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Time and Dose-Dependent Effects of *Labisia pumila* on Bone Oxidative Status of Postmenopausal Osteoporosis Rat Model

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Abstract: Postmenopausal osteoporosis can be associated with oxidative stress and deterioration of antioxidant enzymes. It is mainly treated with estrogen replacement therapy (ERT). Although effective, ERT may cause adverse effects such as breast cancer and pulmonary embolism. *Labisia pumila var. alata* (LP), a herb used traditionally for women's health was found to protect against estrogen-deficient osteoporosis. An extensive study was conducted in postmenopausal osteoporosis rat model using several LP doses and duration of treatments to determine if anti-oxidative mechanisms were involved in its bone protective effects. Ninety-six female Sprague-Dawley rats were randomly divided into six groups of baseline group (BL), sham-operated (Sham), ovariectomised control (OVXC), ovariectomised and given 64.5ìg/kg of Premarin (ERT), ovariectomised and given 20 mg/kg of LP (LP20) and ovariectomised and given 100 mg/kg of LP (LP100). The groups were further subdivided to receive their respective treatments via daily oral gavages for three, six or nine weeks of treatment periods. Following euthanization, the femora were dissected out for bone oxidative measurements which includes superoxide dismutase (SOD), glutathione peroxidase (GPx) and melondialdehyde (MDA) levels. Results revealed that the SOD levels of the Sham and all the treatment groups were significantly higher than the OVX groups at all treatment periods. The GPx level of ERT and LP100 groups at 9th week of treatment were significantly higher than the baseline and OVX groups. The MDA level of the OVX group was significantly higher than all the other groups. The LP 20 and LP100 groups at 9th week of treatment had significantly lower MDA levels than the ERT group. In conclusion,LP supplementation at 100 mg/kg for 9 weeks was able to increase antioxidant enzymes and reduced lipid peroxidation. Hence, LP may prevent bone loss via its antioxidative property.

Key words: Osteoporosis · Menopause · Estrogen · Antioxidant · *Labisia pumila*

INTRODUCTION

Osteoporosis has been associated with many factors primarily aging and hormonal disturbances which mainly affects women. Osteoporosis is a silent, slowly progressive systemic skeletal disease that is characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased bone fragility, resulting in an increased risk of fractures [1]. As defined by World Health Organization (WHO), osteoporosis occurs when bone mineral density (BMD) T score is more than 2.5 standard deviation below the peak bone mass reference standard for young women [2]. This is a common condition affecting 30% of women and 12% of men at some point of their lifetimes [3]. Based on the main causes of osteoporosis which are aging and hormonal disturbances, postmenopausal women are greatly affected by this condition due to tremendous loss of estrogen after menopause.

Aging and hormonal disturbances in postmenopausal women can be related to oxidative stress. Antioxidant systems play an important role in

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suppressing the development of osteoporosis [4]. Due to estrogen reduction following menopause, the body is subjected to high level of free radicals and disruption of oxidative stress defense system. These will later lead to increased production of inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-11, IL-17 and tumor necrosis factor (TNF)-α [5]. These cytokines are potent stimulators of bone resorption which promote osteoclast differentiation and activation as well as inhibit their apoptosis [6]. Consequently, bone resorption activity by osteoclasts will outweigh the bone formation resulting in bone loss.

Since estrogens are the major hormonal regulator of bone metabolism, it therefore has been used in the treatment of postmenopausal osteoporosis. Due to the presence of estrogen receptors on osteoblast and osteoclast cells, estrogen acts directly via the activation of estrogen-receptor complex which will stimulate the osteoblast formation and induce osteoclast apoptosis [7]. Hence, estrogen replacement therapy (ERT) is effective in increasing sex hormone level and improving bone mass as reported in many studies. Although effective, prolonged use of ERT may lead to many adverse effects such as breast cancer, cardiac infarction, stroke and pulmonary embolism [8]. To date, other forms of effective treatment of postmenopausal osteoporosis are the selective estrogen receptor modulators (SERMs) such as Raloxifene and biphosphonates such as alendronate and risedronate [9]. It was reported that Raloxifene is able to prevent bone loss as well as reducing risk of fractures in women with low bone mass [10]. Biphosphonates on the other hand have also been proven to be potent inhibitors of bone resorption. Prolonged use of all these anti-osteoporotic agents however may result in adverse effects such as thromboembolism, cataract, esophagitis and osteonecrosis of jaw bones [11].

Although conventional osteoporosis treatments are available, the use of natural remedies such as tocotrienol [12], soy [13] and blueberry [14] is on the rise. The effectiveness and adverse effects of natural remedies need to be investigated. *Labisia pumila* (LP) or also known by the locals as Kacip Fatimah, Akar Fatimah, Pokok Pinggang and Belangkas Hutan [15] has been widely used by women for many generations. Its water extract is traditionally consumed by women to treat menstrual irregularities, promote uterine contraction and promote sexual health function [16]. LP was also reported to be effective against gonorrhoea, rheumatism and sickness in bones [17]. The mechanisms of LP are still unclear but it has been speculated that the health values of LP are contributed by its phytoestrogenic, antioxidative and anti-inflammatory properties [18]. Previous study done by Nazrun *et al*. (2011) [19] showed that supplementation of LP at the dose of 17.5 mg/kg was able to increase bone formation marker and reduce bone resorption marker in ovariectomized rats. There is a paucity in the literature on antioxidative mechanism of LP although this herb is rich with antioxidant compounds. Since our main concern is on oxidative stress-related osteoporosis, this study was conducted to determine the dose and time-dependent effects of LP supplementation on oxidative stress and antioxidative enzyme markers in the bone of ovariectomized rats.

MATERIALS AND METHODS

Animals and Treatment: The study was approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (ethical approval number: FP/FAR/2011/NAZRUN/30-NOVEMBER/415-NOVEMBER-2011-MAY-2012). 96 female Sprague-Dawley rats aged 3-5 months weighing between 200-250g were obtained from the Universiti Kebangsaan Malaysia Laboratory Animal Research Unit. The rats were housed in plastic cages at temperature of 29 ± 3°C under natural day/night cycle. They were fed with commercial food pellets (Gold Coin, Port Klang, Malaysia) and deionised water *ad libitum*. They were allowed to acclimitized the new environment for a week before the study was started. They were then randomly divided into six main groups with six rats in the baseline group (BL) and eighteen rats in the rest of the groups which consisted of sham-operated (Sham), ovariectomized control (OVXC), ovariectomized and given estrogen (Premarin) at 64.5µg/kg (ERT), ovariectomized and given *Labisia pumila* at 20mg/kg (LP20) and ovariectomized and given *Labisia pumila* at 100mg/kg (LP100). All the treatments were given daily via oral gavages. These groups were subdivided into three, six and nine weeks of treatment periods. Body weights were measured before the start of treatment and weekly until the end of the study.

***Labisia Pumila Var. Alata* (LP) and Estrogen (ERT):** Raw powdered form of LP was supplied by Delima Jelita Herbs (Alor Setar, Kedah). It was obtained from the *Labisia pumila var. alata* whole plant and was grinded and freeze dried into powdered form. The dried powdered

LP extract was sent to Forest Research Institute Malaysia (FRIM) for phytochemical screening to detect the phytochemical constituents. LP was dissolved in deionised water and given via oral gavage at doses of 20 mg/kg or 100 mg/kg rat weight daily at 9 am for 3, 6 or 9 weeks according to their assigned groups. Estrogen (Premarin®, Wyeth-Ayerst, Canada) tablet containing 0.625mg of conjugated estrogen was crushed, dissolved in deionised water and given via oral gavage at the dose of 64.5µg/kg rat weight daily at 9 am for 3, 6 or 9 weeks accoding to their assigned groups.

Bone Sampling: Rats in the BL group were euthanized before the start of the study while other rats were euthanized upon completion of their treatments. Femora were dissected and cleaned from all muscles and soft tissues. They were then wrapped in phosphate-buffered saline-soaked gauze and rewrapped with aluminium foil prior to storage in -70°C freezer until they were ready to be tested for superoxide dismutase (SOD), glutathione peroxidise (GPx) and malondialdehyde (MDA) level.

Measurement of Superoxide Dismutase (SOD) Enzyme: The femur was homogenized firstly by perfusing the bone with phosphate-buffered saline at pH 7.4 to remove any blood cells or clots. Then 0.25 g of bone was weighed and placed in 10mL tube containing 2mL of 20mM HEPES buffer (20mM HEPES buffer pH 7.2, containing 1 mM EGTA, 210 mM Mannitol and 70mM sucrose per gram tissue). The mixture was then homogenized using tissue homogenizer Omni Bead Ruptor 24 (Omni International Inc) prior to centrifugation at 1500 x g for 5min at 4°C. The supernatant was collected to measure the superoxide dismutase enzyme level using Superoxide Dismutase Assay Kit (Cayman Chemical Company, USA) [20]. SOD level was measured spectrophotometrically at 540nm using Microplate Reader (MBC VERSA max, USA).

Measurement of Glutathione Peroxidase (GPx): About 10 mg femur was weighed for homogenization. The tissue was placed in a tube containing 0.2 mL phosphate-buffered saline prior to homogenization using Omni Bead Ruptor 24 for 40 seconds. The homogenized tissue was centrifuged at 14,000 x g for 10 minutes at 4?C. The supernatant was finally collected for GPx assay using Glutathione Peroxidase Assay Kit (Bioassay Systems, USA) [20]. GPx was estimated by measuring the optical density (OD) at 340 nm.

Measurement of Melondialdehyde (MDA): Prior to homogenization, about 25 mg of the femur was weighed and placed into tube containing 250 µL RIPA buffer with protease inhibitors. The mixture was then homogenized using Omni Bead Ruptor 24 for 40 seconds and centrifuged at 1,600 x g for 10 minutes at 4°C. The supernatant was collected for malondialdehyde (MDA) measurement using TBARS Assay kit (Cayman Chemical Company, USA) [20]. The concentration of MDA was measured spectrophotometrically at 540 nm.

Statistical Analysis: The data were analysed using Statistical Package for Social Sciences software (SPSS 19.0, Chicago, USA). Firstly, the data were tested for normality using the Kolmogorov-Smirnov test (n=<100). For normally distributed data, the statistical tests used were the analysis of variance (ANOVA), followed by Tukey's HSD test. For data that were not normally distributed, Kruskal-Wallis and Mann-Whitney tests were used. All the results were expressed as mean ± standard error of the mean (SEM).

RESULTS

Superoxide Dismutase (SOD): At the 3rd, 6th and 9th weeks all the groups showed significantly higher SOD level than the OVX group ($p< 0.05$). The SOD level of the OVX group was significantly lower than the BL group. The SOD level of the LP20 at 6th week was significantly higher than the SHAM and ERT groups, hence it has exhibited the best result compared to the other groups (Figure 1).

Glutathione Peroxidase (GPx): There was no significant results for all the groups at the 3rd week of treatment. At 6th week, SHAM had a significantly higher GPx level compared to the BL and OVX groups. The ERT and LP100 groups at 9th week showed higher GPx level than the BL and OVX groups (Figure 2).

Lipid Peroxidation (MDA): There was no significant results for all the groups at the 3rd week of treatment. The OVX group at the 6th and 9th weeks showed significantly higher MDA levels compared to the BL group. All the groups at the 6th and 9th weeks showed significantly lower MDA levels compared to the OVX group. The LP20 group at the 6th week had a significantly lower MDA level than the SHAM and ERT groups. The LP20 and LP100 at the 9th week had significantly lower MDA levels compared to the ERT group (Figure 3).

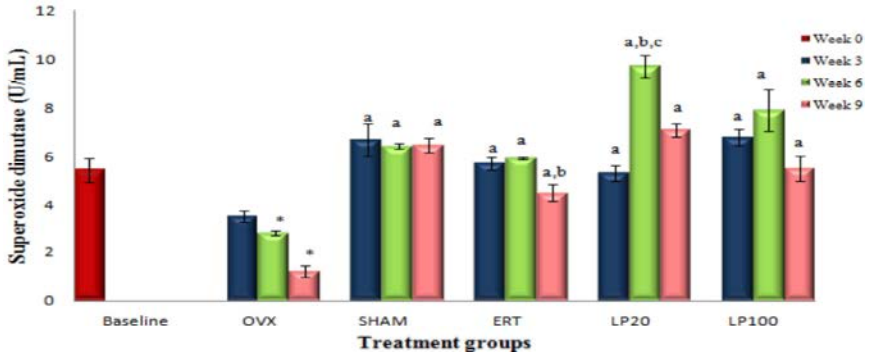

Fig. 1: Mean superoxide dismutase concentration for all the groups after 3, 6 and 9 weeks of treatment.Data presented mean ± SEM ($p< 0.05$). Sham: sham-operated, OVX: ovariectomized control, ERT: ovariectomized and estrogen supplementation, LP20: ovariectomized and supplemented with LP at the dose of 20mg/kg, LP100: ovariectomized and supplemented with LP at the dose of 100mg/kg. * $p < 0.05$ vs Baseline, $^{a}p < 0.05$ vs OVX, $^{b}p < 0.05$ vs SHAM, $^{c}p < 0.05$ vs ERT

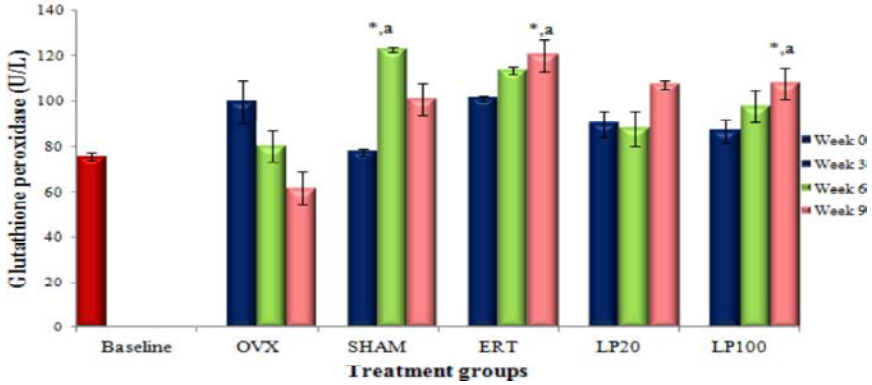

Fig. 2: Mean glutathione peroxidase concentration for all the groups after 3, 6 and 9 weeks of treatment.Data presented mean ± SEM ($p < 0.05$). Sham: sham-operated, OVX: ovariectomized control, ERT: ovariectomized and estrogen supplementation, LP20: ovariectomized and supplemented with LP at the dose of 20mg/kg, LP100: ovariectomized and supplemented with LP at the dose of 100mg/kg. * $p < 0.05$ vs Baseline, $^{a}p < 0.05$ vs OVX

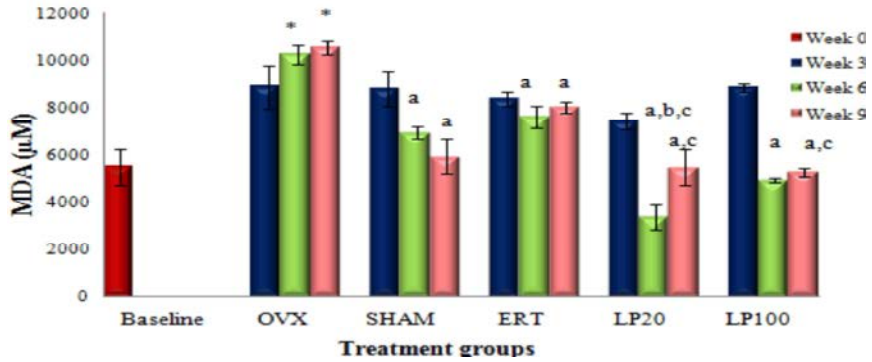

Fig. 3: Mean melondialdehyde concentration for all the groups after 3, 6 and 9 weeks of treatment.Data presented mean ± SEM ($p < 0.05$). Sham: sham-operated, OVX: ovariectomized control, ERT: ovariectomized and estrogen supplementation, LP20: ovariectomized and supplemented with LP at the dose of 20mg/kg, LP100: ovariectomized and supplemented with LP at the dose of 100mg/kg. * $p < 0.05$ vs Baseline, $^{a}p < 0.05$ vs OVX, $^{b}p < 0.05$ vs SHAM, $^{c}p < 0.05$ vs ERT

DISCUSSION

Estrogen has profound effects on bone physiology which keeps the bone turnover rate at balance. Estrogen reduction is the major cause of osteoporosis in postmenopausal women. Ovariectomized rats were used in this study because they exhibit progressive bone deterioration through a process that is similar to postmenopausal osteoporosis. Following ovariectomy, reduction in estrogen levels causes an increase in bone turnover, where bone resorption exceeds bone formation leading to bone loss [21].

It has been reported that oxidative stress and impairment of the antioxidant defense system may be responsible for the bone loss in postmenopausal osteoporosis [22]. Reactive oxygen species (ROS) were shown to be responsible for the development of osteoporosis [23-25]. Previous study by Nazrun *et al.* [19] has found that LP was able to increase the bone formation marker and reduce the bone resorption marker. Another literature reported that there were improvements in histomorphometric parameters of ovariectomized rats supplemented with LP [26]. This has led to our interest in studying the antioxidative effect of LP on bone. To the best of our knowledge, this is the first report on antioxidative mechanism of LP in preventing bone loss

In our present study, two doses of LP (20 mg/kg and 100 mg/kg) were given at three different duration of treatments (3, 6 and 9 weeks) to evaluate the dose and time-dependent effects of LP on antioxidative parameters. LP extract has been shown to be safe with LD50 of more than 5.0 g/kg [27]. According to other studies, LP extract was found to exhibit no-adverse-effect-level (NOAEL) at the dose of 50 mg/kg in sub-acute [28], 1000 mg/kg in sub-chronic [29] and 800 mg/kg in reproductive toxicity studies [27]. In human, the effective doses normally taken by women are around 500-1000mg/kg daily. Therefore, the doses used in this study are considered to be safe.

At the 6^{th} and 9^{th} weeks of treatment, there were significant reductions in SOD levels of the ovariectomised rats compared to baseline rats. Rats in all the groups at the 3^{rd}, 6^{th} and 9^{th} weeks of treatment showed significantly higher SOD levels than the ovariectomised rats of the corresponding week. Rats which received LP supplementation at 20 mg/kg daily (LP20 group) for 6 weeks showed the best result where their SOD level was significantly higher than rats in the OVX, Sham and ERT groups.

Rats supplemented with ERT and LP at 100 mg/kg had significantly higher GPx levels than rats in the OVX and BL groups. The positive effects of LP on the antioxidative enzyme were seen after 6 weeks of treatment. This reiterates the notion that in response to oxidative stress following ovariectomy, the levels of anti-oxidative enzymes were augmented by the antioxidative compunds present in LP. Consistent with this, the MDA level of all the LP-supplemented groups were lowered. The ovariectomised rats on the other hand showed significantly higher MDA levels compared to other groups. Similar changes were seen in in postmenopausal osteoporotic women where their glutathione reductase were significantly reduced while their MDA levels were significantly elevated compared to normal women [30,31].

Following ovariectomy, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) were released in abundance together with cytokines and prostaglandins [32]. This may explain the oxidative stress findings in our study where there were reduction in antioxidative enzymes and elevation of MDA levels of rats in the OVX group. ROS may not only stimulate osteoclastic differentiation and function but is also essential in osteoblast apoptosis [33,34]. Therefore, oxidative stress may lead to increased osteoclastic activity and decreased osteoblastic activity [35]. This will result in cell damage especially via lipid peroxidation, with MDA as the end product [36]. Due to its high reactivity towards amino groups, MDA may inhibit the nucleic acids and proteins synthesis which consequently deactivates the antioxidant enzymes [37]. Hence, the decrease antioxidant enzymes in femur of ovariectomised rats may be related to increase in lipid peroxidation activity in the bone [38].

Antioxidant enzymes play an important role in reducing lipid peroxidation by breaking down the oxidation chain and suppressing free radicals release [39]. Their levels would be reduced with the antioxidative activities as reported by the low SOD and GPx levels in the femur of OVX rats [40]. While, ERT and LP-supplementation were found to maintain the antioxidant enzyme levels. This is in line with the fact that estrogen is able to offer antioxidant protection of lipoproteins and increase the expression of these enzymes in bone cells [41]. On the other hand, LP stimulated the antioxidant enzymes activity and decreased the MDA level via its phytoestrogenic and antioxidative properties.

Previous studies reported that the antioxidative properties of LP are contributed by the presence of flavonoids, ascorbic acid, beta-carotene, anthocyanin and phenolic compounds [42]. In another study done by Norhaiza *et al.* (2009), it was reported that among the constituents in LP, β-carotene showed the best correlation with the antioxidative activities, followed by flavonoids, ascorbic acid, anthocyanin and phenolic content [43]. β-carotene and flavonoids have been shown to be effective in scavenging free radicals by quenching singlet oxygen and consequently inhibiting peroxyl free radicals [44,45]. It was reported that flavonoids and phenolic compounds posses some features that resemble estrogens, allowing them to bind to the estrogen receptors (ERs) [46,44]. This binding will consequently regulate the receptors to stimulate osteoblasts activity [48]. The estrogen-like properties of these compounds may promote osteoblast differentiation and thus bone formation activities [49]. Chen *et al.* [50] reported that phenolic acids were able to reduce osteoclastogenesis, hence reducing bone resorption activity as well as increasing bone mass. There was also a report that rats supplemented with anthocyanin have higher osteoblast differentiation and osteoclast apoptosis [51]. In short, all these reports have strongly supported our study findings on the LP antioxidative effects on bone.

Oxidative stress may increase cytokine production by activating the transcription factors nuclear factor kappa B (NFêB) and activator protein-1 (AP-1) [52,53]. Elevated cytokine levels such as interleukin (IL)-1, IL-6 and IL-11 will result in osteoclasts differentiation and activation, leading to accelerated bone loss. These cytokines may also influence osteoclastogenesis by stimulating self-renewal of osteoclasts and inhibiting their apoptosis [54,55]. LP which contains flavonoid has been shown to inhibit production of nitric oxide (NO), via suppression of NFêB [56,57]. NO is an important regulator of bone metabolism [58,59]. It was reported that NO exerted biphasic effects on bone resorption, whereby high levels is associated with bone loss and vice versa [60]. High NO level contributes to the pathogenesis of osteoporosis by enhancing the ability of IL-1 and TNF to stimulate osteoclast activity [61]. LP may have prevented bone loss by suppressing the NO level via its antioxidative property. This is also supported by a study which have shown that the leaf and root extracts of Labisia pumila var. alata decreased NO production [62].

CONCLUSION

The present study has confirmed that LP was able to protect against estrogen-deficient bone loss in a dose and time-dependent manner. Supplementation of LP at 100 mg/kg for 9 weeks was the best treatment regimen for bone protection as it was able to increase both SOD and GPx levels and reduce lipid peroxidation. Due to its comparable effect to ERT and good safety profile, LP has the potential to be used as an alternative treatment for postmenopausal osteoporosis. The mechanism may be contributed by LP anti-oxidative property. However, further studies are required for a more detailed and conclusive mechanism of LP.

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