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Comparison of the Effects of Tocotrienol and Estrogen on the Bone Markers and Dynamic Changes in Postmenopausal Osteoporosis Rat Model

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ABSTRACT

The standard treatment for postmenopausal osteoporosis is Estrogen Replacement Therapy (ERT) but it was associated with serious adverse effects such as breast cancer and cardiovascular disease. There is a need to find other alternatives for the treatment of post-menopausal osteoporosis. Vitamin E especially tocotrienol was shown to have anti-osteoporosis effects in many animal osteoporotic models but it has never been compared to estrogen. This study aimed to compare the effects of tocotrienol-enriched fraction to estrogen on the bone biochemical markers and dynamic histomorphometric changes using ovariectomised rats as the postmenopausal osteoporosis model. Thirty-two female Sprague-Dawley rats were randomly divided into groups of sham-operated (SHAM), ovariectomised control (OVC), ovariectomised+60 mg kg^{-1} tocotrienol-rich fraction (OV+T) and ovariectomised+64.5 μg kg^{-1} of premarin® (OV+ERT). The rats were treated for two months and the serum osteocalcien and serum C-telopeptide of type 1 collagen (CTX) were measured using ELISA technique, while the femoral dynamic changes were analysed histomorphometrically. The CTX levels were found to be lowered compared to the pre-treatment levels for all the groups. Only the osteocalcin level of OV+ERT group was significantly reduced compared to its pre-treatment level. Dynamic histomorphometric analysis showed that both the OV+ERT and OV+T groups have lower single-labeled surface/bone surface (sLS) but higher double-labeled surface/bone surface (dLS), bone formation rate/bone surface (BFR) and mineral apposition rate (MAR) compared to the BC and OVC groups. The OV+T group showed better effects on most of the dynamic parameters compared to the OV+ERT group. Therefore, in postmenopausal osteoporosis model, tocotrienol-rich fraction has shown better bone protective effects than ERT.

Key words: Osteoporosis, estrogen-deficiency, bone biomarkers, histomorphometry

INTRODUCTION

Osteoporosis is an important age-related disease constituting a major health problem due to its association with high incidence of fracture and significant healthcare costs (Sambrook and Cooper, 2006). Fracture may occur with minimal trauma when the bone loss resulted in low bone mineral density and microarchitectural disruption. In estrogen-deficient state, cytokines are released which

increases the osteoclasts life-span and bone resorbing activity (Kurihara *et al*., 1989; Papanicolaou and Vgontzas, 2000). In response to the increased bone resorption, the bone formation tries to compensate but often failed. The high-turnover state leads to bone loss and perforation of the trabecular plates.

Estrogen Replacement Therapy (ERT) is a well known treatment for postmenopausal osteoporosis. The major physiological effect of estrogen is inhibition of bone resorption by regulating osteoclast number and activity. However, a recent study found that ERT may cause breast cancer, uterine cancer and thromboembolic disease (Ferguson, 2004). Women taking ERT have higher risk of getting uterine cancer and breast cancer compared to those not taking ERT (Lane, 2001). Women with cardiovascular diseases on ERT were also found to have higher rate of cardiovascular complications (Conteras and Parra, 2000). The women's health initiative study found that women who took ERT have slightly higher rates of breast cancer, ovarian cancer, heart attack, stroke, thromboembolism and alzheimer's disease (Rossouw *et al*., 2002; Chlebowski *et al*., 2003; Shumaker *et al*., 2003). Therefore, studies are on the way to find other alternatives which can protect women from postmenopausal osteoporosis but without any serious side-effects.

Animal studies are required to determine the safety and efficacy of a potential new drug before a human drug trial can be carried out. Many studies have used ovariectomised rats as postmenopausal osteoporosis model for its convenience, relevance and appropriateness. Their bone anatomy, trabecular bone remodeling and response to treatment are considered similar to human beings (Abe *et al*., 1993; Mosekilde, 1995; Jee, 1995). Several animal studies has shown that vitamin E especially tocotrienol supplementation was able to protect against bone loss in various animal osteoporosis models (Nazrun *et al*., 2010). Deficiency of vitamin E induced a state of calcium deficiency (Norazlina *et al*., 2002a) and increased free radical activity (Norazlina *et al*., 2002b). The anti-osteoporotic action of vitamin E was believed to be contributed by its ability to assist the endogenous antioxidant defense system protect bone from oxidative stress. In fact, estrogen may also protect bone against osteoporosis by ameliorating free radicals as it also has anti-oxidant properties (Badeau *et al*., 2005).

Tocotrienols and tocopherols are the two forms of vitamin E. The chemical structure of tocotrienol differs from tocopherol by possessing a farnesyl (isoprenoid) rather than a saturated phtyl side chain (Serbinova *et al*., 1991). The unique chemical structure of tocotrienol may have contributed to its efficient anti-oxidant and other unique properties namely anti-cancer, anti-platelet and anti-cholesterol properties (Conte *et al*., 2004; Nesaretnam *et al*., 2004; Hasselwander *et al*., 2002). Recently, tocotrienol were found to exhibit bone anabolic property in nicotine treated rats (Hermizi *et al*., 2009) and normal male rats (Nazrun *et al*., 2010).

So far, there has been no study to compare the anti-osteoporotic effects of tocotrienol to estrogen, the standard treatment of post-menopausal osteoporosis. Therefore, this study was conducted to examine the effects of tocotrienol and estrogen on markers of bone turnover and the dynamic histomorphometric analysis. We hypothesized that tocotrienol may be able to match estrogen in protecting the rat model against bone loss induced by ovariectomy.

MATERIALS AND METHODS

This research was approved by Research and Ethical Committee, Faculty of Medicine, University Kebangsaan Malaysia (FP/FAR/2010/NAZRUN). Thirty-two female Sprague-Dawley rats (four months old) weighing between 180 and 200 g were obtained from the Laboratory Animals Resource Unit, Universiti Kebangsaan Malaysia. The rats were kept two per cage under

12 h natural light-dark cycle and were given drinking water *ad libitum*. The rats were randomly assigned into groups of sham-operated (SHAM), ovariectomised-control (OVC), ovariectomised and given 60 mg kg^{-1} of tocotrienol-rich fraction (OV+T) and ovariectomised and given 64.5 μg kg^{-1} of premarin® (OV+E).

Preparation of treatments: Tocotrienol-Rich Fraction (TRF) was supplied by Carotech Bhd, Ipoh, Malaysia and has the following composition: α-tocotrienol 24.67%, γ-tocotrienol 38.955%, δ-tocotrienol 4.55% and α-tocopherol 20.11%. It was diluted in olive oil (Bertolli, Italy) and given via oral gavages at the concentration of 60 mg kg^{-1} body weight daily at 9 am for 8 weeks. Premarin® (Wyeth-Ayerst, Kanada) tablet containing 0.625 mg of conjugated estrogen was crushed, dissolved in deionised water and given via oral gavages at the dose of 64.5 μg kg^{-1} rat weight daily at 9 am for 8 weeks (Al-Wahabi *et al*., 2007). The SHAM and OVC groups were given oral gavages of vehicle for similar duration of treatment.

Blood and bone sampling: Blood samples were collected before the start and after 8 weeks of treatment from the retro-orbital vein after anesthetizing the rats with ether. After 3 h, the blood was centrifuged at 3000 rpm for 10 min and the serum was stored at -70°C. The rat bones were fluorochrome-labeled with intraperitoneal injections of 20 mg kg^{-1} calcien at 9 days and 2 days before the rats were euthanized. The left femurs were dissected out and fixed with 70% alcohol.

Bone biochemical markers: Bone biochemical markers of serum osteocalcin and C-terminal telopeptide of type 1 collagen (CTX) were measured before and after the treatment using an ELISA reader (VERSAmax, Sunnyvale, USA). The kits used were rat osteocalcin ELISA (Biomedical Technologies, Herlev, Denmark) and RatlapsTM ELISA CTX-1 (Nordic Biosciences, IDS UK).

Bone histomorphometry: Bone histomorphometry is generally defined as the measurement of the shape or form of a bone. The bone dynamic histomorphometric parameters were measured according to The American Society of Bone Mineral Research Histomorphometry Nomenclature Committee 1987 (Parfitt *et al*., 1987). The left femurs were dissected out and fixed with 70% ethanol. After one week, the femora were cut sagitally at the epiphyseal and metaphyseal area. The femora were then embedded in methyl methacrylate (Osteo-Bed Bone Embedding Kit; Polysciences, USA) and sectioned at 9 μm thickness using a microtome (Leica RM2155, Wetzlar, Germany). The dynamic bone histomorphometric parameters were analyzed using an image analyzer Pro-Plus (Media Cybernatics, Silver Spring, MD, USA), a fluorescence microscope (Nikon Eclipse 80 μ, Japan) and a Weibel grid (Freere and Weibel, 1967). The dynamic bone histomorphometric parameters include single-labeled surface/bone surface (sLS/BS), double-labeled surface/bone surface (Dls/BS), mineralizing surface/bone surface (MS/BS), bone formation rate/bone surface (BFR/BS) and Mineral Apposition Rate (MAR). The measurements were performed at the metaphyseal region which is rich in trabecular bone. This secondary spongiosa area is located 3 to 7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex, excluding the endocortical region (Baldock *et al*., 1998).

Statistical analyses: The results were expressed as Mean±standard deviation (SD). Data analysis was performed using the statistical package for social sciences software (SPSS 19; SPSS, Chicago, IL, USA). Data were tested for normality using the Kolmogorov-Smirnov test. The statistical tests

used were analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for normally distributed data and the Kruskal-Wallis and Mann-Whitney tests for data that were not normally distributed. The significant level was determined at $p<0.05$.

RESULTS

Biochemical markers: The post-treatment level of serum CTX (bone resorption marker) was significantly lower than the pre-treatment level for the all the groups. However, there was no significant difference between the groups (Fig. 1). As for serum osteocalcin (bone formation marker), the only significant finding was the lower post-treatment level in the OV+E group compared to its pre-treatment level (Fig. 2). There was no significant difference between the groups.

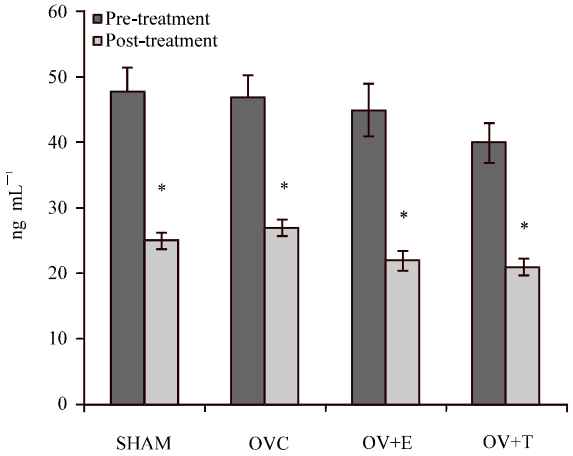

Fig. 1: Serum cross linked-telopeptide of type I collagen (CTX) levels for all the groups and after treatment * indicate significant difference between pre-treatment and post-treatment levels within the group

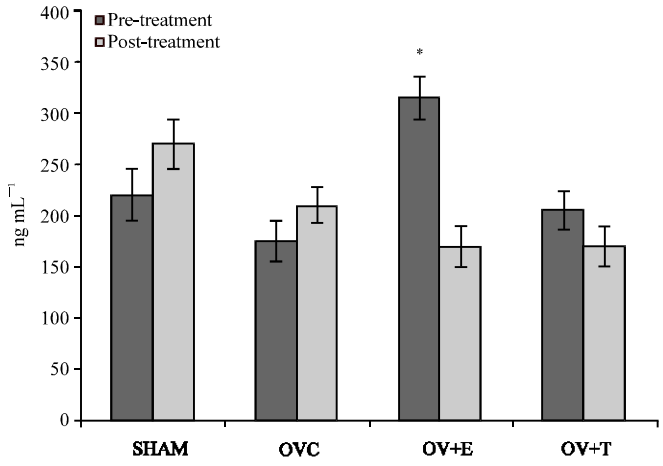

Fig. 2: Serum osteocalcein levels for all the groups before and after treatment * indicate significant difference between pre-treatment and post-treatment levels within the group

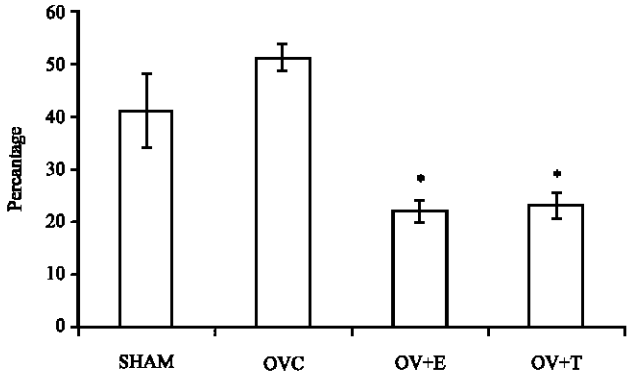

Fig. 3: Single labeled surface per bone surface (sLS/BS) for all the groups. Data was presented as Mean+SEM. Significant level was taken at $p<0.05$. *Indicate significantly different compared to OVC group

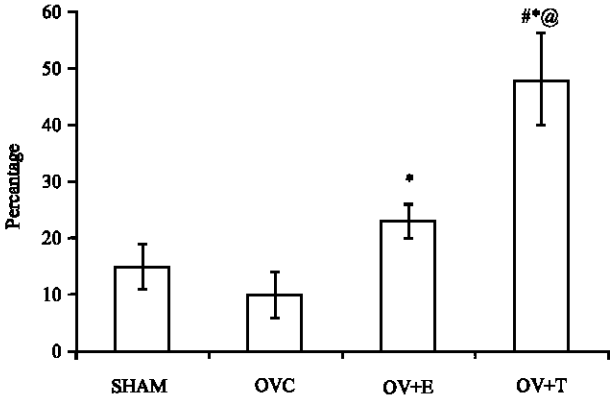

Fig. 4: Double labeled surface per bone surface (dLS/BS) for all the groups. Data was presented as Mean+SEM. Significant level was taken at $p<0.05$. *Indicate significantly different compared to OVC group, #Indicate significantly different compared to SHAM group and @ indicate significantly different compared to OV+E group

Single-labeled surface/bone surface (sLS/BS): There was no significant difference in the sLS/BS of the SHAM and OVC groups. The sLS/BS of the Ov+E and OV+T groups were significantly lower than the OVC group. There was no other significant difference between the other groups (Fig. 3).

Double-labeled surface/bone surface (dLS/BS): There was no significant difference in the dLS/BS of the SHAM and OVC groups. The dLS/BS of both the Ov+E and OV+T groups were significantly higher than the OVC group. The dLS/BS of the OV +T group was also significantly higher than the rest of the groups (Fig. 4).

Mineralizing surface/bone surface (MS/BS): There was no significant difference in the MS/BS for all the groups (Fig. 5).

Bone formation rate/bone surface (BFR/BS): There was no significant difference in the BFR/BS of the SHAM and OVC groups. The BFR/BS of the OV+E and OV+T groups were

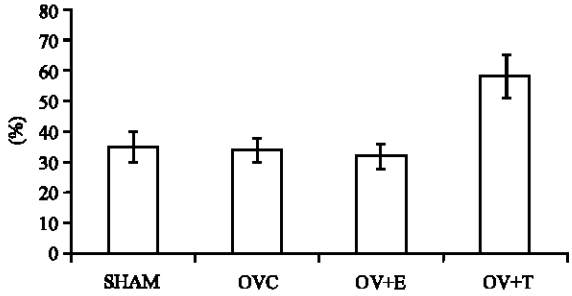

Fig. 5: Mineralising surface per bone surface (MS/BS) for all groups. Data was presented as Mean+SEM. Significant level was taken at $p<0.05$

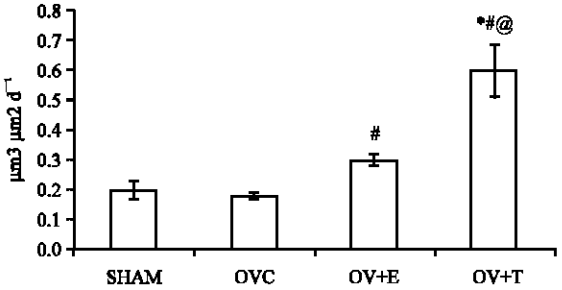

Fig. 6: Bone formation rate (BFR) for all the groups. Data was presented as Mean+SEM. Significant level was taken at $p<0.05$, *Indicate significantly different compared to SHAM group, #Indicate significantly different compared to OVC group and @Indicate significantly different compared to OV+E group

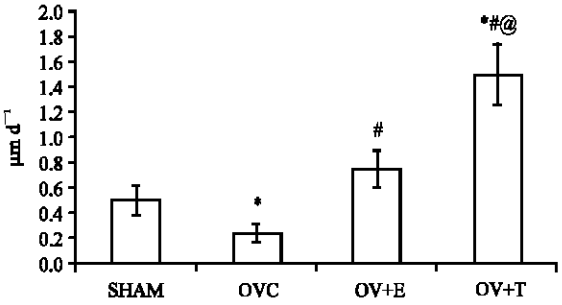

Fig. 7: Mineral apposition rate (MAR) for all the groups. Data was presented as Mean+SEM. Significant level was taken at $p<0.05$, *Indicate significantly different compared to SHAM group, #Indicate significantly different compared to OVC group and @Indicate significantly different compared to OV+E group

significantly higher than the SHAM and OVC groups. When the OV+E group and OV+T group were compared, the OV+T group had higher BFR/BS (Fig. 6).

Mineral Apposition Rate (MAR): The MAR of the OVC group was significantly lower than the SHAM group. The MAR of the OV+E and OV+T groups were significantly higher than the OVC groups. When the OV+E and OV+T groups were compared, the OV+T group had higher BFR/BS (Fig. 7).

DISCUSSION

Ovariectomised rat is an accepted model for postmenopausal bone loss as ovariectomy-induced osteopenia in rats produce similar skeletal responses to post-menopausal woman (Abe *et al*., 1993; Mosekilde, 1995). They began to show reduction in bone mineral density two months after ovariectomy with greater bone loss occurring at regions rich in trabecular bone (Ima-Nirwana *et al*., 1998). They are usually compared to sham-operated rats to rule out the influence of surgical stress from ovariectomy procedure on the bone parameters.

Osteocalcin, a protein specifically synthesized by the osteoblasts is regarded as a specific marker for osteoblastic activity and bone formation (Eastell *et al*., 1993; Akesson *et al*., 1995). C-telopeptide of type-I collagen (CTX) which accounts for more than 90% of the bone organic matrix is used as bone resorption marker (Calvo *et al*., 1996; Garnero and Delmas, 1998). During renewal of the skeleton, bone matrix is degraded and consequently fragments of type I collagen is released into circulation.

The serum CTX was significantly reduced after treatment for all the groups but was not different from each other. Reduction in marker may indicate slowing of the bone turnover as the rats reached maturity at the end of the study. The estrogen deficiency induced by ovariectomy should have caused CTX elevation (Garnero *et al*., 1996) but this was not seen in this study. Treatment with an anti-osteoporotic agent should cause reduction in the CTX level (Rosen *et al*., 2000). However, as all the groups had lower CTX levels, it is difficult to determine if estrogen or TRF has anti-resorptive action. Factors such as food intake and time of sample collection can affect the variability of CTX levels (Qvist *et al*., 2002). Furthermore, in a study using ovariectomised rat model, tocotrienol-rich palm vitamin E was found to be ineffective in reducing tartrate resistant acid phosphatase (TRAP), another marker of bone resorption (Norazlina *et al*., 2000).

As, for serum osteocalcin, the bone formation marker, the high turnover rate expected in ovariectomised rats was also not observed with this marker. Osteocalcin may not truly reflect bone formation as it was found to be a poor predictor of bone loss in perimenopausal women (Vestergaard *et al*., 2001). The only significant finding was the lower post-treatment level compared to pre-treatment level in the OV+E group. This meant that only estrogen has managed to slow down the bone turnover rate by reducing both the bone resorption and formation. There was also reduced post-treatment osteocalcin level with TRF supplementation but was not significant. In this study, the high bone turnover state of ovariectomised rats was not seen in the biochemical markers changes. However, it must be noted that none of the biochemical markers has proven useful as a single diagnostic index of osteoporosis and that they are not substitutes for individual bone mass measurements (Seibel, 2006).

The bone dynamic histomorphometry allows changes in the bone cellular activity over time to be studied by labeling the bone twice with a flourochrome. In this study, the deleterious effect of ovariectomy was only reflected as the significant reduction in MAR of OVC group compared SHAM group. The sLS/BS and dLS/BS parameters also showed characteristic of bone loss-induced by ovariectomy but were not significant.

The bone protective effects of estrogen and TRF in ovariectomised rats were seen in the sLS/BS, dLS/BS, BFR/BS and MAR parameters. These parameters were found to be significantly higher than that of the ovariectomised control group except for the sLS/BS which was significantly lower. Therefore, both estrogen and tocotrienol-rich fraction (TRF) was effective in reversing the deleterious bone changes induced by ovariectomy. This was expected of estrogen as it was already accepted as the standard treatment of postmenopausal osteoporosis.

A study on rat model has shown that treatment with tocotrienol was able to reverse the adverse effects of nicotine on the histomorphometic parameters of the trabecular bone (Hermizi *et al*., 2009). In rats exposed to ferric nitrilotriacetate, an oxidizing agent, tocotrienol had protected the bone against histomorphometric changes induced by free-radicals (Ahmad *et al*., 2005).

For the first time in this study, the efficacies of estrogen and TRF on the bone dynamic parameters were compared. Both were found to have similar efficacy on sLS/BS, but TRF was superior in dLS/BS, BFR/BS and MAR. In addition, TRF promoted better histomorphometric parameters compared to SHAM group, thus exhibiting bone anabolic actions. This confirmed the findings in other studies which found that vitamin E exhibited anabolic actions in nicotine treated rats (Hermizi *et al*., 2009) and normal male rats (Nazrun *et al*., 2010).

CONCLUSION

TRF has comparable or more effective anti-osteoporotic effects than estrogen in postmenopausal osteoporosis rat model. TRF had also exhibited bone anabolic actions in some of the histomorphometric parameters. Therefore, TRF has potential as an alternative anti-osteoporotic agent but without any adverse effects of an estrogen.

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