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Tocotrienols as an Anti-Osteoporotic Agent: The Progress So Far

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ABSTRACT

Osteoporosis is a metabolic bone disease affecting both men and women especially postmenopausal women. Osteoporosis has been associated with oxidative stress and therefore the protective effects of antioxidants such as vitamin E were studied. Lately, there has been a growing interest in tocotrienol, a potent vitamin E with anti-cholesterol, anti-cancer and perhaps anti-osteoporotic properties. We have conducted studies on the effects of tocotrienol on various animal models of osteoporosis and discovered its ability to prevent osteoporosis. In most of the studies, tocotrienol mixtures as well as its isomers such as gamma-, alpha- or delta-tocotrienol were compared to alpha tocopherol, the most abundant and widely commercialized vitamin E. The techniques that were used included Enzyme-linked immunosorbent assay (ELISA), bone histomorphometry, Dual energy X-ray absorptiometry (DEXA) and biomechanical testing. Most of the results revealed that tocotrienols were more efficacious than alpha-tocopherol in protecting the bone from various inducers of osteoporosis. These convincing results warrant further studies in pursuing the idea that in future, tocotrienol would be accepted as part of the treatment regime for osteoporosis. The role of tocotrienols in studies using various osteoporotic models was discussed in light of its potential as an anti-osteoporotic agent.

Key words: Vitamin E, resorption, anti-oxidant, calcium, osteocalcin

INTRODUCTION

Osteoporosis is a bone disease characterized by low bone mineral density with high risk of fractures. It occurs when there is an imbalance between bone resorption and bone formation during the bone remodeling process. Free radicals play an important role in bone remodeling by promoting differentiation and bone resorptive activity of osteoclast. However, high levels of free radicals may lead to oxidative stress and need to be converted to less reactive forms by anti-oxidant enzymes (Khalkali-Ellis *et al.*, 1997). Osteoporotic subjects were found to have low anti-oxidants (Maggio *et al.*, 2003) and high levels of reactive oxygen species (Sontakke and Tare, 2002), meaning that they were under increased oxidative stress. Furthermore, similar to lipids in the arterial wall, lipids that have accumulated in human osteoporotic bone (Yeung *et al.*, 2005) were found to be oxidized (Parhami *et al.*, 1997). These oxidized lipids promote bone resorption (Garrett *et al.*, 1990) by recruitment and differentiation of osteoclast precursors and also by inhibiting osteoblast differentiation (Parhami *et al.*, 2000).

Naturally, antioxidants such as vitamin E would help the endogenous antioxidant defense system protect bone from oxidative stress. Vitamin E occurs in eight isoforms of α -, β -, γ - and δ -tocopherols or tocotrienols. Tocotrienol differs from tocopherol by possessing a farnesyl (isoprenoid) rather than a saturated phytanyl side chain (Serbinova *et al.*, 1991). It was also found that vitamin E deficiency induced a state of calcium deficiency (Norazlina *et al.*, 2002a) that could be related to increased free radical activity (Norazlina *et al.*, 2002b).

For the last decade, our research team has been conducting studies and publishing articles on the effects of tocotrienols on bone metabolism. Our studies focused on the bone protective effects of tocotrienol against various stressors of osteoporosis. The tocotrienol used in our studies were extracted from palm oil and consists of either palm vitamin E extract, palm tocotrienol mixtures or isomers of tocotrienols. In most of our studies, α -tocopherol was used as comparison.

It is a requirement that a potential new drug must undergo animal studies before human drug trial can be started. There are a few animal models available but rat has favourable characteristics as a model for bone studies. The rat's bone anatomy, trabecular bone remodeling and response to treatment are similar to human beings (Abe *et al.*, 1993; Mosekilde, 1995). Therefore, the rat model is considered adequate for extrapolating changes in bone mass to humans (Jee, 1995). Rats can be subjected to various osteoporosis stressors to simulate the real osteoporotic condition in humans. The rat models that were used in our studies included the hyperthyroid, adrenalectomised, orchidectomised, ovariectomised, nicotine-treated and ferric nitrilotriacetate-treated models.

The current pharmacological treatment for osteoporosis aims at either replacing hormones that are deficient or by inhibiting bone resorption. None directly deals with the possible oxidative stress condition associated with osteoporosis. In this review, we discussed the impact of vitamin E especially tocotrienol on the various types of osteoporosis in animal models and the mechanisms that may be involved.

Hyperthyroid-induced osteoporosis: the hyperthyroid rat model: Thyroid hormones are important for normal bone growth, metabolism and turnover in children and adults. Excess thyroid hormones increase bone formation and resorption with a net increase in resorption and bone loss which may lead to osteoporosis (Leb *et al.*, 1994). Hyperthyroidism can be induced in rats by giving thyroxine via daily intraperitoneal injections. This model has been used to test treatment modalities in thyroxine-induced osteopenia and osteoporosis (Ongphiphadhanakul *et al.*, 1992; Balena *et al.*, 1993). Hyperthyroidism is associated with high bone turnover and increased lipid peroxidation and free radical formation. Since, hyperthyroidism is associated with enhanced lipid peroxidation (Asayama *et al.*, 1987), the damaging effects of oxidative stress on bone may be ameliorated with palm vitamin E supplements. We have used the hyperthyroid model to study the effects of palm vitamin E extract on bone metabolism in hyperthyroid rats (Ima-Nirwana *et al.*, 1999). The hyperthyroid state was confirmed by elevated triiodothyronine (T3) and tetraiodothyronine (T4) levels compared to the euthyroid rats. At the end of the 3-week treatment period, serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) levels, the bone biomarkers for bone formation and bone resorption respectively, were measured.

We would expect the bone biomarkers of the rats given thyroxine to be elevated because of the high bone turnover in hyperthyroid state (Garnero *et al.*, 1994). However, the ALP and TRAP levels were found to be similar between hyperthyroid rats compared to euthyroid rats. This meant that the bone turnover of the hyperthyroid rats was not as high as expected. We have noted that the ALP measured in this study was not specific to bone, therefore any differences in bone ALP may

be masked by ALP derived from other sources such as the liver, giving the unexpected results. The same reason however, cannot be used for TRAP as it is bone specific. Both the bone biomarkers were significantly lowered in both euthyroid and hyperthyroid rats supplemented with palm vitamin E compared to euthyroid and hyperthyroid control rats. These results reflected that both the osteoblastic and osteoclastic activities were reduced by palm vitamin E. The reduction in TRAP was more significant ($p < 0.01$) compared to the reduction in ALP ($p < 0.05$). This meant that palm vitamin E had caused a more significant reduction in bone resorption than bone formation, suggesting a net positive bone remodeling. The bone calcium content of the left femur and the fifth lumbar vertebra were also not significantly different between the euthyroid and hyperthyroid control rats. Similarly, the bone calcium content of the femur and the fifth lumbar vertebra in the euthyroid and hyperthyroid rats given palm vitamin E were not significantly different from the euthyroid and hyperthyroid control rats. Therefore, the 3 weeks duration of treatment with thyroxine was not sufficient for osteopenia or osteoporosis to set in but palm vitamin E had promoted positive bone remodeling.

Glucocorticoid-induced osteoporosis: The adrenalectomised rat model: Corticosteroids include glucocorticoids and mineralcorticoids but only the former was found to affect bone metabolism (Ima-Nirwana and Fakhrurazi, 2002). Patients on long term glucocorticoid use are at risks of its side-effects including osteoporosis. Glucocorticoids are known to affect bone in many ways. Its catabolic effects may result in loss of bone mass while its anabolic effects may result in loss of bone protein. It can also promote urinary excretion of calcium, inhibit calcium absorption from the intestines, reduce plasma testosterone, estrogen and calcitonin levels and cause secondary hyperparathyroidism (Jenkinson and Balla, 1993). In laboratory rats, long-term use of glucocorticoid led to osteoporosis by decreasing bone formation activity and increasing bone resorption activity (Ortoft and Oxlund, 1996). At the cellular level, high levels of glucocorticoids drastically reduced proliferation of osteoblast precursors leading to glucocorticoid-induced osteoporosis (Scutt *et al.*, 1996). Histomorphometric studies indicated reduced trabecular size, while dynamic indices reflected lower bone formation rate in rats (Chavassieux *et al.*, 1993).

In present studies, dexamethasone, a potent synthetic member of the glucocorticoid was given to rats (Ima-Nirwana and Fakhrurazi, 2002; Ima-Nirwana and Suhaniza, 2004). Adrenalectomy was performed on these rats to remove the endogenous glucocorticoids from the circulation. This is to remove the circadian rhythm of endogenous glucocorticoids and to ensure a constant concentration of dexamethasone in the circulation. Present results showed that adrenalectomised rats receiving high doses of dexamethasone ($120 \mu\text{g kg}^{-1} \text{ b.wt.}$) failed to achieve the bone mineral density of control rats. These rats also have shorter femur length and lower femoral bone calcium content. This was consistent with other reports (Jenkinson and Balla, 1993; Scutt *et al.*, 1996; Goldstein *et al.*, 1999) and confirmed that excess glucocorticoids impaired bone mineralisation which can lead to osteoporosis.

Dexamethasone was shown to reduce superoxide dismutase activity and increased thiobarbituric acid reactive substances (TBARS) in erythrocytes (Orzechowski *et al.*, 2000). The association of lipid peroxidation with dexamethasone has raised the possibility that oxidative stress may be involved in the pathogenesis of steroid-induced osteoporosis. We have conducted studies to determine if palm vitamin E was able to protect bone from steroid induced osteoporosis. In one of the studies, palm vitamin E was found to be effective in maintaining bone mineral density, femur length and bone calcium content when supplemented to dexamethasone-treated rats

(Ima-Nirwana and Fakhrurazi, 2002). This showed that palm vitamin E was able to protect bone against the deleterious effects of excessive glucocorticoid treatment. The palm vitamin E used in this study consisted of α -tocopherol, α -tocotrienol, δ -tocotrienol and γ -tocotrienol. Therefore, one or more of these vitamin E isomers could be responsible for the protective effect. In a later study using a similar model of dexamethasone induced osteoporosis, γ -tocotrienol was found to be able to increase the fourth lumbar vertebra bone calcium content after 8 weeks of treatment (Ima-Nirwana and Suhaniza, 2004), while α -tocopherol failed to produce any significant changes. This study has confirmed that γ -tocotrienol was responsible for the bone protection against dexamethasone-induced osteoporosis. The mechanism involved may be via the more potent antioxidant property of γ -tocotrienol.

However, several studies have shown mixed results on the effects of dexamethasone on lipid peroxidation. Dexamethasone did not have any effects on the generation of the superoxide anion by osteoclasts (Berger *et al.*, 1999). While another study found that dexamethasone decreased TBARS in lymphoid organs but raised TBARS in the soleus and gastrocnemius muscles of the rat (Pereira *et al.*, 1999). Therefore, palm vitamin E may be able to protect bone against excessive glucocorticoids by a mechanism other than its antioxidant effects.

Testosterone-deficient osteoporosis: The orchidectomised rat model: Male osteoporosis has received more attention lately due to the increasing number of men having osteoporosis as a result of increased longevity. It is estimated that 20% of osteoporotic cases involved men and their risk of osteoporotic fractures is about 1 in 5 after the age of 50 (NOF, 2004). The main cause of osteoporosis in men is testosterone deficiency as testosterone is required to maintain bone density (Looker *et al.*, 1997). The rate of bone loss in testosterone deficient men was found to be similar to postmenopausal women (Stepan *et al.*, 1989). Similar bone mineral density loss was demonstrated in the testosterone deficient rat model induced by orchidectomy (Gunnness and Orwoll, 1996; Ima-Nirwana *et al.*, 1998; Rosen *et al.*, 1995).

Orchidectomised rats are under oxidative stress as they were found to have suppressed antioxidant status and elevated plasma malondialdehyde levels (Deyhim *et al.*, 2006, 2007). Orchidectomy was also associated with increased lipid peroxidation in the heart, while administration of testosterone reversed this to a certain extent (Sreelatha *et al.*, 1993). Testosterone treatment was also found to decrease levels of lipid peroxidation products in the liver due to alcohol and paracetamol toxicity (Jaya *et al.*, 1995). The raised lipid peroxidation associated with testosterone deficiency may be responsible for bone loss in orchidectomised rats. Palm vitamin E may be as effective as testosterone in reducing lipid peroxidation and reversing bone loss in orchidectomised rats. This hypothesis was tested in our study where orchidectomised rats were supplemented with palm vitamin E for eight months before their bone parameters were measured (Ima-Nirwana *et al.*, 2000). The bone mineral density of the orchidectomised, unsupplemented control rats was found to be reduced in all the femur regions and lumbar vertebrae except the distal femur and third to fifth lumbar vertebrae, consistent with other studies (Gunnness and Orwoll, 1996; Rosen *et al.*, 1995; Ima-Nirwana *et al.*, 1998). Palm vitamin E supplementation was able to protect orchidectomised rats from bone loss as they have similar bone mineral density in all the skeletal regions compared to sham-operated rats. We found that the bone calcium content of the fifth lumbar vertebra was also significantly reduced in orchidectomised rats. Other researchers have found similar bone calcium loss in the tibial bone after orchidectomy (Shoutens *et al.*, 1984). In our study the bone calcium loss was fully restored by palm vitamin E supplementation.

The bone biochemical markers, alkaline phosphatase and tartrate resistant acid phosphatase were not raised in orchidectomised rats. Other studies did not find any significant changes in serum alkaline phosphatase activity after 3 and 4 months of orchidectomy (Rosen *et al.*, 1995). Palm vitamin E supplementation did not seem to have any effects on the bone biochemical markers of orchidectomised rats. The lack of significant changes in the bone biochemical markers may be due to the long duration of the study (8 months) which have allowed these biochemical markers to reach steady state.

In conclusion, palm vitamin E was effective in preventing loss in bone mineral density and bone calcium in testosterone-deficient male rats. The mechanism of action is probably via its anti-oxidant properties.

Smoking induced osteoporosis: The nicotine rat model: Smoking can decrease bone mineral density and is considered as one of the risk factors for osteoporosis and fractures. A study concluded that one in eight hip fractures is attributable to cigarette smoking. Hip fracture risk among smokers is greater at all ages but rises from 17% at age 60 to 71% at age 80 and 108% at age 90 (Law and Hackshaw, 1997). Nicotine is one of the active components found in cigarettes which was found to reduce bone mineral density (Daniell, 1976), inhibit osteoblast-like function (Fang *et al.*, 1991) and delay bone healing (Porter and Hanley, 2001). *In vitro* and *in vivo* studies have linked nicotine exposure to oxidative stress (Wetscher *et al.*, 1995; Suleyman *et al.*, 2002; Kalpana and Menon, 2004). This information has led us to study the effectiveness of vitamin E as an antioxidant in preventing nicotine induced bone loss.

Our own studies have found that nicotine increased the levels of the bone resorbing cytokine, interleukin-1, (Norazlina *et al.*, 2004) and reduced bone calcium content (Ima-Nirwana *et al.*, 2005). Another study using a similar model found that nicotine also induced secretion of other bone resorbing cytokines such as interleukin-6 and tumor necrosis factor- α from osteoblasts (Kamer *et al.*, 2006). Histomorphometrically, we found that nicotine decreased trabecular bone volume, osteoblast surface, double-labeled surface, mineral appositional rate and bone formation rate. Nicotine also increased single-labeled surface, osteoclast surface and eroded surface (Hermizi *et al.*, 2007, 2009). These findings confirmed that nicotine induced bone loss. Palm tocotrienol mixture was able to prevent nicotine induced elevation of interleukin-1 and interleukin-6 but failed to restore the nicotine-induced bone calcium loss (Norazlina *et al.*, 2007). In a later study, vitamin E supplementations in the form of tocotrienol enhanced fraction and γ -tocotrienol were able to reverse all the histomorphometric changes induced by nicotine (Hermizi *et al.*, 2009). In most of the parameters measured in the above studies, vitamin E containing tocotrienol such as palm tocotrienol mixture, tocotrienol enhanced fraction and γ -tocotrienol were found to be superior than α -tocopherol (Hermizi *et al.*, 2007; Norazlina *et al.*, 2007).

Post-menopausal osteoporosis: The ovariectomised rat model: Postmenopausal women undergo changes due to low estrogen levels and are exposed to osteoporosis. Ovariectomy in rats induced changes similar to those seen in women with menopause (Mosekilde and Mosekilde, 1990). Bone loss in rats and humans share many similar characteristics such as greater loss of cancellous than cortical bone. Since bone changes in post-ovariectomised rats are similar to those in post-menopausal women, the ovariectomised rat is a suitable model for postmenopausal bone loss (Kalu, 1991). This was demonstrated in our own study in which the rats began to show reduction in bone mineral density 2 months after ovariectomy in the lumbar vertebrae and distal femur, which are rich in cancellous bone (Ima-Nirwana *et al.*, 1998).

Physiological levels of 17α -estradiol have been shown to protect human low density lipoprotein against oxidation (Sack *et al.*, 1994), while low estrogen levels in postmenopausal women has been associated with oxidative stress (Basu *et al.*, 2001; Maggio *et al.*, 2003). Estrogen can also be considered 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). It is clear from all these studies that estrogen deficiency will expose the bone to free radical attacks and eventually osteoporosis. We have conducted studies to determine if palm vitamin E could provide an antioxidant cover in preventing post menopausal osteoporosis using an ovariectomised rat model.

In one of our studies, the bone mineral density of ovariectomised rats was lowered at all the skeletal regions studied except the femoral midshaft when compared to intact rats (Norazlina *et al.*, 2000). This pattern of bone loss is consistent with other reports (Omi and Ezawa, 1995). The femoral midshaft is resistant to bone loss because it is almost totally made up of compact, cortical bone, which has a lower bone remodeling rate compared to trabecular bone. Supplementation with palm vitamin E or α -tocopherol was able to bring up the bone mineral density to the intact rats value. This suggests that both the palm vitamin E and α -tocopherol were able to reverse bone loss induced by estrogen deficiency.

In this study, the ovariectomised rats were not found to have lower bone calcium content compared to the intact rats. There was also no significant difference in the bone calcium content of the ovariectomised rats supplemented with palm vitamin E or α -tocopherol compared to intact rats. This is in contrast to that shown by a previous study where bone calcium decreased after ovariectomy (Kalu *et al.*, 1989). This inconsistent finding was probably because the younger rats we used were still skeletally growing as demonstrated by our earlier study (Ima-Nirwana *et al.*, 1998). Besides that, bone calcium deposition in young age is influenced not only by reproductive hormones but also other hormones such as the growth hormones. The role of vitamin E in bone calcification of growing female rats was not fully understood. Therefore, we have conducted a study to determine the importance of vitamin E in bone calcification and found that growing female rats receiving vitamin E-deficient diet had bone calcium loss (Norazlina *et al.*, 2002a). As anticipated, supplementation of palm vitamin E to these vitamin E-deficient rats was able to improve bone calcium content of their femur and lumbar vertebra. Whereas, supplementation with α -tocopherol failed to improve the bone calcium content of these vitamin E-deficient rats. The findings suggested that vitamin E is required for normal bone calcification. Only palm vitamin E supplementation was effective in reversing the bone calcium loss due to vitamin E deficiency. The palm vitamin E extracts used consisted of tocotrienols and α -tocopherol. We believed that the protective effects on bone were contributed by the tocotrienols rather than α -tocopherol as we have found α -tocopherol to be ineffective in reversing bone calcium loss due to vitamin E deficiency.

Results on the biochemical markers of bone formation (ALP) and bone resorption (TRAP) indicated that both palm vitamin E and α -tocopherol supplementations were able to increase ALP level while α -tocopherol supplementation alone was able to reduce TRAP level in the ovariectomised rats. However, the bone biochemical markers may have already reached a steady state and may not be totally accurate because of the long duration of the study (10 months). The conclusion from the study is that vitamin E was able to reverse the negative effects of estrogen deficiency on bone mineral density and bone calcium, probably through its antioxidant properties. Palm vitamin E was more superior to α -tocopherol in protecting bone from estrogen deficiency.

Based on these encouraging results we went on to determine the effects of supplementation of pure palm tocotrienol mixture (without tocopherol) and a pure α -tocopherol on the bone resorbing cytokines, interleukin-1 and interleukin-6 in the ovariectomised rats. Both the palm tocotrienols and α -tocopherol were able to prevent the increase in interleukin-1 and interleukin-6 seen in ovariectomised control rats. However, we could not find any significant differences between palm tocotrienols and α -tocopherol (unpublished data). Bone histomorphometric findings done simultaneously confirmed the above observations. Trabecular volume, trabecular thickness and trabecular number were all decreased but trabecular separation was increased by ovariectomy. Supplementation with palm tocotrienols and α -tocopherol were able to prevent these ovariectomy-induced changes. Present findings did not reveal any significant differences between the effects of palm tocotrienols and α -tocopherol (unpublished data). Both the osteoclast and osteoblast surfaces were increased by ovariectomy, indicating increased cellular activities due to high bone turnover. Palm tocotrienols and α -tocopherol were found to prevent the increase in osteoclast surface but allowed the increase in osteoblast surface (unpublished data). This may be the mechanism by which vitamin E protects bone in estrogen deficiency, by increasing osteoblastic bone formation relative to osteoclastic bone resorption, resulting in positive bone remodeling. Similar protective effects were seen in the hyperthyroid rat model (Ima-Nirwana *et al.*, 1999) where palm vitamin E had caused a more significant reduction in the bone resorption marker than the bone formation marker, leading to positive bone remodeling. These findings also supported the earlier findings whereby both palm tocotrienols and α -tocopherol had reduced the levels of the bone resorbing cytokines, interleukin-1 and interleukin-6. However, we consistently did not observe significant differences between the palm tocotrienols and α -tocopherol in this rat model.

Oxidative stress: The ferric-nitilotriacetate rat model: All the various risk factors of osteoporosis listed above were linked to oxidative stress. Risk factors of osteoporosis such as smoking (Law and Hackshaw, 1997), hypertension (Cappuccio *et al.*, 1999) and diabetes mellitus (Christensen and Svendsen, 1999) were all associated with oxidative stress. This had led to the belief that free radicals may be responsible for osteoporosis. There are studies which have found osteoporotic subjects to be under oxidative stress. Osteoporotic patients were found to have increased malondialdehyde levels and reduced antioxidant enzymes (Sontakke and Tare, 2002; Maggio *et al.*, 2003). A high concentration of 8-iso-prostaglandin $F_{2\alpha}$, a biomarker of oxidative stress, was associated with a reduction in bone mineral density (Basu *et al.*, 2001). These findings further demonstrated a strong relationship between oxidative stress and bone loss.

Physiologically, free radicals play an important role in bone remodeling whereby oxygen-derived free radicals stimulates the formation and activation of osteoclasts, which in turn release reactive oxygen species to digest bone (Yang *et al.*, 1996; Garrett *et al.*, 1990; Malcolm, 2002). However, excess free radicals as in oxidative stress, may lead to imbalance in bone remodeling leading to osteoporosis. Therefore, potent antioxidants such as palm vitamin E are essential to help the internal anti-oxidant defenses maintain the free radicals at acceptable levels.

All the rat models of osteoporosis that we have studied so far have some oxidative stress involvement in the process of bone loss. Therefore, we have conducted studies using an oxidative stress model, whereby the oxidative stress generated by ferric-nitilotriacetate (FeNTA) directly caused bone loss. Ferric ions (Fe^{3+}) in FeNTA produce oxidative stress in rats by generating reactive oxygen species through the Fenton reaction (Gutteridge *et al.*, 1982). The FeNTA model would increase our understanding regarding the role of palm vitamin E in osteoporosis induced by oxidative stress.

Our studies have shown that the bone-resorbing cytokines, interleukin-1 and interleukin-6 were elevated in the FeNTA-induced oxidative stress model. This was accompanied by elevated levels of deoxypyridinoline cross-links, a marker of osteoclast activity (Ahmad *et al.*, 2005). Other negative effects in the oxidative stress model included deterioration of the structural, static and dynamic parameters of bone histomorphometry (Ebina *et al.*, 1991; Ahmad *et al.*, 2005), impairment of bone mineralisation (Takeuchi *et al.*, 1997) and decreased bone calcium content (Yee and Ima-Nirwana, 1998).

Free-radicals may damage bone cells directly by lipid peroxidation (Ebina *et al.*, 1991; Yee and Ima-Nirwana, 1998) or indirectly by stimulating the formation and bone-resorbing activity of osteoclasts (Garrett *et al.*, 1990). Histomorphometric studies in the FeNTA-oxidative stress model have shown that there was impairment of the osteoblasts' function to form bone (Ebina *et al.*, 1991; Takeuchi *et al.*, 1997; De Vernejoul *et al.*, 1984) as well as decreased osteoblast recruitment and collagen synthesis (De Vernejoul *et al.*, 1984). There was also growth suppression of the osteoblast-like cell Hu09 and inhibition of alkaline phosphatase secretion (Takeuchi *et al.*, 1997). Besides being toxic to osteoblasts, free radicals generated by FeNTA may also stimulate osteoclast differentiation, as shown by the increased osteoclast number in the FeNTA oxidative stress model (Ahmad *et al.*, 2005; Ebina *et al.*, 1991). Furthermore osteoclasts themselves are activated by free-radicals (Garrett *et al.*, 1990) and they in turn produced free-radicals to resorb bone (Beckman *et al.*, 1989).

Free radicals may activate nuclear factor $\kappa\beta$ (NF $\kappa\beta$), a transcription factor responsible for the synthesis of bone-resorbing cytokines such as interleukin-1 and interleukin-6 (Baldwin, 2001). Our study has shown that both palm tocotrienols and α -tocopherol were able to prevent interleukin-1 elevation in the FeNTA-oxidative stress model (Ahmad *et al.*, 2005). Histomorphometric parameters in the same model indicated that palm tocotrienols were able to prevent the increase in osteoclast number and eroded surface but α -tocopherol was only effective in preventing the increase in osteoclast number. Only palm tocotrienols were able to prevent the reduction in osteoblast number and bone formation. Our studies on the oxidative stress model have demonstrated the role of vitamin E in protecting bone against free radical activity, thereby reducing the risk factors of osteoporosis. Palm tocotrienols were more superior than α -tocopherol in most of the parameters measured.

Mechanism of protection of vitamin e against osteoporosis: Looking at the various models of osteoporosis in our studies, oxidative stress has played an important part in the pathogenesis of bone loss leading to osteoporosis. This explains why vitamin E, an antioxidant was able to offer protection against osteoporosis in these models. Analysis of the results from our studies has given us some insight on the mechanism involved in the protection of vitamin E against osteoporosis.

Vitamin E may protect bone by interrupting the free radical chain reaction and therefore preventing damage due to lipid peroxidation (Burton *et al.*, 1983; Nur Azlina *et al.*, 2005; Asmadi *et al.*, 2005). Free radicals can activate transcription factor NF κ B to produce bone resorbing cytokines interleukin-1 and interleukin-6. Vitamin E may act as a free radical scavenger and neutralize free radicals before it could activate transcription factor NF κ B. This was seen in our studies which demonstrated reduction in the levels of bone resorbing cytokines when supplemented with vitamin E (Ahmad *et al.*, 2005; Norazlina *et al.*, 2007). Vitamin E may have also scavenged free radicals before they could activate osteoclasts and therefore keeping their numbers and activities down. Vitamin E may have combined with the phospholipids in the membrane of osteoblasts to protect from *in situ* lipid peroxidation (Hammer and Wills, 1978).

Our studies proved that palm tocotrienols were more effective than α -tocopherol in protecting bone from free radical-induced damage in the FeNTA-oxidative stress model (Ahmad *et al.*, 2005). We hypothesize that in doing so, vitamin E supported the anti-oxidant enzymes in the bone and suppressed the MDA level in the bone. We have conducted a study to measure the level of thiobarbituric acid-reactive substance (TBARS), an index of lipid peroxidation and the level of the antioxidant enzymes, glutathione peroxidase and superoxide dismutase, in the femur (Maniam *et al.*, 2008). The TBARS level was reduced and the glutathione peroxidase activity was increased in the femur of the palm tocotrienol supplemented group compared to the age-matched control group. These findings were not observed in the α -tocopherol group. This study has demonstrated that tocotrienol directly boosted the anti-oxidant defense of the bone and further strengthened our belief that vitamin E protects bone against osteoporosis via its anti-oxidant properties.

The more superior tocotrienol action compared to tocopherol may be contributed by its more potent anti-oxidant property. Tocotrienol interacts better with the lipoprotein in the membrane lipid and are uniformly distributed in the membrane layer compared to tocopherol (Suarna *et al.*, 1993; Serbinova *et al.*, 1991). Palm tocotrienol has been shown to exert better anti-oxidant activities in rat bone compared to alpha-tocopherol (Maniam *et al.*, 2008).

However, tocotrienol has been shown to have other unique properties. The slight structural change in the phtyl side chain of vitamin E has enabled tocotrienol to have special properties not found in tocopherol such as anti-cholesterol (Hasselwander *et al.*, 2002) and anti-cancer properties (Conte *et al.*, 2004; Nesaretnam *et al.*, 2004). Therefore, the anti-osteoporotic actions of tocotrienol may also be contributed by mechanisms other than the anti-oxidant properties.

There are only two molecular studies which can be used to explain the mechanism of tocotrienol in preventing osteoporosis. Wu *et al.* (2008) have shown that tocotrienol can suppress COX-2 expression in RAW 264.7 cells (exposed to LPS), therefore preventing the development of diseases related to inflammation. A RAW cell is the same cell that is used in cell culture studies as pre-osteoclast cells by adding RANKL. This indirectly implies that the molecular mechanism of tocotrienol in suppression of osteoclastic activity is by suppressing COX-2 expression. COX-2 is an inducible enzyme expressed during inflammation which suggests the role of tocotrienol as an anti-inflammatory agent in protecting bone against excessive osteoclastic activity. There is another study which has shown that tocotrienol inhibits HMG CoA reductase activity (Pearce *et al.*, 1992), the rate-limiting enzyme in the mevalonate pathway, which is essential for osteoclast differentiation. This offers another mechanism on how tocotrienol can suppress bone resorption.

Tocotrienol has been accepted to have anti-cholesterol and anti-cancer properties. Our studies have discovered another unique property of tocotrienol, i.e., anti-osteoporosis. Based on all the results, we can conclude that tocotrienol has the potential to be used as anti-osteoporotic agent and is a good candidate for human studies.

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