ORIGINAL ARTICLE

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

Nazrun AS[1], Khairunnur A[2], Norliza M[1], Norazlina M[1], Ima Nirwana S[1].

[1] *Departments of Pharmacology, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur*
[2] *Departments of Biomedical Sciences, Faculty of Allied Health Sciences. Universiti Kebangsaan Malaysia, Kuala Lumpur*

ABSTRAK

Stres oksidatif dikaitkan dengan osteoporosis pos-menopaus yang meningkatkan risiko patah tulang. Tokotrienol sawit ialah antioksidan yang poten dan berpotensi untuk digunakan bagi merawat osteoporosis pos-menopaus. Tujuan kajian ialah untuk menentukan samada suplementasi tokotrienol sawit boleh mengurangkan stress oksidatif dan meningkatkan kekuatan tulang pada model tikus terovariektomi. Tikus dibahagikan kepada empat kumpulan : (i) bedah sham (SHAM) (ii) kawalan ovariektomi (OVX) (iii) ovariektomi dan diberi 60mg/kg α-tokoferol melalui gavaj oral (OVX+ATF) (iv) ovariektomi dan diberi 60mg/kg tokotrienol sawit melalui gavaj oral (OVX+PTT). Selepas rawatan selama lapan bulan, sampel darah diambil untuk mengukur status oksidatif (MDA, SOD dan GPX) sementara tulang femur diuji kekuatan dan resistan biomekanikal terhadap kepatahan. Ovariektomi menyebabkan stres oksidatif seperti yang ditunjukkan dengan peningkatan aras MDA dan penurunan aras aktiviti GPX. Tokotrienol sawit menunjukkan perlindungan terhadap stres oksidatif aruhan ovariektomi seperti yang ditunjukkan dengan penurunan aras MDA dan peningkatan aktiviti GPX dan SOD pada kumpulan OVX+PTT. Apabila dibandingkan, α-tokoferol hanya berupaya meningkatkan SOD tetapi tidak setinggi tokotrienol sawit. Ujian biomekanikal menunjukkan bahawa ovariektomi tidak memberi kesan signifikan kepada kekuatan tulang selepas rawatan selama lapan minggu. Suplementasi tokotrienol sawit selama lapan minggu berkesan untuk mencegah stres oksidatif pada tikus pos-menopaus.

Kata kunci: vitmain 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-

Address for correspondence and reprint requests: Professor Dr Ima Nirwana Soelaiman. Department of Pharmacology, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur. Tel: 03-92897281 / 5514 Fax; 603- 26938205 E-mail: imasoel@medic.ukm.my

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.

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 glutathion 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 $F_{2\alpha}$, 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 bio-mechanical 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 thi-obarbituric acid (TBARS) to form a pin-kish 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 supe-roxide dismutase (SOD) activities were measured by using RANSEL and RANSOD kits (RANDOX Lab Ltd, Co Antrim, UK) respectively.

The femurs were thawed at room tem-perature one hour before the start of the biomechanical test. The femurs were subjected to three point bending configu-ration using an Instron® machine 5560 and were analysed using Bluehill® 2 soft-ware (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 per-formed using Statistical Package for So-cial Sciences (SPSS 12.0.1, Chicago, IL, USA) software. Statistical test used was ANOVA followed by Tukey's hsd for nor-mally 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).
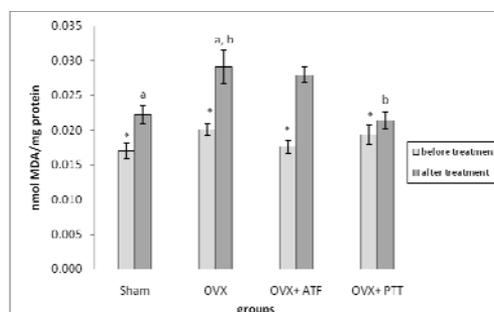
RESULTS

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 re-duce 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 toco-trienols raised the SOD. The OVX+PTT group had higher levels of SOD com-pared 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 sup-plementation with palm tocotrienols pre-vented 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,

Same alphabet indicates significant difference between the groups. *indicates significant difference before and after treatment within the same group. Significance is taken at $p<0.05$.
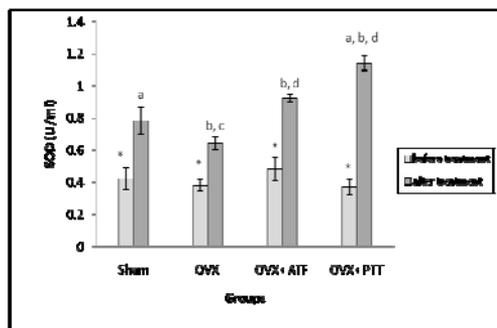
Sham : sham-operated
OVX : ovariectomised and given vehicle (olive oil)
OVX+ATF: ovariectomised and 60mg/kg body weight of α-tocopherol
OVX+PTT: ovariectomised and 60mg/kg body weight of palm tocotrienols.

Figure 1: The plasma malondialdehyde (MDA) levels for all the groups before and after treatment.

Same alphabet indicates significant difference between the groups. *indicates significant difference before and after treatment within the same group. Significance is taken at p<0.05.
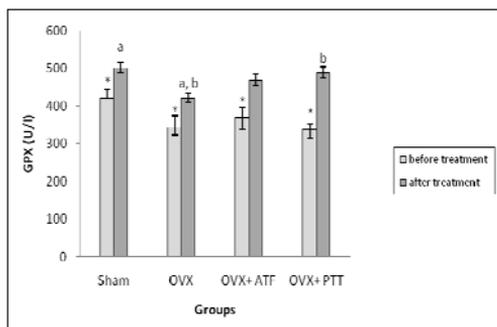
Sham : sham-operated
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OVX+PTT: ovariectomised and 60mg/kg body weight of palm tocotrienols.

Figure 2: The erythrocyte superoxide dismutase levels for all the groups before and after treatment.

Same alphabet indicates significant difference between the groups. *indicates significant difference before and after treatment within the same group. Significance is taken at p<0.05.

Sham : sham-operated
OVX : ovariectomised and given vehicle (olive oil)
OVX+ATF: ovariectomised and 60mg/kg body weight of α-tocopherol
OVX+PTT: ovariectomised and 60mg/kg body weight of palm tocotrienols.

Figure 3: The plasma glutathione peroxidase level before and after treatment.

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 K_2 could partially prevent the bone loss in orchidectomised young rats by normalizing the raised bone resorption (Iwamato et al. 2003). An analogue of vitamin D_3 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-

Table 1: The rat femur bone biomechanical parameters for all the groups after treatment.

Group	Maximum load (N)	Maximum Stress (MPa)	Stiffness (N/mm)	Young's modulus (E)
Sham	117.89±12.01	166.78±11.23	175.23±18.02	57.94±14.91
OVX	107.5±4.20	148.68±8.02	195.7±12.81	67.56±8.85
OVX+ATF	117.83±8.05	170.12±9.85	253.41±8.02	78.38±6.43
OVX + PTT	108.14±3.43	155.47±7.21	243.55±8.32	83.07±12.04

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 (H_2O_2) which is then converted by GPX to water.

GPX also degrade H_2O_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 H_2O_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, where-as 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 dam-

age 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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REFERENCES

Ahmad, N.S., Khalid, B.A.K., Luke, D.A., Ima-Nirwana, S. 2005. Tocotrienol Offers Better Protection than Tocopherol from Free Radical-Induced Damage of Rat Bone. *Clin Exp. Pharm. Physiol.* **32**:761-770.

Ahmad, N.S., Luke, D.A., Khalid, B.A.K., Ima-Nirwana, S. 2005. Vitamin E protects from free-radical damage to femur of rats treated with ferric nirilotriacetate. *Curr. Top. Pharm* **9**(2) :107-115.

Bai, X.C., Lu, D., Bai, J., Zheng, H., Ke, Z.Y., Li, X.M., Luo, S.Q. 2004. Oxidative stress inhibit osteoblast differentiation of bone cells by ERK and NF-κβ. *Biochem. Biophys. Res. Comm.* **314**:197-207.

Basu, S., Michaelsson, K., Olofsson, H., Johansson, S., Melhus, H. 2001. Association between oxidative stress and bone mineral density. *Biochem. Biophys. Res. Comm.* **288**(1):275-279.

Cooper, C., Campion, G., Melton. L.J. 1992. Hip fractures in the elderly: a world-wide projection. *Osteo. Int.* **2**:285-259.

Devareddy, L., Khalil, D., Korlagunta, K., Hooshamand, S., Bellmer, D., Arjmandi, B.H. 2006. The effects of fructo-olligosaccharides in combination with soy protein on bone in osteopenic ovariectomised rats. *Menopause* **13**(4):629-699.

Dubey, R.K., Jackson, E.K., Gillespie, D.G., Zacharia, L.C., Imthurn, B., Keller, P.J. 2000. Clinically used estrogens differentially inhibit human aortic smooth muscle cell growth and mitogen-activated protein kinase activity. *Arterioscler. Thromb. Vasc. Biol.* **20**(4):964-972.

EFFO and NOF. 1997. Who are candidates for prevention and treatment for osteoporosis? *Osteoporos. Int.* **7**:1.

Ferretti, J.L., Capozza, R.F., Mondelo, N., Zanchetta, J.R. 1993. Interrelationships between densitometric, geometric, and mechanical properties of rat femora inferences concerning mechanical regulation of bone modeling. *J Bone Mineral Res* **8**:1389–1396.

Garrett, I.R., Boyce, B.F., Oreffo, R.O.C., Bonewald, L., Poser, J., Mundy, G.R. 1990. Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone *in vitro* and *in vivo. J. Clin. Invest.* **85**:632-639.

Gullberg, B., Johnell, O., Kanis, J.A. 1997. World-wide projections for hip fracture. *Osteo. Int.* **7**:407

Ha, B.J. 2004. Oxidative stress in ovariectomy menopause and role of chondroitin sulfate. *Arch Pharm Res* **27**(8):867-872.

Hermizi, H., Faizah, O., Ima-Nirwana, S., Ahmad Nazrun, S., Luke, D.A., Norazlina, M. 2007. Negative effects of nicotine on bone resorbing cytokines and bone histomorphometric parameters in male rats. *Journal of Bone and Mineral Metabolism* **25**:93-98.

Hidoka, S., Okamoto, Y., Uchiyama, S., Nakatsuma, A., Hashimoto, K., Ohnishi, T.,

Yamaguchi, M. 2006. Royal jelly prevents osteoporosis in rats: beneficial effects in Ovariectomy model and in bone tissue culture model. *eCAM* **3**(3):339-348.

Ima Nirvana, S., Kiftiah, A., Zainal, A.G., Norazlina, M., Gapor, M.T., Khalid, B.A.K. 2000. Palm vitamin E prevents osteoporosis in orchidectomised growing male rats. *Nat. Prod. Sciences* **6**(4):155-160.

Iwamato, J., Takeda, T., Yeh, J., Ichimura, S., Toyama, Y. 2003. Effects of vitamin K2 on cortical and cancellous bones in orchidectomised young rats. *Maturitas* **44**:19-27.

Kamat, J.P., Sarma, H.D., Devasagayam, T.P.A., Nesaretnam, K., Basiron, Y. 1997. Tocotrienols from palm oil as effective inhibitors of protein oxidation and lipid peroxidation in rat liver microsomes. *Mol Cell Biochem* **170**:131-138.

Kanis, J.A., Johnell, O., Oden, A. 2000. Long-term risk of osteoporotic fracture in Malmo. *Osteo. Int.* **11**:669-674.

Kimmel, D.B. 1996. Animal models for in vivo experimentation in osteoporosis research. In: Marcus R, Feldman D, Kelsey J. (eds) *Osteoporosis.* Academic Press, San Diego, pp 671–690.

Lean, J.M., Davies, J.T., Fuller, K. 2003. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. *J Clin Invest* **112**(6):915-923.

Lean, J.M., Jagger, C.J., Kirstein, B., Fuller, K., Chambers, T.J. 2005. Hydrogen Peroxide Is Essential for Estrogen-Deficiency Bone Loss and Osteoclast Formation. *Endocrinology* **146**(2):728-735.

Ledwozyw, A., Michalak, J., Stepien, A., Kandziolka, A. 1986. The relationship between plasma trigliserides, cholesterol, total lipids and lipid peroxidation products during atherosclerosis. *Clin Chim Acta* **293**:275-284.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J Biol Chem* **193**:265-275.

Maggio, D., Barabani, M., Pierandrei, M., Polidori, M.C., Catani, M., Mecocci, P., Senin, U., Pacifici, R., Cherubini, A. 2003. Marked decrease in plasma antioxidants in aged osteoporotic women: results of a cross-sectional study. *J Clin Endocrinol Metab* **88**(4):1523-1527.

Melton III, L.J., Atkinson, E.J., O'Connor, M.K., O'Fallon, W.F., Riggs, B.L. 1998. Bone density and fracture risk in men. *J. Bone Miner. Res.* **13**:1915-1922.

Muthusami, S., Ramachandran, I., Muthusamy, B., Vasudevan, G., Prabhu, V., Subramaniam, V.,

Jagadeesan, A., Narasimhan, S. 2005. Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. *Clin Chim Acta* **360**(1-2):81-86.

Norazlina, M., Ima Nirwana, S., Kifitah, A., Zainal, A.G., Gapor, M.T., Khalid, B.A.K. 2000. Palm oil is comparable to alpha tocopherol in maintaining bone mineral density in ovariectomised female rats. *Exp Clin Endocrinol Diabetes*. **108**:1-6.

Oh, K.O., Kim, S.W., Kim, J.Y., Ko, S.Y., Kim, H.M., Baek, J.H., Ryoo, H.M., Kim, J.K. 2003. Effect of Rehmannia glutinosa Liborsh extracts on bone metabolism. *Clinica Chimica Acta* **334**:185-195.

Ozgocmen, S., Kaya, H., Fadilillioglu, E., Aydogan, R., Yilmaz, Z. 2007. Role of antioxidant systems, lipid peroxidation and nitric oxide in postmenopausal osteoporosis. *Mol Cell Biochem* **295**:45-52

Peng, Z., Tuukkanen, J., Zhang, H., Jämsä, T., Väänänen, H.K. 1994. The mechanical strength of bone in different rat models of experimental osteoporosis. *Bone* **15**:523–532.

Puglia, C.D., Powell, S.R. 1984. Inhibition of cellular antioxidants: a possible mechanism of toxic cell injury Environ. *Health Perspect.* **57**:307-311.

Raab, D.M., Smith, E.L., Crenshaw, T.D., Thomas, D.P. 1990. Bone mechanical properties after exercise training in young and old rats. *Jour App Physiol* **68**:130–134.

Randell, A., Sambrook, P.N., Nguyen, T.V. 1995. Direct clinical and welfare costs of osteoporotic fractures in elderly men and women. *Osteoporos. Int.* **5**:427-432.

Suzuki, Y.J., Tsuchiya, M., Wassall, S.R. 1993. Structural and dynamic membrane properties of α-tocopherol and α-tocotrienol: implication to the molecular mechanism of antioxidant potency. *Biochemistry* **32**:10692-10699.

Serbinova, E., Kagan, V., Han, D., Packer, L. 1991. Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha tocotrienol. *Free Rad Biol Medic* **10**(5):263-275.

Shevde, N.K., Plum, L.A., Dame, M.C., Yamamoto, H., Pike, W.J., DeLuca, H.F. 2002. A potent analogue of 1α,25-dihydroxyvitamin D3 selectively induces bone formation. *Biochem* **99**:13487-13491.

Sontakke, A.N., Tare, R.S. 2002. A duality in the roles of reactive oxygen species with respect to bone metabolism. *Clin Chim Acta* **318**(1-2):145-148.

Tuukkanen, J., Peng, Z., Väänänen, H.K. 1994. Effect of running exercise on the bone loss induced by orchidectomy in the rat *Calcif Tiss*

Int **55**:33–37.

Xie, F., Wu, C., Zhang, Y., Yao, X., Cheung, P., Chan, A., Wong, M. 2005. Increase in Bone Mass and Bone Strength by *Sambucus williamsii* HANCE in Ovariectomised Rats. *Biol Pharm Bull* **28**(10):1879-1885.

Yee, J.K., Ima Nirwana, S. 1998. Palm vitamin E protects against ferric-nitrilotriacetate–induced impairment of bone calcification. *Asia Pac.Jour. Pharmacol.* **13**:35-41.