The effects of *Labisia pumila var. alata* on bone markers and bone calcium in a rat model of post-menopausal osteoporosis

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**A R T I C L E   I N F O**

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**A B S T R A C T**

**Aim of the study:** Postmenopausal osteoporosis is mainly treated with estrogen replacement therapy (ERT). However, ERT causes side effects, mainly breast cancer, uterine cancer and thromboembolic problems. *Labisia pumila var. arata* (LPva), a herb with phytoestrogenic effects has the potential to be used as an alternative agent to ERT. This study was conducted to determine the effects of LPva on bone biochemical markers and bone calcium content in ovariectomised rats.

**Materials and methods:** Thirty two Wistar rats were divided into 4 groups, with 8 rats in each group. The first group was sham operated (Sham), the second group was ovariectomised (OVX), the third (LPva) and fourth group (ERT) were also ovariectomised and given LPva 17.5 mg/kg and Premarin® 64.5 µg/kg, respectively. Blood samples were taken before and after treatment to measure osteocalcin and C-terminal telopeptide of type 1 collagen levels using ELISA while the fifth lumbar bone samples were taken to measure bone calcium content using the Atomic Absorption Spectrophotometer (AAS).

**Results:** The osteocalcin levels were significantly higher in both the LPva and ERT groups compared to the OVX group. The CTX levels were significantly lower in both the LPva and ERT groups compared to the OVX group. However, only the ERT group had significantly higher bone calcium level compared to the OVX group.

**Conclusion:** The supplementation of 17.5 mg/kg of LPva to ovariectomised rats for 8 weeks was able to prevent the changes in bone biochemical markers but failed to prevent the bone calcium loss induced by ovariectomy.

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1. Introduction

Osteoporosis is characterised by low bone mineral density resulting in fragile bones with increased risk of fracture. According to the World Health Organisation (WHO), osteoporosis occurs when bone mineral density falls to more than 2.5 standard deviations (SD) below the standard reference for maximum bone mineral density of the young adult female. According to statistics, 75 million people in the United States of America, Europe and Japan suffer from osteoporosis. Women were found to have a higher risk of getting osteoporosis than men with the ratio of 1.6:1. The sudden drop in estrogen in post-menopausal women can lead to osteoporosis. There is a 45% reduction in trabecular bone number (TbN) while the remaining trabecular bone hypertrophies to compensate for the loss. Micro fractures occur and the biomechanical strength is compromised, exposing the bone to serious fracture (Fordham, 2004). In the year 2000, there are about 9 million cases of fracture due to osteoporosis worldwide. About 1 in 3 women aged more than 50 years old experienced an osteoporotic fracture in their lifetime (International Osteoporosis Foundation, 2009).

Estrogen replacement therapy (ERT) is the main form of treatment and prevention of post menopausal osteoporosis. Estrogen (17β-estradiol) given alone or combined with progesterone was able to prevent postmenopausal bone loss effectively (Al-Azzawi, 2008). Estrogen binds to the estrogen receptor on the osteoclast surface, which causes release of chemical mediators and reduction of osteoclastic activity, therefore inhibiting bone resorption (Arcangelo and Peterson, 2005). Estrogen also promotes absorption of calcium, activation of vitamin D and synthesis of calcitonin in order to prevent osteoporosis (Raisz, 2005). Studies have shown that ERT lowered the risk of osteoporosis and reduced the risk of vertebral fracture by 34% and skeletal fracture in general by 24% (Rossouw et al., 2002; Speroff and Fritz, 2005). However, ERT...
has been found to increase the risks of breast cancer, uterine cancer as well as cardiovascular and thromboembolic diseases (Raisz, 2005). These problems occurred in a small but important number of women within the first 1–4 years of using ERT. According to the North American Menopause Society (2007), no particular form or dose of ERT has been proven to be safer compared to others. In response to these findings, efforts are on the way to find alternative anti-osteoporotic agents with comparable effectiveness to estrogen but with minimal side-effects.

*Labisia pumila*, a herbal plant from the family of Myrsinaceae may have some potential to be an alternative agent to estrogen. There are three types of *Labisia pumila*, i.e. *Labisia pumila var. alata* (LPva), *Labisia pumila var. pumila* (LPvp) and *Labisia pumila var. lanceolata* (LPvl) (Arifin, 2005). Traditionally, *Labisia pumila* extract is prepared by boiling the roots, leaves or the whole plant in water and the extract is taken orally (Burkill, 1935; Zainon et al., 1999; Runi, 2001). It is used to accelerate labour, shrink the uterus, improve the menstrual cycle and for weight loss (Arifin, 2005). Its’ exclusive use in women has led to the belief that it is a phytoestrogen, a compound with similar chemical structure to estrogen (Jamia et al., 2003) and therefore able to relieve menopausal symptoms (Bhathena and Velasquez, 2002). The ethanolic extract of LPva has a weak but specific estrogenic effect on human endometrial adenocarcinoma cells of the Ishikawa-var I line, resulting in enhanced secretion of alkaline phosphatase (Jamia et al., 1998). The water extracts of LPva were able to displace estradiol bound to estrogen receptors (Husniza, 2002). Furthermore, LPva increased uterine weight in ovariectomised and dihydrotestosterone-induced polycystic ovarian syndrome rats, thus exhibiting estrogenic properties (Fazliana et al., 2009; Manerras et al., 2010). LPva was also able to initiate lipolysis in adipose tissue in a manner similar to that reported for estrogen (Ayida et al., 2007).

The estrogenic activites of LPva may also be benefical for the treatment of postmenopausal osteoporosis. Therefore, we carried out a study to determine the effects of LPva using ovariectomised rats as the model of postmenopausal osteoporosis. Bone biomarkers were measured as indicators of bone formation and resorption, while bone calcium content was used as an indicator of bone loss in this model. The effects of LPva were compared to a group of ovariectomised rats given ERT as the gold standard of treatment for postmenopausal osteoporosis.

2. Materials and methods

2.1. Animal model

32 female Wistar rats aged 3 months were obtained from the Universiti Kebangsaan Malaysia Laboratory Animal Research Unit and divided randomly into 4 groups. The rats were housed in plastic cages at temperature of 29 ± 3 °C under natural day/night cycle. They were fed commercial food pellets (Gold Coin, Port Klang, Malaysia) and deionised water *ad libitum*. They were allowed to adjust to the new environment for a week before the study was started.

2.2. LPva extract

LPva freeze dried extract was supplied by Phytes Biotek Sdn Bhd. (Malaysia) (Batch No: KF071107). The extract was obtained from the root of the plant. The brownish powdered extract was dissolved in deionised water and given via oral gavage at the dose of 17.5 mg/kg rat weight daily at 9 am for 8 weeks (Ayida et al., 2007). Premarin® (Wyeth-Ayerst, Kanada) tablet containing 0.625 mg of conjugated estrogen was crushed, dissolved in deionised water and given via oral gavage at the dose of 64.5 μg/kg rat weight daily at 9 am for 8 weeks (Ayida et al., 2007).

2.3. Study design

Rats in the first group were sham-operated (Sham group) while the rats in the rest of the groups were ovariectomised. The second group was the ovariectomised control group (OVX group). The third (LPva) and fourth (ERT) groups were ovariectomised and given 17.5 mg/kg LPva or 64.5 μg/kg Premarin, respectively daily via oral gavage for 8 weeks. Premarin is often used as an estrogen replacement therapy in postmenopausal women, therefore the ERT group acted as the positive control. The duration of the study was thought to be adequate for changes in bone parameters based on the finding that bone mineral density was reduced 8 weeks after ovariectomy (Ima-Nirwana et al., 1998). Body weights were measured before the start of treatment and then weekly until the end of the study. The study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (ethical clearance number: PP/FAR/2009/NAZRUN/14-JULY/267-JULY-2009-MAY-2010).

2.4. Blood and bone sampling

Blood samples were collected before the start and after 8 weeks of treatment from the retro-orbital vein after anesthetizing the rats with ether. After 3 h, the blood was centrifuged at 3000 rpm for 10 min and the serum was stored at –70 °C. At the end of the treatment period, the rats were sacrificed humanely and the fifth lumbar vertebrae were dissected out and cleansed of all soft tissues.

2.5. Biochemical analysis

Bone biochemical markers of serum Osteocalcin and C-terminal telopeptide of type 1 collagen (CTX) were measured before and after the treatment period using an ELISA reader (VERSAmax, Sunnyvale, USA). The kits used were Rat Osteocalcin ELISA (Biomedical Technologies, Herlev, Denmark) and RatlapsTM ELISA CTX-1 (Nordic Biosciences, IDS UK).

2.6. Bone calcium content measurement

The bones were dried in an oven at 100 °C for 24 h, then ashed in a furnace at 800 °C for 12 h. The ash was weighed and dissolved in 3 ml nitric acid and then diluted in lanthanum chloride. Calcium chloride was measured with an Atomic Absorption Spectrophotometer (Shimadzu AA-680) at 422.7 nm.

2.7. Statistical analysis

The results were expressed as mean ± SEM. The statistical significance of the data was determined using one-way analysis of variance (ANOVA) and post hoc Tukey’s test. The software used was the “Statistical Package for Social Sciences” (SPSS) version 16.0. The level of significance was taken as p < 0.05.

3. Results

3.1. Body weights

After 8 weeks of treatment, all the groups had significantly higher body weights compared to their initial body weights. The final body weights were not significantly different between groups except for the Sham group which had a significantly lower body weight than all the other groups (Fig. 1).
3.2. Bone biochemical markers

There was no significant difference in osteocalcin levels for all the groups before the start of the treatment. After 8 weeks of treatment, the osteocalcin level of the OVX group was significantly lower compared to the Sham, LPva and ERT groups. This indicated that ovariectomy had resulted in low osteocalcin levels but treatment with LPva or estrogen had successfully maintained the level of this bone formation marker. There was no significant difference between the LPva and ERT groups (Fig. 2).

As for the serum CTX levels, there were no significant difference between all the groups before the start of treatment. After 8 weeks of treatment, the CTX level of the OVX group was significantly elevated compared to the Sham, LPva and ERT groups. This meant that ovariectomy had resulted in elevation of CTX level but treatment with LPva or estrogen had prevented the elevation of this bone resorption marker. There was no significant difference between the LPva and ERT groups (Fig. 3).

3.3. Bone calcium content

Ovariectomy was found to cause significant loss in bone calcium which was consistent with osteoporosis due to estrogen deficiency. When the ovariectomised rats were given estrogen replacement, the bone calcium was preserved as observed in the ERT group. LPva supplemented to ovariectomised rats failed to preserve the bone calcium as was shown by estrogen replacement (Fig. 4).

4. Discussion

Hormone or estrogen replacement therapy (HRT/ERT) has been used for prevention and treatment of postmenopausal osteoporosis but may cause serious side-effects such as breast cancer, uterine cancer and thromboembolic disease (Ferguson, 2004). Women taking ERT have twice the risk of getting uterine cancer while those taking ERT for more than 15 years have one half higher risk of getting breast cancer compared to those not taking ERT (Lane, 2001). Heart and Estrogen/Progestin Replacement Study (HERS) has shown that women with cardiovascular diseases have higher rate of cardiovascular complications during the first year of taking ERT (Conteras and Parra, 2000). The Women’s Health Initiative study found that women who took HRT have slightly higher rates of breast cancer, ovarian cancer, heart attack, stroke, thromboembolism and Alzheimer’s disease (Rossouw et al., 2002; Chlebowski et al., 2003; Shumaker et al., 2003).

In light of the side-effects of ERT and estrogenic properties of LPva, we have investigated the potential of LPva as an alternative treatment for postmenopausal osteoporosis.
shown to be safe with LD50 of more than 5.0 g/kg (Wan Ezumi et al., 2007). LPva extract was found to exhibit no-adverse-effect-level (NOAEL) at the dose of 50 mg/kg in sub-acute toxicity study (Singh et al., 2009), 1000 mg/kg in subchronic toxicity study (Taneja, 2008) and 800 mg/kg in reproductive toxicity study (Wan Ezumi et al., 2007). The ovariectomised rat was used in this study as its bone loss share many similar characteristics to postmenopausal women and therefore is a suitable model for postmenopausal osteoporosis (Mosekilde and Mosekilde, 1990; Kalu, 1991).

The body weight of the rat increased steadily throughout the study. As was seen in other studies, ovariectomised rats had significantly higher body weight compared to sham-operated rats due to fat deposition caused by estrogen deficiency (McElroy and Wade, 1987; Devlin and Ferguson, 1988; Azman et al., 2001). Both LPva and estrogen failed to prevent the weight gain induced by ovariectomy which was opposite to the findings in a study by Fazlilana et al. (2009). These different findings may be contributed by the lower doses of LPva and estrogen used in our study. The doses of 17.5 mg/kg of LPva and 64.5 µg/kg of ERT used in our study were based on Ayida et al. (2007) and were shown to be able to modulate postmenopausal adiposity. Fazlilana et al. (2009) had used several doses of LPva ranging from 10 to 50 mg/kg but found that only the dose of 50 mg/kg was able to suppress the weight gain of ovariectomised rats. While, the dose of estrogen used in the same study was 0.625 mg/kg. However, our lower estrogen dose was acceptable as more importantly, this dose was able to prevent bone calcium loss and bone marker changes induced by ovariectomy.

In terms of the bone calcium content, our data showed that LPva failed to match estrogen in preventing the loss of bone calcium in the ovariectomised rats although the trend was there. Perhaps, higher doses of LPva is required before it is able to prevent the bone calcium loss.

In this study, we managed to demonstrate the bone biochemical marker changes characteristics of osteoporosis in ovariectomised rats. Osteocalcin is a non-collagenous protein synthesised by mature osteoblast and is generally regarded as a specific marker for osteoblastic activity and for bone formation (Delmas, 1992; Eastell et al., 1993; Akesion et al., 1995). At the end of the study, the osteocalcin level was found to be lowered in ovariectomised rats, corresponding to reduced bone formation. Treatment with LPva or estrogen to ovariectomised rats was able to return the osteocalcin to the level seen in the sham-operated group, indicating normalisation of bone formation.

CTX are peptide fragments from collagen degradation by cathepsin enzyme that is released by osteoclasts during bone resorption. These collagen crosslinks enter the blood circulation which can be measured as biochemical markers of bone resorption (Calvo et al., 1996; Garnero and Delmas, 1998). It is sensitive and specific in detection of osteoporosis as it has a low coefficient of variation (Rosen et al., 2000). The CTX level was raised in our ovariectomised rat model, consistent with other studies which have shown that the CTX level was raised after menopause (Garnero, 1996; Melton, 1997). The CTX level was found to be reduced when anti-osteoporotic treatment is started (Rosen et al., 2000). In our study, the CTX level was lowered with LPva or estrogen treatment given to ovariectomised rats. This is the first report on the finding that LPva was as effective as estrogen in preventing the changes in the bone markers induced by ovariectomy. Therefore, it has potential as an alternative to estrogen in the treatment of postmenopausal osteoporosis.

The protective effects of LPva seen in our study may be contributed by its phytoestrogenic actions. However, its estrogenic activities have been shown to be too weak (Jami et al., 2003) to match the effects of estrogen on the bone formation and resorption markers. Therefore LPva may affect bone via other mechanisms of action. It has been reported to contain benzoquinoid derivatives, alkenyl resorcinols and triterpenoid compound (Houghton et al., 1999) but there are no studies to indicate that any of these compounds could be responsible for the effects of LPva.

Osteoporosis has been shown to be associated with oxidative stress (Ahmad et al., 2005). Low estrogen levels in postmenopausal women have been associated with oxidative stress (Busu et al., 2001; Maggio et al., 2003) while physiological levels of estrogen have been shown to protect low density lipoprotein against oxidation (Sack et al., 1994). Furthermore, estrogen can also be considered as an antioxidant as it was found to exhibit antioxidant protection of lipoproteins in the aqueous system (Badeau et al., 2005) and was also shown to increase the expression of glutathione peroxidase in osteoclasts (Lean et al., 2005). Vitamin E, a potent anti-oxidant was able to protect ovariectomised rats against osteoporosis (Norazlina et al., 2000). LPva extract demonstrated a strong antioxidant activity comparable to that of ascorbic acid, one of the strongest known antioxidants (Choi et al., 2010). Perhaps, LPva protected the ovariectomised rats in this study via its antioxidant effects. Another possible mechanism is one that is related to TNF-α, a bone resorbing cytokine which promotes bone resorption by activating mature osteoclasts or by stimulating proliferation and differentiation of osteoclasts (Pfeilschifter et al., 1989; van der Pluijm et al., 1991; Lerner and Ohlin, 1993). It has been shown that blocking the effects of TNF-α prevented post-ovariectomy bone loss (Kitazawa et al., 1994). This was also seen in ovariectomised transgenic mice that over expressed a soluble form of the TNF receptor that blocks TNF binding to membrane receptors (Ammann et al., 1997). LPva extract was found to suppress the TNF-α level to below the baseline level in cultured HaCaT cells (Choi et al., 2010). This inhibitory effect on TNF-α may be another possible mechanism for preventing bone resorption in the estrogen deficient state. Relevant to this, a study on postmenopausal women found that etanercept, a specific blocker of TNF-α action was able to blunt the increase in serum CTX levels (Charatcharoenwitthaya et al., 2007). This is similar with our finding that LPva was able to blunt the increase in serum CTX levels of our rat model of postmenopausal osteoporosis.

Based on its effects on the bone markers of ovariectomised rats which is comparable to estrogen and its safety profile, LPva has the potential to be utilised as an alternative treatment for postmenopausal osteoporosis. Higher doses of LPva may be needed to match estrogen in terms of preventing the bone calcium loss. Further studies are required to determine its anti-osteoporotic mechanisms.

Conflict of interest

The authors declare to have no conflict of interests whatsoever. The authors alone are responsible for the content and writing of the paper.

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