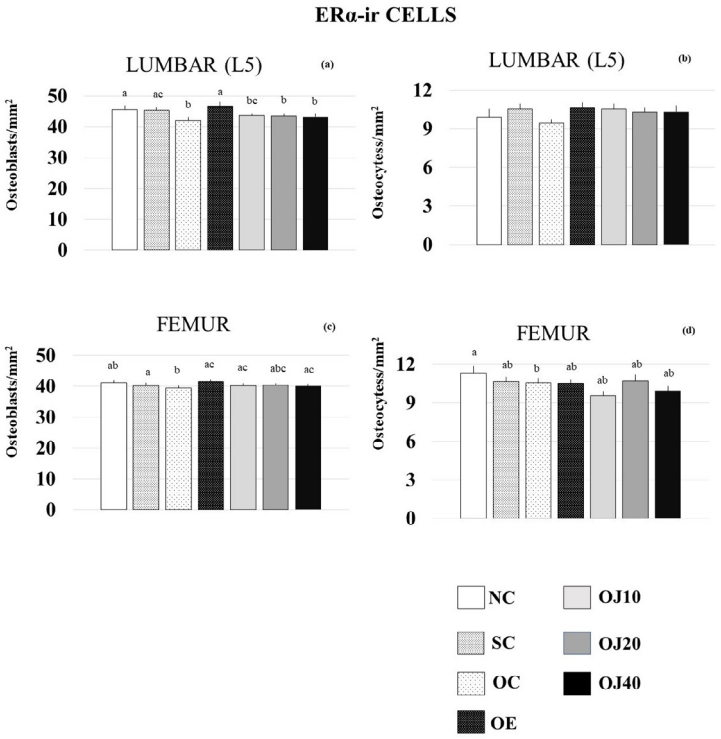


Fig. 4A.- The number of ERα-ir osteoblasts and osteocytes of the lumbar vertebra (L5) and the femur. Data expressed as mean ± SEM. Columns superscript with different letters are significantly different at $p < 0.05$. NC = baseline control group; SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; OJ40 = orchidectomized rat receiving YCJ 40 mL/kgBW/day.

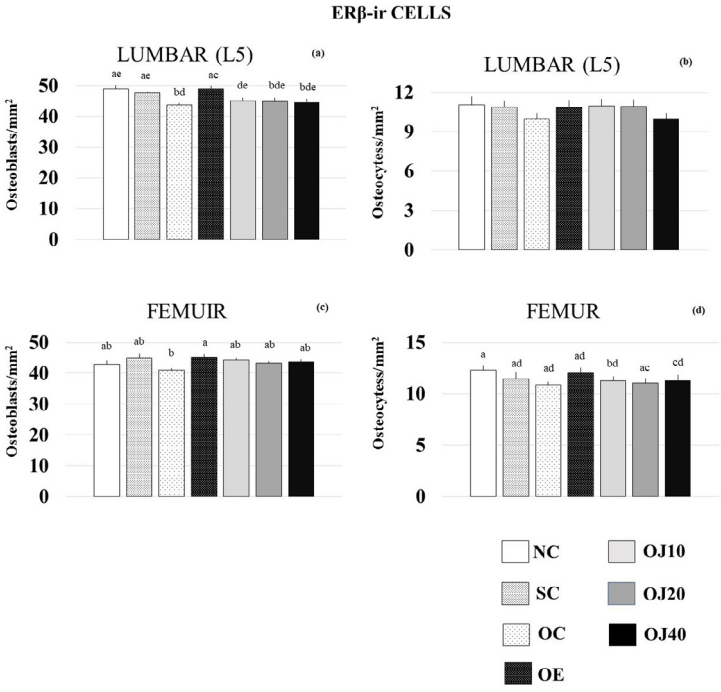
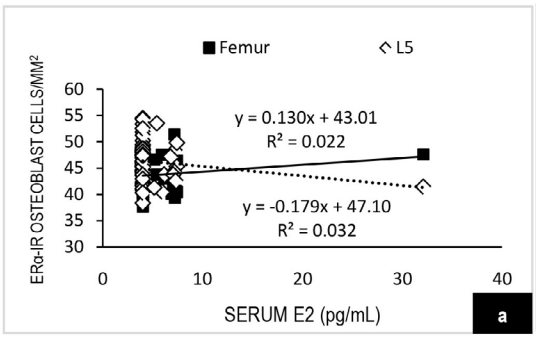
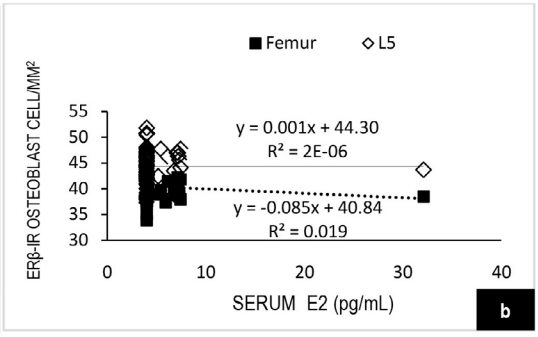


Fig. 4B.- The number of ERβ-ir osteoblasts and osteocytes of the lumbar vertebra(L5) and the femur. Data expressed as mean ± SEM. Columns superscript with different letters are significantly different at $p < 0.05$. NC = baseline control group; SC = sham-operated group; OC = orchidectomized group; OE = orchidectomized rat receiving estradiol benzoate (EB) 2.5 µg/kgBW/day; OJ10 = orchidectomized rat receiving YCJ 10 mL/kgBW/day; OJ20 = orchidectomized rat receiving YCJ 20 mL/kgBW/day; OJ40 = orchidectomized rat receiving YCJ 40 mL/kgBW/day.



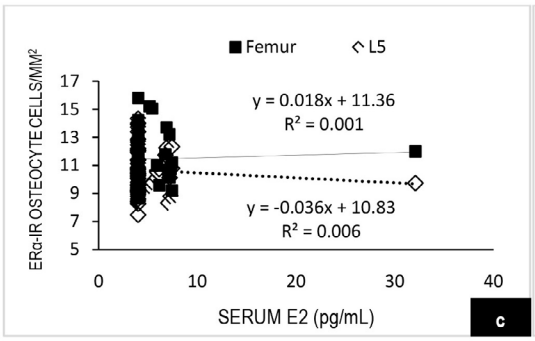


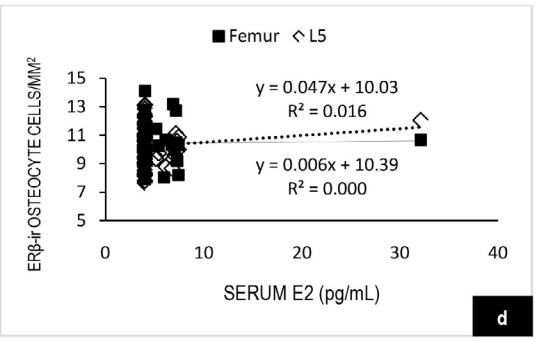
Femur (R^2 = 0.022, p-value = 0.22)

Lumbar (R^2 = 0.032, p-value = 0.14)

Femur (R^2 = 0.019, p-value = 0.26)

Lumbar (R^2 = 0.2^{-6}, p-value = 0.99)



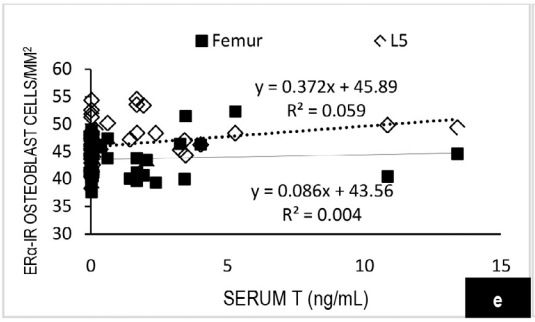


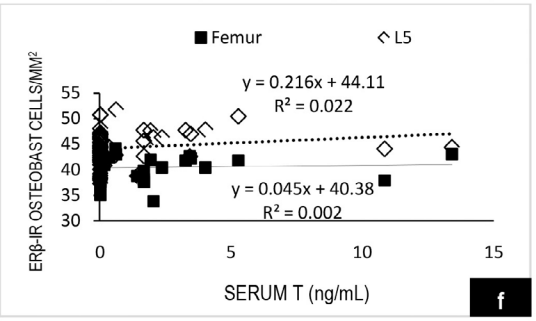
Femur (R^2 = 0.001, p-value = 0.99)

Lumbar (R^2 = 0.006, p-value = 0.51)

Femur (R^2 = 0.000, p-value = 0.89)

Lumbar (R^2 = 0.016, p-value = 0.30)



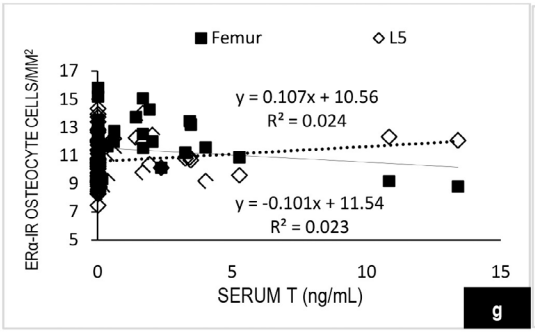


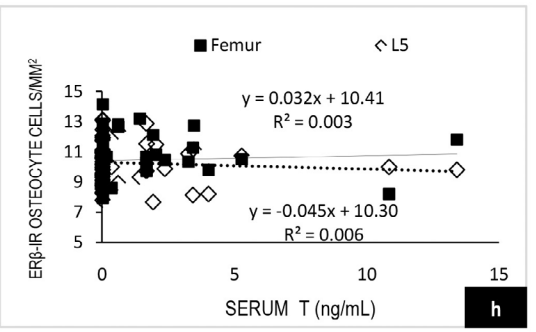
Femur (R^2 = 0.004, p-value = 0.60)

Lumbar (R^2 = 0.059, p-value = 0.05)

Femur (R^2 = 0.002, p-value = 0.69)

Lumbar (R^2 = 0.022, p-value = 0.22)





Femur (R^2 = 0.023, p-value = 0.22)

Lumbar (R^2 = 0.024, p-value = 0.20)

Femur (R^2 = 0.003, p-value = 0.63)

Lumbar (R^2 = 0.006, p-value = 0.52)

Fig. 5.- Plot of numbers of serum E2 level against ERα-ir osteoblast cells (a), ERβ-ir osteoblast cells (b), ERα-ir osteocyte cells (c), and ERβ-ir osteocyte cells (d); serum testosterone (T) level against ERα-ir osteoblast cells (e), ERβ-ir osteoblast cells (f), ERα-ir osteocyte cells (g), ERβ-ir osteocyte cells (h) from the same rats and from all animal groups.

OJ40 group, the percentage bone volume of ORX receiving YCJ, was not significantly different from all control (NC, SC, OE, and OC) groups. This result agrees with Ramli et al. (2012) that treated 10-12 months old Sprague Dawley rats *with Eurycoma longifolia* containing quassinoids phytoestrogen 90 mg/kg for six days. The treatment helped prevent bone density and volume loss at metaphysis of the distal femur of the ORX group, as shown by the insignificant difference in bone density from the sham group (Ramli et al., 2012).

In contrast, results from the Micro-CT scan of L5 indicated that there was no significant difference in all bone parameters, when the ORX groups given YCJ were compared with the control groups (Fig. 2B, a-h). Furthermore, femur % bone volume of OJ40 was significantly lower than that of the control (NC, SC, and OE) groups, but that of the OJ10 and OJ20 groups showed no significantly difference when compared with the same control groups. This indicated that YCJ is not a dose-related effect and is likely to have a SERM activity.

Several studies suggested that the Conn.D may be the parameter most affected by osteoporosis (Ramli et al., 2012). The present study demonstrated the ability of YCJ to help prevent volume loss of the femur by increasing its trabecular bone Conn.D, but not of the L5 vertebra. This is in agreement with Shaltiel et al. (2013), that reported estrogen deficiency triggering bone mass loss, causing osteoporosis by influencing vertebra less than femur and tibia. In addition, it was found that testosterone alone prevented the decline of the trabecular bone mass but not the cortical bone mass by dwindling osteoclastogenesis (Mohamad et al., 2016). Estrogen mediates androgen's bone-sparing effects only after its aromatization process. The gene expression and activity of aromatase have been indicated in male rodents and humans (Noirrit-Esclassan et al., 2021). Osteoporosis occurred in estrogen receptor-α knock-out and aromatase knock-out mice indicating that in bone, estrogen works through ERα (Noirrit-Esclassan et al., 2021). These previous studies support our current work. Furthermore, it was confirmed by immunohistochemistry study showing YCJ affecting cell number of ERα-osteoblasts in ORX+YCJ groups rather than ERα-osteocytes, and these results are obviously seen in femur rather than L5 (Fig. 4A, a-d). In contrast, either in femur or L5, immunoreactivity of ERβ-osteoblasts or osteocytes was not affected by YCJ treatments (Fig. 4B, a-d).

The regression correlation graphs (Fig. 5, a-h) between the numbers of ERα-osteoblasts and ERα-osteocytes with serum E2, or serum testosterone (T) indicated that the number of ERα-osteoblasts correlated with serum E2 and serum testosterone much more than ERα-osteocytes, and much more than ERβ-osteoblasts and osteocytes. Altogether, immunohistochemistry results and the regression correlation graphs confirmed that YCJ action was produced via ERα rather than ERβ and via osteoblasts rather than osteocytes. It is of interest to note that our result indicated that in bone tissue sections ERs staining occurred in cytoplasm and nucleus, consistent with ER mode of action, indicating the possibility of a non-genotropic pathway (Sonavane, 2022).

Estrogen plays a crucial part in male skeletal growth (Rochira et al., 2015). In this study, a dose of 2.5 µg/kg BW/day estradiol benzoate (EB) (OE group) that was intraperitoneally injected for three days a week preserved the trabecular bone. Even though some are not significantly different, the dose also countered ORX effects for all measured parameters, more than YCJ treatments. Previous studies showed that E2 is pro-osteoblastic that generate a net increase in bone-building process (Khalid and Krum, 2016), has an anti-apoptotic property in osteoblast, where ovariectomy prompts an increase in apoptosis of osteocyte and osteoblast (Rochira et al., 2015), and E2 also regulates the osteoblast anti-apoptotic protein Bcl2 (Komori, 2015; Pantschenko et al., 2005). Contrariwise, Kousteni et al. (2001) reported that E2 may inhibit dexamethasone, TNFα-induced apoptosis, or etoposide in osteoblasts. E2 also stimulates the transcription of alkaline phosphatase, a marker of osteoblast differentiation (Krum et al., 2008b). There are not many known direct targets of E2 in normal osteoblasts, and much less is reported on the targets of estrogen receptors, particularly ERβ (Farman, 2019; Khalid and Krum, 2016). Even at the physiological level of testosterone, low estrogen levels are associated with an in-

crease fracture risk in men. Therefore, although the mechanisms of osteoporotic fractures are multifactorial, estrogen deficiency plays crucial roles in the pathogenesis of osteoporosis in men and women (Khosla and Pacifici, 2021).

This study carried out immunohistochemical analyses to examine the part of estrogen receptors that facilitate osteogenic response to estrogen. The results revealed that osteoblasts and osteocytes expressed ERα and ERβ at significant quantities in the cancellous bone. These results agree with previous reports by (Khalid and Krum, 2016). These findings indicate a direct osteoblast ERα-dependent activation which contributes to bone formation induced by estrogen, was consistent with a report that estrogen has a direct action on osteoblasts in inhibiting their apoptosis (Rochira et al., 2015).

Several mechanisms contribute to the protective effects of E2 in bone. In the present study, speculatively, YCJ may induce osteoblast transcription of Fas Ligand (FasL) via ERα activation. FasL separates from the cell surface by MMP3, and this soluble FasL then stimulates osteoclast apoptosis (Garcia et al., 2013, Krum et al., 2008a). Another action is mediated by the suppression of osteoclasts involving the RANKL/(OPG) ratio regulation. Receptor activator of nuclear factor κB ligand (RANKL) is a crucial cytokine in osteoclast formation and differentiation processes, and in bone resorption activation and enhancement. Conversely, osteoprotegerin (OPG), a decoy receptor produced by osteoblastic lineage cells, counteracts RANKL thus, prevents bone loss. Therefore, the RANKL-OPG ratio is crucial for osteoclastogenesis. E2 has been indicated to escalate the transcription of OPG (Farman, 2019) and to influence RANKL positioning at the osteoblast surface (Martin et al., 2015). It was reported that in a human osteoblastic cell line, 17β-estradiol activates the ERα which in turn increases OPG mRNA levels and protein secretion (Jia et al., 2017). Windahl et al. (2013) found that in male mice osteocyte, ERα is essential for trabecular bone formation and therefore, trabecular bone volume. In the present study, however, it was found that YCJ affects the parameters tested with ERα-osteocytes were less than ERα-osteoblasts, either in femur or L5.

Our previous studies have shown that YCJ is likely to have acted as SERM. For example, flavonoids exist in most of the phytoestrogens with healing properties (Panche et al., 2016), and our previous studies found that YCJ accelerated wound healing (Radenahmad et al., 2012; 2015). Previous studies with the SERM and raloxifene prevented both postmenopausal and andropausal osteoporosis (Farman, 2019; Pankova and Tsvetkova, 2015; Zhou et al., 2021).

The results of body weights of rats in the same rat experiment groups were published by Balit et al. (2018). We found that after 10 weeks of ORX, the OE group's body weight on the day of termination was considerably lower than on the day of initiation (Table 2). These findings suggest that in the OE group, testosterone shortage after ORX and estrogen supplementation by injection with EB contributed to the weight loss. Pratchayasakul et al. (2011) reported that the body weight of both normal and high fat diet male mice treated with estrogen was considerably lower than the vehicle treatment group.

The body weight of all three OJ groups (OJ10, OJ20, and OJ40) was lower at the end of the experiment after force feeding with YCJ for 10 weeks compared with the onset albeit not statistically significant. YCJ might not influence the accumulation of fat in ORX rats, even though YCJ contains a high proportion of 44.9 percent glucose and 43.9 percent fructose (Santoso et al., 1995), these monosaccharides might have been converted to glycogen in the liver or fat in the body.

YCJ had no effects on male accessory organs, e.g., seminal vesicle, prostate gland, and have positive effects on serum cholesterol and mineral levels e.g., phosphorus, sodium, calcium, potassium, and magnesium; blood sugar, renal and liver function tests (Balit et al., 2018). Therefore, YCJ may also be a good selection for male osteoporosis therapy. In this study, however, YCJ mimicked the estrogen conserving effects but could not achieve the estrogenic level in all parameters. YCJ significantly preserved the microstructure of the osteoporotic bones, but this effect is not dose-related. It is interesting to see whether higher doses of YCJ would produce the same results as EB treatment.

YCJ contains various phytoestrogens, but has β-sitosterolis as its main component (58%). It also contains other sterols i.e., stigmastatrienol, stigmasterol, α-spinasterol, and fucosterol (Pungmatharith, 1988). As plant-derived estrogen, phytoestrogens possess different characteristics from estrogen but is like SERMs, having higher binding for ERβ (Pankova and Tsvetkova, 2015). The ERα is found predominantly in cortical bone, whereas ERβ in cancellous bone. In our study, preserving the effects of YCJ acts predominantly on ERα-osteoblasts. Therefore, the preventive effects for osteoporosis are not as straightforward as they should be. In addition, the lower doses (10, 20, 40 mL/kg BW) used in this study as well as in another study (Matsushita et al, 2017) using 15 mL/kg BW dose might account for the slight results compared with the apparent results of our previous studies at 100 mL/kg BW/day (Matsushita et al., 2017; Sayoh et al., 2008; Suwanpal et al., 2011). In bone, the function of ERβ in males is less than ERα, while in female ERβ controls ERα functions (Farman, 2019). Supplementation may prevent the periosteal apposition of cortical bone but not trabecular bone. Therefore, we speculate that YCJ supplementation reversed bone loss, possibly via ERα, not via ERβ, as speculated by Matsushita (2017). ERα is definitely the major core of estrogen osteoprotective actions, and ERβ is insufficient to compensate for ERα (Noirrit-Escalssan et al., 2021)

There have been many research works on molecular mechanisms of phytoestrogens in preventing osteoporosis. For example, Domazetovic et al. (2020) found that in humans, blueberry juice (BJ) protects osteoblast-like SaOS-2 cells from Runt-related transcription factor 2 and alkaline phosphatase as oxidative damage components associated with bone formation and remodeling. It did so by stimulating the expression a potential molecular target for anti-osteoporotic medicines, the sirtuin type 1 deacetylase. It has been suggested that blueberry polyphenols may be advantageous for bone regeneration because, TSP (total soluble polyphenols) quantitative studies revealed that BJ contains high bioavailability of anthocyanins having exceptional antioxidant properties (Domazetovic et al, 2020). Genistein, a type of isoflavone, enhances osteoprotegerin protein expression in bone-forming osteoblasts. This isoflavone facilitates osteoblastic differentiation and proliferation by inhibiting the transformation of a receptor activator of the nuclear factor-κB ligand (RANKL) into an osteoclast in bone marrow stem cells via the Wnt3a/beta-catenin signaling pathway (Hsiao et al., 2020; Kim, 2021; Yu et al., 2015). Astragalin (AG), also known as kaempferol-3-O-glucoside, enhances osteoblastic differentiation and bone formation through synergistic interactions that involve both BMP and MAPK pathways. BMP (bone morphogenetic protein) is a significant member of the TGF (transforming growth factor) family of proteins. MAPKs (mitogen-activated protein kinases) are a type of serine/threonine kinase that are involved in a variety of cellular processes include differentiation, proliferation, and inflammation (Liu et al. (2019). Whether YCJ action is the same as phytoestrogens' action mentioned above needs further verification in future studies. Further investigation is vital for a full comprehension on the impact of YCJ on osteogenic cells.

Since calcium is an essential mineral in bones, absorption of calcium in the intestine performs a significant part in calcium uptake into the body as facilitated by the essential function of vitamin D and by the work of calbindin, a calcium-binding protein (Wongdee et al., 2019). Our parallel work in the same rats with the same model and experimental design investigated CB, VDR, ERα and ERβ using immunohistochemistry techniques. We have found that exogenous estrogen (EB) and YCJ prevent osteoporosis by increasing CB-, VDR-, ERα- and ERβ-reactive cells in the gastrointestinal tract of orchidectomized rats (Hayeelateh et al., 2022). Therefore, estrogen/phytoestrogen-YCJ may influence calcium-binding protein, CB, and VDR cell production, resulting in bone calcium deposition by increasing ERα- and ERβ-reactive cell numbers, %bone volume, and other factors Micro-CT parameters in the femur and L5 vertebra as occurred in this study.

In summary, long-term YCJ consumption slightly improved indices of bone mass or bone histomorphometry in ORX rats, contending suggestion of the full benefits of YCJ for the prevention of os-

teoporosis in andropausal or aging men. Considering, however, the results of our previous studies (Suwanpal et al., 2011; Yusuh et al., 2010), YCJ may be beneficial in lessening bone loss in men at early andropause at a young age, which we hypothesize may delay the need and lessen the time duration of anti-apoptotic treatment as reported by Morii et al. (2015) and Matsushita et al. (2017) who worked on osteoporotic female rats.

CONCLUSION

YCJ had a noticeable effect and delayed the bone loss at trabecular structures associated with osteoporosis. Its effects, however, did not achieve the level of the estrogen-protective effect on the microfracture incidence, the basis of osteoporotic fractures. YCJ, nevertheless, may provide a potential male osteoporosis treatment, considering that the feminizing effects of estrogen do not counsel its use in males.

This study revealed that (1) EB injection at 2.5 µg/kg BW/day for three days per week is enough to restore all reactive cells as detected by anti-ERα- and anti-ERβ-ir antibodies and all parameters detected by Micro-CT scans. (2) YCJ treatment at various doses restored the declined numbers of ERα- and ERβ-ir cells caused by orchidectomy to normal in either L5 vertebra or femur. (3) Immunohistochemistry technique revealed that the effects of YCJ were comparable to EB treatment effects. In most cases, the optimal dose was 10 mL/kg BW/day for ERα- of both osteoblasts and osteocytes and 40 mL/kg BW/day for ERβ-of both osteoblasts and osteocytes. (4) YCJ action influenced via ERα rather than ERβ and osteoblasts rather than osteocytes. (5) Using Micro-CT scan, YCJ treatment, at the doses tested in this study, rebuilt the connectivity density and % bone volume of L5 vertebra and femur. These findings indicate that feeding YCJ might account for, at least in part, for a partial protective role of estrogen replacement in preventing or reducing the risk of osteoporosis in male rats.

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