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Comparison of the Effects of Tocopherol and Tocotrienol on Osteoporosis in Animal Models

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Abstract: Studies have shown the potential of vitamin E in protecting bone against osteoporosis. Osteoporotic animal models were treated with vitamin E in the form of tocotrienols or tocopherols with mixed but overall good results in the bone parameters measured. The common issue raised was which of the two forms of vitamin E has greater potential as anti-osteoporotic agent. For the past decade, we have dedicated our bone researches on examining the role of vitamin E in bone metabolism of various animal models of osteoporosis. In some of our studies both tocopherol and tocotrienol were used as supplements, allowing their effectiveness to be compared. The bone parameters measured included bone resorbing cytokines, bone biochemical markers, bone calcium content, bone histomorphometry and bone strength. The tocopherols were represented by the alpha isomer, while the tocotrienols were represented by several different preparations of tocotrienols. Generally, the tocotrienols were found to be more superior to α-tocopherol in its effects on the bone parameters with a few exceptions. In this review, we explore the capabilities of both forms of vitamin E in their protective effects against osteoporosis. We hope it will shed some light on their potential as anti-osteoporotic agents and facilitate future directions of study on vitamin E.

Key words: Vitamin E, anti-oxidant, remodeling, histomorphometry, osteocalcin

INTRODUCTION

Osteoporosis is a major public health problem due to high fracture rates, decrease in quality of life and high healthcare costs (Sambrook and Cooper, 2006). There are many factors which can cause osteoporosis such as menopause, aging, thyroid diseases, chronic steroid intake and calcium deficiency. The current treatments for osteoporosis are mainly anti resorptive agents which inhibit further osteoclatic activity such as estrogen replacement therapy, bisphophanate and calcitonin. There are studies which have associated osteoporotic conditions with oxidative stress (Sontakke and Tare, 2002; Maggio *et al*., 2003). Therefore, antioxidants may be an alternative treatment for the prevention and treatment of osteoporosis. Vitamin E is a group of potent, lipid-soluble, chain-breaking antioxidants. Based on the chemical structure, vitamin E can be classified into tocopherol and tocotrienol. Tocotrienol differs from tocopherol by possessing a farnesyl (isoprenoid) rather than a saturated phtyl side chain (Serbinova *et al*., 1991). Vitamin E occurs in eight isoforms of α-, β-, γ-and δ-tocopherols or tocotrienols. Once taken orally, vitamin E is absorbed in the intestine and enters the circulation via the lymphatic system. It is then absorbed together with lipids and transported to the liver with the chylomicrons (Traber and Sies, 1996). α-Tocopherol will mostly appear in the plasma as it is selected by the hepatic α-tocopherol transfer protein (α-TTP) (Hosomi *et al*., 1997). Vitamin E is metabolized by side chain degradation initiated by cytochrome P450 (CYP)-catalyzed ω-hydroxylation followed by β-oxidation and then excreted in the urine (Brigelius-Flohe, 2003).

The focus of our studies was to investigate the effects of tocotrienols on bone metabolism. We realised that tocopherol and tocotrienol will always be compared in terms of their efficiency. With that in mind, we have included a tocopherol-treated group to allow comparison with the tocotrienols. Tocopherol was represented by α-tocopherol which is the most abundant and widely commercialized vitamin E. We used several forms of tocotrienols, namely pure γ-tocotrienol, pure tocotrienol mixture of γ, α and δ isomers and tocotrienol-enriched fraction of palm vitamin E containing small amount of α-tocopherol. Palm vitamin E is made up of γ, α and δ-tocotrienols as well as a small quantity of α-tocopherol. It is more practical and viable for human supplementation as it is easier and cheaper to extract compared to the pure tocotrienol isomers.

The doses of vitamin E that were selected ranged from 30 to 100 mg kg^{-1} rat weight. These doses were given via oral gavages to our rat models of osteoporosis

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and their effects on the bone parameters were evaluated. The rat was chosen as the animal model for bone studies and was subjected to various osteoporotic stressors to simulate the real osteoporotic conditions in humans. For example the rats were given thyroxine, nicotine, glucocorticoid or ferric nitrilotriacetate to induce osteoporosis. In other conditions, the rats were subjected to orchidectomy or ovariectomy to remove the hormonal influences on bone and therefore exposing them to osteoporosis.

The rat bone anatomy, bone remodeling and response to treatment are similar to human beings (Abe *et al*., 1993; Mosekilde, 1995). These allow changes in the bone mass of the rat model to be extrapolated to humans (Jee, 1995). The rat models that were used in present studies included the hyperthyroid, orchidectomised, ovariectomised, nicotine, glucocorticoid and ferric nitrilotriacetate (FeNTA) models. The bone parameters measured were bone resorbing cytokines, biochemical markers, bone anti-oxidant status, bone calcium, bone mineral density and bone histomorphometry. To the best of our knowledge, there is no review that compares the actions of different forms of vitamin E on various bone parameters. In this review, we compared the actions of tocopherol and tocotrienol on several bone parameters in the rat models of osteoporosis. Unfortunately, in the studies using hyperthyroid and orchidectomised models (Ima-Nirwana *et al*., 1999, 2000), there were no tocopherol groups available to compare with tocotrienol. We hope this review would allow other interested researchers to evaluate the efficacy of both forms of vitamin E with respect to alternative treatments for osteoporosis.

Effects of tocopherol and tocotrienol on bone resorbing cytokines: Cytokines are proteins or glycoproteins which act as the biological mediator for most cells. Interleukin-1 (IL-1) and Interleukin-6 (IL-6) are cytokines that are involved in bone resorption. Their levels in the serum can be used to represent local production by the bone marrow (Cohen *et al*., 1999). The IL-1 is mainly secreted by monocytes and is a potent bone resorbing factor *in vivo* (Boyce *et al*., 1989) and *in vitro* (Gowen *et al*., 1983). It stimulates osteoclastic formation and activities which leads to bone resorption (Suda *et al*., 1992). The IL-6, which is mainly secreted by osteoblast, promotes the formation of osteoclast and plays an important role in the pathogenesis of estrogen-deficient osteoporosis (Kurihara *et al*., 1989; Papanicolaou and Vgontzas, 2000). These two cytokines maybe related as IL-1 has been shown to stimulate osteoblast to secrete IL-6 (McSheehy and Chambers, 1986), while an *in vitro* study found the opposite, where IL-6 induced the secretion of IL-1 by stromal cells (Schaafsma *et al*., 1989). However, another study found that the human bone marrow stromal fibroblast could secrete IL-6 on its own without the presence of other cytokines (Guba *et al*., 1992).

We have studied the actions of tocopherol and tocotrienol on bone resorbing cytokines in ovariectomised, FeNTA and nicotine models. In ovariectomised rats, there were significant elevations of IL-1 and IL-6 but when palm tocotrienols or α-tocopherol was supplemented to these ovariectomised rats, the increments of IL-1 and IL-6 were suppressed (unpublished data). There was no significant difference between palm tocotrienols and α-tocopherol in terms of their actions on IL-1 and IL-6 in the ovariectomised rats.

In the FeNTA model, IL-1 and IL-6 were found to be elevated (Ahmad *et al*., 2005). Studies have shown that oxidative stress can increase IL-1 and IL-6 levels through activation of cytokine-encoding genes like STAT3 or Nuclear factor-kappaB (Carballo *et al*., 1999; Visseren *et al*., 2002). These studies suggested a link between the generation of reactive oxygen species by FeNTA and the elevated levels of bone resorbing cytokines. Our studies found that both the vitamin Es were able to suppress the elevation of IL-1 and IL-6 induced by FeNTA. Both the tocopherol and tocotrienol may have achieved this by preventing free-radicals generated by FeNTA from activating monocytes and osteoblasts, cells that mainly produce IL-1 and IL-6 respectively. Specifically, only palm tocotrienol mixture at doses of 60 mg kg^{-1} b.wt. and above was able to suppress the level of IL-1, while both palm tocotrienol mixture and α-tocopherol at doses of 30 mg kg^{-1} b.wt. and above were able to suppress the level of IL-6. Therefore, palm tocotrienol mixture was better than α-tocopherol in suppressing bone resorbing cytokines in the FeNTA model. We also need to bear in mind that the cells that mainly secrete these two cytokines have different morphology and may respond differently to these two forms of vitamin E (Ahmad *et al*., 2005).

Studies using the nicotine-treated rat model have suggested that nicotine might have a direct action on bone metabolism by influencing the bone remodeling process. *In vitro* studies have demonstrated that nicotine inhibited osteoblast activities and growth (Fang *et al*., 1991; Ramp *et al*., 1991) while it stimulated osteoclast resorption (Henemyre *et al*., 2003). Other studies have linked nicotine exposure to oxidative stress (Wetscher *et al*., 1995) and increased lipid peroxidation (Helen *et al*., 2000). In term of bone resorbing cytokines, our study has shown that nicotine significantly increased the IL-1 and IL-6 levels (Norazlina *et al*., 2004). Palm

tocotrienol mixture was able to prevent nicotine-induced elevation of IL-1 and IL-6, while α-tocopherol had no significant effect on both the cytokines levels (Norazlina *et al*., 2007). Once again, the palm tocotrienol mixture was more effective compared to α-tocopherol in terms of its action on bone resorbing cytokines.

Based on the results from the three rat models, we can conclude that tocotrienol was more superior than tocopherol in terms of its ability to suppress the bone resorbing cytokine.

Effects of tocopherol and tocotrienol on bone biomarkers: Bone metabolism may be investigated using serum or urine markers that originate from bone. One of the markers is osteocalcin, a small protein specifically synthesized by the osteoblasts which is used as a marker for bone formation. A major part of the *de novo* synthesized osteocalcin is bound to the hydroxyapatite matrix in bone but about 20% is set free in the blood stream. This circulating osteocalcin is generally regarded as a specific marker for osteoblastic activity and for bone formation (Delmas, 1992; Eastell *et al*., 1993; Akesson *et al*., 1995). Alkaline phosphatase is another marker for bone formation which is secreted by osteoblasts. Its activity in the serum is a reflection of osteoblastic activity (Delmas, 1992).

Bone resorption can be assessed by measuring urinary pyridinium crosslinks of collagen (DPD) (Black *et al*., 1988). The DPD is found in significant amounts in type I collagen which represents 90% of the organic matrix of bone (Eyre *et al*., 1988). In the process of bone degradation, DPD is released into the circulation and excreted in urine. Another marker for bone resorption is Tartrate-Resistance Acid Phosphatase (TRAP) which is secreted by osteoclasts. A measurement of its level in the serum is specific for osteoclastic activity (Delmas, 1992). Relationships between tocopherol and tocotrienol supplements to bone markers were studied in FeNTA and ovariectomised models.

In the FeNTA-induced oxidative stress model, the high bone turnover rate was maintained till the end of the study as shown by the elevated levels of deoxypyridinolline cross-links (DPD) and osteocalcin (Ahmad *et al*., 2005). The DPD levels were significantly decreased when FeNTA- treated rats were supplemented with palm tocotrienol mixtures at doses as low as 30 mg kg^{-1}. Whereas, for α-tocopherol, reduction of the DPD levels was seen only at doses higher than 60 mg kg^{-1}. The high osteocalcin levels in FeNTA-treated rats were lowered with supplementation of α-tocopherol or palm tocotrienol mixture at 100 mg kg^{-1}. Therefore, both α-tocopherol and palm tocotrienol mixture equally suppressed osteocalcin levels, but palm tocotrienol mixture was more potent at suppressing DPD levels. This suggests that tocotrienol was more potent than tocopherol in reducing bone resorption in the FeNTA model.

In our study on the ovariectomised rat model, both the ovariectomised and intact rats were treated with α-tocopherol or palm vitamin E. It was found that the level of alkaline phosphatase, a bone formation marker, was elevated in the ovariectomised rats supplemented with 30 mg kg^{-1} of palm vitamin E compared to intact rats. The alkaline phosphate level was also elevated in ovariectomised rats supplemented with similar dose of α-tocopherol (Norazlina *et al*., 2000). This indicates that both α-tocopherol and palm vitamin E were equally effective in increasing bone formation in ovariectomised rats. As for TRAP, a bone resorption marker, its level was found to be significantly lower in the ovariectomised rats supplemented with 30 mg kg^{-1} of α-tocopherol, whereas supplementation with palm vitamin E have no effects on the TRAP level (Norazlina *et al*., 2000). Surprisingly, this indicates that only α-tocopherol was able to reduce bone resorption in ovariectomised rats. Thus for ovariectomised rats, both tocotrienol and tocopherol had increased bone formation while only tocopherol had decreased bone resorption.

In summary, both tocopherol and tocotrienol had favourable effects on bone biomarkers in both the ovariectomised and FeNTA rat models. However, tocotrienol appeared better at reducing bone resorption in the FeNTA model compared to tocopherol, but the opposite was see in the ovariectomised rat model.

Effects of tocopherol and tocotrienol on bone calcium content: Calcium is the most abundant mineral found in bone. Disturbance in bone metabolism and bone turnover can affect bone calcium content and bone calcification. We have compared the actions of tocopherol and tocotrienol on bone calcium content in glucocorticoid, nicotine and ovariectomised rat models.

In the glucocorticoid model, dexamethasone was administered to adrenalectomised rats to induce osteoporosis. Two forms of vitamin E were used to supplement the rats, γ-tocotrienol and α-tocopherol. Based on the results, γ-tocotrienol was found to increase the bone calcium content of the fourth lumbar vertebra (Ima-Nirwana and Suhaniza, 2004), while α-tocopherol failed to produce any significant changes. This study has confirmed that tocotrienol was effective in protecting bone against dexamethasone-induced osteoporosis while tocopherol was ineffective.

In a study using the ovariectomised rat as a model for post menopausal osteoporosis, intact rats were compared to ovariectomised rats. We failed to demonstrate significant femoral or vertebral bone calcium loss in ovariectomised rats compared to intact rats (Norazlina *et al*., 2000). This was not expected as a previous study has shown reduced bone calcium after ovariectomy (Kalu, 1991). The bone calcium of ovariectomised rats supplemented with either palm vitamin E or α-tocopherol was also not different from their intact counterparts. These inconsistent findings were probably because of the young age of the rats used in the study. They were still skeletally growing under the influence of not just the reproductive hormones but other hormones as well, such as the thyroid and growth hormones (Ima-Nirwana *et al*., 1998). The only fact that we can gather from the study is that both tocotrienol and tocopherol were not able to influence bone calcium deposition in young ovariectomised rats. However, vitamin E is required for optimized bone calcification and its deficiency may lead to bone calcium loss. This was demonstrated in growing female rats given a vitamin Edeficient diet (Norazlina *et al*., 2002). Supplementation of palm vitamin E to these vitamin E-deficient rats was able to improve bone calcium content of their femur and lumbar vertebra. Supplementation with α-tocopherol failed to produce the same effect. Therefore, this suggests that both tocopherol and tocotrienol were not able to influence bone calcification in the vitamin E-replete state but tocotrienol was able to reverse bone calcium loss in the vitamin E deficiency state.

In nicotine treated rats, the femoral bone calcium content were reduced while there was no effect on the fourth lumbar bone calcium. Both palm tocotrienol mixture and á-tocopherol had prevented nicotine-induced bone calcium loss from the femoral bones (Norazlina *et al*., 2010). In addition, the femur of rats treated with α-tocopherol end up having higher bone calcium than the normal control rats indicating not only protective effects but also an anabolic action of α-tocopherol. This is another unexpected outcome in which α-tocopherol was better than the tocotrienol.

In the rat models used, tocotrienol was found to be more effective in maintaining bone calcium than tocopherol except for the surprising anabolic action of α-tocopherol on femoral bone calcium in nicotine-treated rats.

Effects of tocopherol and tocotrienol on bone mineral density: Dual Energy X-ray Absorptiometry (DEXA) is considered the gold standard as it is the most extensively validated test for predicting fracture outcomes (Nelson *et al*., 2002). The bone mineral density of ovariectomised rats was found to be lowered at all the skeletal regions except the femoral midshaft when compared to intact rats (Norazlina *et al*., 2000). This pattern of bone loss is consistent with other studies (Omi and Ezawa, 1995). The femoral midshaft is expected to be 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 prevent the decline in bone mineral density due to ovariectomy. This suggested that tocopherol was as effective as tocotrienol in preventing loss in bone mineral density induced by ovariectomy.

Effects of tocopherol and tocotrienol on bone histomorphometric parameters: Bone histomorphometry was used to study the histological structure and to assess bone formation and resorption. It provides a two dimensional measurement out of the three dimensional bone structure (Key *et al*., 1990). Bone histomorphometry was used to diagnose metabolic bone disease like Paget's disease, hyperparathyroidism and osteoporosis (Compston and Crouchier, 1991). We have compared the effects of tocopherol and tocotrienol on bone histomoprhometric parameters innicotine, FeNTA and ovariectomised rat models.

In the study on nicotine-treated rats, three forms of vitamin E were used, tocotrienol-enriched fraction, γ-tocotrienol and α-tocopherol (Hermizi *et al*., 2009). Nicotine treatment to rats for 2 months had compromised their bone histomorphometric parameters. Cessation of nicotine for 2 months failed to return the histomorphometric parameters to control levels. We found that treatment with all three forms of vitamin E were able to reverse the adverse effects of nicotine on the histomorphometic parameters of the trabecular bone. In fact, at the end of the study, vitamin E-treated groups had better trabecular bone properties than the control group, thus, exhibiting anabolic actions. Comparing the effects of tocotrienol and tocopherol on the bone histomorphometric parameters, tocotrienol was noted to be slightly superior to tocopherol.

In the ovariectomised rat model, the bone histomorphometric parameters such as trabecular volume, trabecular thickness and trabecular number were all decreased, while trabecular separation was increased. At the same time, both the osteoclast and osteoblast surfaces were increased by ovariectomy, indicating increased cellular activities due to high bone turnover. Supplementation with palm tocotrienols and α-tocopherol

had prevented these ovariectomy-induced changes. Based on these results, tocopherol was as effective as tocotrienol in preventing the adverse histomorphometric changes induced by ovariectomy (unpublished data).

In FeNTA-oxidative stress model, there were deteriorations in the structural, static and dynamic parameters of bone histomorphometry (Ebina *et al*., 1991; Ahmad *et al*., 2005). Other studies have also shown impairment of osteoblast function and decreased osteoblast recruitment (Ebina *et al*., 1991; Takeuchi *et al*., 1997; De Vernejoul *et al*., 1984). Palm tocotrienol supplementation at 100 mg kg^{-1} had protected the bone volume from the deleterious effects of FeNTA but the same dose of α-tocopherol was not able to do the same. Only supplementation with palm tocotrienol had suppressed the high osteoclast number-induced by FeNTA. Supplementation with palm tocotrienol was found to have positive effects on osteoblast as shown by the normalization of the osteoblast number (Ahmad *et al*., 2005). Therefore, tocotrienol was more effective than tocopherol in protecting bone from the histomorphometric changes induced by FeNTA.

Based on the results of the various models used, tocotrienol was better than tocopherol in its action on bone histomorphometric parameters. In the nicotine model, both forms of vitamin E had exhibited anabolic action on bone.

DISCUSSION

Present studies using various models of osteoporosis in rats for the past decade, has enabled us to compare the effectiveness of tocopherol and tocotrienol on the various bone parameters measured. This information may help in deciding which form of vitamin E has greater potential for further development as an anti-osteoporotic agent. We think that the representation of tocopherol by its alpha isomer form is relevant as it is the most commercially available vitamin E. However, we only managed to use the gamma isomer of tocotrienol in a few of our studies due to technical and financial difficulties in obtaining pure isomers of tocotrienol. Thus, we consider pure tocotrienol mixtures, tocotrienol-enriched fraction and palm vitamin E to be a fair representation of the tocotrienols. In fact, palm vitamin E and tocotrienol-enriched fraction are presently available commercially in the market.

Generally, tocotrienol had offered better bone protection than tocopherol based on the results of its action on the bone resorbing cytokines, bone biomarkers, bone calcium content and bone histomorphometric parameters. These convincing results maybe contributed by the higher antioxidant potency of tocotrienol compared to tocopherol (Serbinova *et al*., 1991). It is believed that in all models of osteoporosis used, oxidative stress may have contributed significantly to the underlying pathology of bone loss. This may explain why an antioxidant like vitamin E had successfully protected these models against further bone loss. Most studies have demonstrated the anti-oxidative effects of vitamin E by measuring the oxidative parameters in the serum or organs other than bone. In order to provide evidence of the protective effects of vitamin E in bone, we have measured the bone lipid peroxidation and anti-oxidant enzymes directly. As expected, vitamin E was found to suppress lipid peroxidation and increase the glutathione peroxidase activity in bone. Palm tocotrienol was found to be more effective than α-tocopherol (Mamiam *et al*., 2008). This suggested that tocotrienol has offered protection against free radical damage in bone compared to tocopherol. However, tocotrienol has also been shown to have other unique properties other than anti-oxidative effects such as anti-cancer and anti-cholesterol effects (Conte *et al*., 2004; Nesaretnam *et al*., 2004; Hasselwander *et al*., 2002). Therefore, tocotrienol may have other undiscovered mechanisms of protection against osteoporosis.

There were several conditions where tocotrienol was not better than tocopherol. In the ovariectomised rat model, both the vitamin E were equally effective in maintaining the bone mineral density from the deleterious effects of ovariectomy. The ovariectomised rat was the only model available for this parameter, thus not enabling further comparison between the two forms of vitamin E in other models of osteoporosis. Apart from that, there were several unexpected results where tocopherol was better than tocotrienol. Again in the ovariectomised rat model, only α-tocopherol was able to lower TRAP level whereas, palm vitamin E was ineffective. Perhaps the palm vitamin E that represented tocotrienol was not potent enough as it was not purely tocotrienols but contained some α-tocopherol. In another situation, both forms of vitamin E were effective in preventing femoral bone calcium loss in nicotine-treated rats. However, only α-tocopherol had exhibited an anabolic action by further increasing femoral bone calcium content. In the same model, the tocotrienol-enriched fraction, γ-tocotrienol and α-tocopherol exhibited bone anabolic actions on the bone histomorphometric parameters. These findings have led us to investigate further on the bone anabolic actions of vitamin E. In a later study, we found that only γ-tocotrienol supplementations to normal male rats were able to raise their bone histomorphometric parameters and biomechanical strength, while α-tocopherol supplementations improved only parts of the parameters

measured (Nazrun *et al*., 2010). Therefore, we have confirmed that both vitamin E have some bone anabolic properties with tocotrienol exhibiting better effects than tocopherol.

In view of all these findings, we can conclude that both tocotrienol and tocopherol have the potential to be developed as an anti-osteoporotic agent. However, tocotrienol has been shown to be superior than tocopherol and is a better candidate for human studies.

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