Effects of Palm Tocotrienols on Oxidative Stress and Bone Strength in Ovariectomised Rats

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ABSTRACT

Oxidative stress has been associated with postmenopausal osteoporosis which predisposes to risk of fracture. Palm tocotrienol is a potent antioxidant and has the potential to be used for treatment of post-menopausal osteoporosis. The aim of the study is to determine if palm tocotrienol supplementation could alleviate oxidative stress in ovariectomised rat model and improve its bone strength. The rats were di-

Kata kunci: vitamin E, tikus terovariektomi, osteoporosis, histomorfometri tulang, biomekanikal

ABSTRACT

Oxidative stress has been associated with postmenopausal osteoporosis which predisposes to risk of fracture. Palm tocotrienol is a potent antioxidant and has the potential to be used for treatment of post-menopausal osteoporosis. The aim of the study is to determine if palm tocotrienol supplementation could alleviate oxidative stress in ovariectomised rat model and improve its bone strength. The rats were di-
vided into four groups: (i) sham-operated group (SHAM) (ii) ovariectomised-control group (OVX) (iii) ovariectomised and given 60mg/kg α-tocopherol by oral gavage (OVX + ATF) (iv) ovariectomised and given 60mg/kg palm tocotrienols by oral gavage (OVX + PTT). After eight weeks of treatment, blood samples were taken to measure oxidative status (MDA, SOD and GPX) while the femurs were biomechanically tested for strength and resistance to fracture. Ovariectomy was shown to induce oxidative stress as shown by the raised MDA levels and reduced GPX activity. Palm tocotrienols seemed to offer protection against the ovariectomy-induced oxidative stress as shown by the suppression of MDA levels and raised GPX and SOD activities in the OVX+PTT group. In comparison, α-tocopherol was only able to raise the SOD but not as high as palm tocotrienols. The biomechanical tests have shown that ovariectomy has not affected the bone strength significantly after eight weeks. Palm tocotrienols supplementation for eight weeks was effective in preventing oxidative stress in a post-menopausal rat.

Key words: vitamin E, ovariectomised-rats, osteoporosis, bone histomorphometry, biomechanical

INTRODUCTION

Osteoporosis is a condition of decreased bone mass which leads to fragile bones with increased risk of fractures. It has affected 75 million people in Europe, USA and Japan (EFFO & NOF 1997). One in 3 women and 1 in 5 men over 50 years old will experience osteoporotic fractures (Melton et al. 1998, Kanis et al. 2000, Randell et al. 1995, Gullberg et al. 1997). It is projected that more than 50% of all osteoporotic hip fractures will occur in Asia by the year 2050 (Cooper et al. 1992). Post menopausal osteoporosis, which occurs in estrogen-deficient women, is the commonest form of osteoporosis. There is increasing evidence that oxidative stress may be partly responsible for post-menopausal osteoporosis. Estrogen itself is an antioxidant with radical-scavenging properties and its deficiency has been associated with oxidative stress (Dubey et al. 2000). Oxidative stress has been shown to stimulate osteoclast differentiation and function (Garrett et al. 1990) and inhibit osteoblast differentiation (Bai et al. 2004). Ferric nitrilotiracetate, an oxidizing agent which causes oxidative stress was shown to reduce bone calcium content in young growing rats (Yee & Ima Nirwana 1998), increase osteoclast numbers and reduce bone volume and thickness (Ahmad et al. 2005).

Many human studies have linked oxidative stress to osteoporosis. In subjects with osteoporosis, the malondialdehyde (MDA) levels were increased while the antioxidant enzymes, superoxide dismutase and glutathione peroxidase were reduced compared to the control group (Sontakke et al. 2002). While in another recent study, osteoporotic women were found to have high MDA levels and low catalase and gluthatione peroxidase activities (Ozgocmen et al. 2007). Elderly women with osteoporosis were found to have lower endogenous and exogenous anti-oxidants (Maggio et al. 2003). A population based study found an association between the increases of prostaglandin F2α, a bio-marker of oxidative stress to reduction in bone mineral density (Basu et al. 2001).

With all the evidence pointing to the in
volvement of oxidative stress in osteoporosis, studies on the ability of potent antioxidants like vitamin E to protect bone from osteoporosis in estrogen deficient women need to be carried out. It is also important to determine if the benefit of vitamin E intake would extend to better bone strength in order to reduce the risk of osteoporotic fracture. A biomechanical test is a useful tool to evaluate bone strength which is associated with susceptibility to fracture. It can only be carried out in animal models as bone samples need to be subjected to force until they break to get the accurate result. Previous studies have used the rat as a standard rodent model for bone biomechanical studies (Ferretti et al. 1993, Kimmel 1996, Peng et al. 1994, Raab et al. 1990, Tuukkanen et al. 1994).

Vitamin E is a fat soluble vitamin with a chain-breaking ability. There are two forms of vitamin E, tocopherol and tocotrienol which can be further subdivided into α, β, γ and δ. Tocotrienol is a unique vitamin E which is abundant in palm oil of *Elaeis guineensis* species. Tocotrienol has better antioxidant ability compared to tocopherol because of its higher recycling efficacy (Serbinova et al. 1991, Kamat et al. 1997). Vitamin E is thought to protect bone via its antioxidant properties by helping the internal anti-oxidative defense against free-radicals and scavenging the lipid peroxidation radicals, therefore preventing oxidative stress.

The aim of the study is to determine the protective effects of palm tocotrienol on the oxidative status and bone biomechanical strength of rat model at eight weeks post-ovariectomy.

**METHODS**

Sixty-four female Wistar rats aged three months, weighing between 200-250 grams were housed in groups of three at 27°C with 12:12 hour light-dark cycle. The rats were fed ‘rat chow’ Gold Coin (Port Klang, Malaysia) and drinking water made available *ad libitum*.

There were eight rats in each group and they received the following treatments: Group 1 (Sham) rats were sham-operated. Group 2 (OVXC) rats were ovariectomised and given vehicle (olive oil) orally. Group 3 (OVX+ATF) rats were ovariectomised and given 60 mg/kg rat weight of α-tocopherol orally. Group 4 (OVX+PTT) rats were ovariectomised and given 60 mg/kg rat weight of palm tocotrienols orally.

Alpha-tocopherol acetate (Sigma, St. Louis, MO, USA) or palm tocotrienol mixture (Palm Oil Research Institute of Malaysia (PORIM) was diluted in olive oil ( Bertolli, Lucca, Italy) to obtain a concentration of 60 mg/kg rat weight, each in 0.1 ml volume. The diluted vitamin E was given orally to rats using gavage needle 6 days / week for three weeks. The palm tocotrienols has the following composition: α-tocotrienol (30.7%), γ-tocotrienol (55.2%) and δ-tocotrienol (14.1%). Olive oil was chosen as the diluents because it contains only 0.0051 % α-tocopherol and no tocotrienols.

Rats were bilaterally ovariectomised using dorsal approach under anaesthesia. For sham-operated rats, the ovaries were identified but left intact. The rats were allowed to recover for two weeks before treatment was started.

Blood samples were collected into an EDTA tube from the retro-orbital vein of the anesthetized rats. The blood was centrifuged at 3000 rpm at 4°C for 10 minutes and the plasma was stored at -70°C. The erythrocytes were washed with normal saline, centrifuged at 3000 rpm for 10 minutes and stored at -20°C. The left femurs were cleaned, wrapped in gauze soaked with phosphate-buffered solution and aluminum foil and stored at -70°C.

The plasma malondialdehyde (MDA) levels were measured according to the method of Ledwozyw *et al.* (Ledwozyew
et al. 1986). The MDA reacts with thiobarbituric acid (TBARS) to form a pinkish complex which was measured using a spectrophotometer (Shimadzu UV-160A, Kyoto, Japan) at a wavelength of 515 nm. The total protein in the plasma is measured according to the method of Lowry et al. (1951). The MDA value (nmol) is divided with the total protein in plasma (mg). The plasma glutathione peroxidase (GPX) and erythrocyte superoxide dismutase (SOD) activities were measured by using RANSEL and RANSOD kits (RANDOX Lab Ltd, Co Antrim, UK) respectively.

The femurs were thawed at room temperature one hour before the start of the biomechanical test. The femurs were subjected to three point bending configuration using an Instron® machine 5560 and were analysed using Bluehill® 2 software (High Wycombe, UK). The data obtained were used to plot load versus displacement and stress versus strain graphs. Bone stiffness and Young’s Modulus were then derived from the graphs.

The results were expressed as mean values ± S.E.M. Data analysis was performed using Statistical Package for Social Sciences (SPSS 12.0.1, Chicago, IL, USA) software. Statistical test used was ANOVA followed by Tukey’s hsd for normally distributed data and Kruskal-Wallis and Mann-Whitney test for data that was not normally distributed. The results were presented as mean values ± standard error of the mean (SEM). This study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC).

RESULTS

There was a significantly higher level of MDA in the OVXC group compared to the Sham and OVX+PTT groups (Figure 1). This indicated that ovariectomy has led to an increase in lipid peroxidation activity. However, oral supplementation with palm tocotrienols at 60mg/kg was able to reduce the lipid peroxidation activity in the ovariectomised rats.

After treatment, the SOD levels of the OVXC group remained unchanged from the Sham group but were significantly lower than the OVX+ATF and OVX+PTT groups. Ovariectomy did not affect SOD levels but oral supplementation of 60mg/kg of α-tocopherol or palm tocotrienols raised the SOD. The OVX+PTT group had higher levels of SOD compared to the OVX+ATF group (Figure 2).

After treatment, the GPX levels of the OVXC group were significantly lower than the Sham and OVX+PTT groups. These results showed that ovariectomy had reduced the GPX level but oral supplementation with palm tocotrienols prevented the reduction in the GPX level (Figure 3).

As for the biomechanical parameters, a pattern was seen although not significant, whereby ovariectomy had weakened the femur ability to withstand load and stress,
but all these were improved with vitamin E supplementations. Vitamin E supplementations had also improved femur stiffness and Young’s modulus value (Table 1).

**DISCUSSION**

The current treatments for post-menopausal osteoporosis are estrogen replacement therapy, raloxifene, bisphosphonates and calcitonin. Alternative treatments using herbs and natural products have been suggested such as *Rehmania glutinosa*, royal jelly, *sumbucus williamsii* HANCE, chondroitin sulfate and fructo-oligosaccharides (Oh et al. 2003, Ha 2004, Devareddy et al. 2006, Xie et al 2005, Hidoka et al. 2006). Several studies have also shown the potential of certain vitamins as alternative treatments for osteoporosis. High doses of vitamin K2 could partially prevent the bone loss in orchidectomised young rats by normalizing the raised bone resorption (Iwamato et al. 2003). An analogue of vitamin D3 has been shown to promote bone growth in ovariectomised rats and cultured osteoblast (Shevde et al. 2002). Vitamin E especially palm tocotrienols were able to protect rat bones against the deleterious effects of ovariectomy (Norazlina et al. 2000), orchidectomy (Ima Nirwana et al. 2000), ferric-nitrilotriacetate (Ahmad et al. 2005) and nicotine (Hermizi et al. 2007).

Our previous study (unpublished) has confirmed that osteoporotic changes were detected histologically as early as four weeks post-ovariectomy in the rat model. In the present study we found that after eight weeks of treatment, ovariectomised-rats have increased oxidative stress compared to the sham-operated rats. This was shown by the increase in the lipid peroxidation product, MDA and the decrease in the antioxidant enzyme glutathione peroxidase (GPX). There also appeared to be a decrease in the super-
oxide dismutase (SOD) levels but the changes were not significant. Among the reactive oxygen species, hydrogen peroxide is the most prominent free radicals as it can readily pass through cell membranes and cannot be excluded from cells. SOD converts superoxide to hydrogen peroxide ($\text{H}_2\text{O}_2$) which is then converted by GPX to water.

GPX also degrade $\text{H}_2\text{O}_2$ that originates from the oxidation of polyunsaturated fatty acids (Puglia & Powell 1984). Therefore, the reduction in GPX level would be more significant as it is actively involved in converting $\text{H}_2\text{O}_2$ to water. Muthusami et al. (2005) was able to demonstrate that ovariectomy had reduced both SOD and GPX activities. However, these anti-oxidant enzymes were measured in the bone, whereas we have measured the SOD level in the plasma. Estrogen deficiency in postmenopausal women leads to oxidative stress as shown by a decrease in thiol antioxidants, exposing the bone to oxidative damage (Lean et al. 2003). Lean et al. (2005) has found that the antioxidants, N-acetyl-cysteine and ascorbate were as effective as estrogen in normalizing the glutathione and thioredoxin reductase activities in rodents in order to prevent ovariectomy-induced bone loss. We have shown in this study that vitamin E, a potent antioxidant, has demonstrated a similar protective role against ovariectomy-induced oxidative stress by suppressing MDA levels and raising GPX and SOD activities. Palm tocotrienols were clearly the better vitamin E in protecting the bones against oxidative damage post-ovariectomy. As previously shown, α-tocotrienol has better antioxidant activity than α-tocopherol (Serbinova et al. 1991, Suzuki et al. 1993). The superior antioxidant activity of tocotrienols may be contributed by its more uniform distribution in the membrane lipid bilayer, more efficient interaction of the chromanol ring with lipid radicals and higher recycling efficiency from chromanoxyl radicals (Serbinova et al. 1991).

In the present study, palm-tocotrienols were able to prevent lipid peroxidation in ovariectomised rats. Both α-tocopherol and palm tocotrienols were found to raise the SOD level with the latter having a more profound effect. While for the GPX level, only palm tocotrienols were able to restore the GPX level of ovariectomised rats back to the sham-operated level. These results have demonstrated the ability of vitamin E to reactivate the antioxidant enzymes and guard against insult caused by oxidative stress.

Based on our previous study (unpublished) that the femur should be osteoporotic as early as four weeks post-ovariectomy, we have assessed the bone strength by running a biomechanical test. In the present study, patterns of reduced bone strength in all the biomechanical parameters were seen in the ovariectomised–control rats. However, even after eight weeks post-ovariectomy, they were not sufficiently low enough to record any significant difference. A study by Xie et al. (Xie et al. 2005), demonstrated significant reduction in the biomechanical parameters in rats sixteen weeks post-ova-
riectomy. Therefore, we conclude that osteoporosis at eight weeks post-ovariectomy was not severe enough to affect biomechanical properties although osteoporosis should have set in four weeks earlier. The different strain of rats used in the study by Xie et al. (Xie et al. 2005), which had used Sprague-Dawley compared to Wistar in our study may have also accounted for the difference in the findings.

In conclusion, palm tocotrienols given as oral supplementation at 60 mg/kg were able to prevent the oxidative stress induced by ovariectomy in a rat model. The efficacy of palm tocotrienols was greater than α-tocopherol. Palm tocotrienols have the potential to be used for the treatment and prevention of post-menopausal osteoporosis. A longer post-ovariectomy period is required to assess the ability of vitamin E in improving the bone biomechanical strength in the post-menopausal rat model.

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