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Bone Oxidative Changes during Early Fracture Healing of Postmenopausal Osteoporosis Rat Model

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ABSTRACT

Ovariectomised rat is an accepted model of postmenopausal osteoporosis. Fracture can be induced in this model to simulate osteoporotic fracture. The aim of the study was to determine the anti-oxidative changes during early fracture healing in postmenopausal osteoporosis rat model. The lipid peroxidation levels and anti-oxidant enzyme activities during the early phase of osteoporotic fracture healing were measured directly in the fractured bone of ovariectomised rats. The effects of antioxidant supplementation i.e., vitamin E, on the oxidative parameters were also measured. Thirty two female Sprague-Dawley rats were divided into groups of Sham-Operated (SO), Ovariectomised-Control (OVC), ovariectomised +60 mg kg^{-1} of α-tocopherol (ATF) and ovariectomised +60 mg kg^{-1} of Tocotrienol-rich fraction (TT). The rats were left untreated for 2 months to allow ovariectomy-induced osteoporosis before their right femora were fractured at the mid-diaphysis. After 2 weeks of treatment, the Thiobarbituric Acid Reactive Substances (TBARS) level and Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPX) enzyme activities of the fractured femora were measured. There was a significant drop in the SOD activity of OVC group compared to SO group. Tocotrienol was able to significantly lower the TBARS level and raise the GPX activity compared to OVC group. While, α-tocopherol was able to significantly raise the SOD activity compared to OVC group. Therefore, only SOD activity was affected during early fracture healing in this rat model. Tocotrienol was able to reduce lipid peroxidation during early fracture healing of osteoporotic bone probably by enhancing its GPX activity. Anti-oxidants were beneficial in improving the bone oxidative status during early fracture healing.

Key words: Ovariectomy, fracture, osteoporosis, vitamin E, tocotrienol, oxidative stress

INTRODUCTION

Osteoporosis may result in fragile bone which easily fractures with trivial force. The high incidences of osteoporotic fractures and their associated morbidity can lead to surgical and medical management cost escalation (Keen, 2003). In the United States, 5 to 10% of the 6 million fractures that occur every year showed delayed or impaired fracture healing. Delay in callous maturation during fracture healing may be caused by infection, malnutrition or osteoporosis (Dai and Hao,

2007). The International Osteoporosis Foundation (IOF) has reported high death and disability rates from osteoporotic fractures in Russia, Central Asia and Eastern Europe. The healing of osteoporotic fractures is crucial to avoid premature death, personal suffering, lost of productivity and long-term dependence on family members (IOF, 2010).

Due to ethical issues, animal osteoporotic models are more appropriate than human subjects in fracture healing studies (Raisz, 1988). Fracture healing can be assessed directly in the fractured bone of animal models by taking out the bone sample, which is impossible to be done in human subject. Besides that, human studies are limited by difficulty to attain control group and create homogeneous study groups (Giannoudis *et al*., 2007).

In this study, fractures were induced on ovariectomised rats to simulate osteoporotic fracture in postmenopausal women and the oxidative parameters were measured during the early phase of osteoporotic fracture healing. The early phase of fracture healing which occurs within 2 weeks after fracture is the phase where oxidative stress is likely to occur as excess superoxide radicals is produced to remove the debris (Reilly *et al*., 1991). The overproduction of oxygen free radicals can cause cell damage and impair bone fracture healing (Foschi *et al*., 1988, 1990; Gokturki *et al*., 1995). The estrogen deficiency condition in ovariectomised rat and postmenopausal women were linked to excess production of free-radicals (Maggio *et al*., 2003; Sontakke and Tare, 2002; Muthusami *et al*., 2005) which can aggravate oxidative stress during the early phase of fracture healing. This was demonstrated by Xu *et al*. (2003) which found reduction in the density of callous, a cartilaginous bridge across a fracture, at the early phase of femoral fracture healing of ovariectomised rats. There are numerous studies using osteoporotic animal model focusing on the effects of estrogen deficiency on bone metabolism (Joldersma *et al*., 2001; Liu and Kalu, 1990; Salih *et al*., 1993). However, there is still a lack of understanding on the effects of osteoporosis on fracture healing (Namkung-Matthai *et al*., 2001; Walsh *et al*., 1997).

Antioxidants such as vitamin E may influence the metabolism of various tissues including bone. Vitamin E alone or in combination with vitamin C were found to offer protection against the hazardous effects of ethanol (Oyinbo *et al*., 2006; Onyesom *et al*., 2007), cadmium (Cinar *et al*., 2011) and chemotherapy (Singh *et al*., 2011) on various tissues. Vitamin E was also shown to prevent osteoporosis in various osteoporotic models (Nazrun *et al*., 2010) but there has been no report yet on its effect on fracture healing.

It is important to measure the oxidative parameters within the fractured bone to determine the influence of anti-oxidants such as vitamin E on fracture healing. Maniam *et al*. (2008) have shown that vitamin E affected the lipid peroxidation and anti-oxidant enzymes within the rat bone. The aim of the study was to investigate the effects of vitamin E intake on lipid peroxidation and anti-oxidant enzymes during fracture healing of osteoporotic bone using rat model. The early phase of fracture healing was chosen as this is the critical period during which lots of free radicals are released.

MATERIALS AND METHODS

Experimental animals and treatment: The study was conducted from April 2009 to February 2010. The rats were purchased from the University Animal House. α-Tocopherol acetate was purchased from Sigma (St. Louis, MO, USA) while palm tocotrienol mixture was provided by the Malaysian Palm Oil Board (MPOB). Thirty-two female Sprague Dawley rats weighing between 250 to 300 g were used. The study had been approved by the UKM Animal Ethics Committee (FP/FAR/2008/NAZRUN/13-FEB/217-FEB-2008-FEB-2010). The rats were divided into 4 groups.

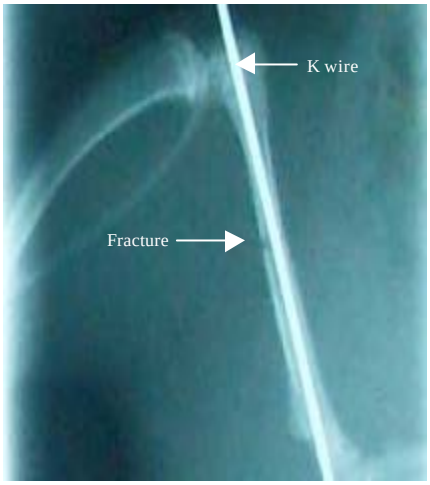

Fig. 1: X-ray of the right femora was done to confirm that fracture had occurred at mid-diaphysis and the K wire was inserted correctly

Rats in the first group were Sham-Operated (SHAM) while rats in the rest of the groups were ovariectomised. Ovariectomised rats were used as the model for postmenopausal osteoporosis. Briefly, the rats were anaesthetized with Ketapex and Xylazil, 1:1 (Troy Laboratories, Australia). The lower abdomen of the rats were shaved and incised. The fallopian tubes and ovaries were identified and catgut suture was used to tie the fallopian tubes below the ovaries. The ovaries were then removed. These rats were left untreated for 2 months to allow the bones of ovariectomised rats to become osteoporotic as by Nazrun *et al*. (2010).

Fracture technique: The right femurs of all the rats were then fractured aseptically according to Vialle *et al*. (2004). X-rays of the right femora were taken immediately using X-ray machine (Proteus XR/a, GE UK) to ensure that fractures had occurred at mid-diaphysis and the K wires were inserted correctly (Fig. 1). Post-fracture, the ovariectomised rats were randomly divided into 3 groups of ovariectomised-control (OVC), ovariectomised + 60 mg kg^{-1} α-tocopherol acetate (ATF) and ovariectomised +60 mg kg^{-1} Tocotrienol mixture (TT) groups. They were allowed unrestricted weight bearing after recovery from anesthesia. The rats were kept one per cage at room temperature of 27°C and 12 h natural light/dark cycles. The rats had free access to rat-chow (Gold Coin, Malaysia) and deionised water.

Vitamin E supplementation: α-Tocopherol acetate (Sigma, St. Louis, MO, USA) and palm tocotrienol mixture (Malaysian Palm Oil Board (MPOB), Kajang, Selangor, Malaysia) were diluted in olive oil (Bertolli, Italy) to obtain the concentration of 60 mg kg^{-1} body weight. The palm tocotrienol mixture (tocotrienol-rich fraction) has the following composition: α-tocotrienol 14.62%, γ-tocotrienol 32.45%, δ-tocotrienol 23.39% and α-tocopherol 18.43%. The α-tocopherol (ATF) and Tocotrienol mixture (TT) groups were supplemented with 0.1 mL of α-tocopherol or palm tocotrienol mixture solutions per 100 g b.wt., respectively via daily oral gavages for 14 days. The SHAM and OVC groups were given oral gavages of olive oil (vehicle) for similar duration of treatment.

The rats were euthanized after 14 days of treatment and the fractured right femurs were dissected out. The K wires were extracted carefully to avoid damage to the femur and stored at -70°C until analysed for biochemical parameters.

Estimation of bone lipid peroxidation (TBARS): Lipid peroxidation in the fractured bone was estimated by measuring malondialdehyde according to the method by Baltaci *et al.* (2004) with slight modifications. The grounded femur was vigorously mixed with 150 mM of cold (4°C) Potassium Chloride (KCl) solution to obtain a 10% (w/v) homogenate. The homogenates were then centrifuged at 3700x g at 4°C for 15 min. (Sigma Laborzentrifugen-3K30, Osterode, Germany). 3 mL of 1% orthophosphoric acid (H_3PO_4), 1 mL of 0.675% TBA and 0.5 mL of supernatant were mixed and kept in boiling water bath for 45 min. Four milliliter of n-butanol was added after the mixture cooled down. The values were read using spectrophotometer at 532 nm against n-butanol using Shimadzu UV-1601 (Tokyo, Japan). Results were presented as 'thiobarbituric acid reactive substances' (TBARS) in nmol mg^{-1} protein instead of malondialdehyde.

Estimation of bone glutathione peroxidase (E.C.1.11.1.9): Glutathione peroxidase in the fractured femur was estimated by the method of Sazuka *et al.* (1989).

Estimation of bone superoxide dismutase (E.C.1.15.1.1): Superoxide dismutase in the fractured femur was estimated by the method of Marklund and Marklund (1974).

Protein estimation: The protein content of the fractured femur was estimated by the method of Lowry *et al.* (1951) with slight modifications. Four milliliter of 1 M NaOH was added to 100 mg of ground femora. The mixtures were incubated for 30 min. Fifty microliter of homogenates were added to 450 μL of NaOH and these diluted homogenates were subjected to the standard protocol of the Lowry assay for protein determination.

Statistical analysis: SPSS version 11 was used for statistical analysis. Data were tested for normal distribution using Kolmogorov-Smirnov normality test. For normally distributed data, the statistical test used was ANOVA followed by Tukey's Honestly Significance Difference test. Data that was not normally distributed was analyzed using Mann-Whitney followed by Kruskal-Wallis. The results were presented as Means±SEM. The level of significance was taken as $p<0.05$.

RESULTS

The rats recovered well after fracture procedure and surgery without any complications. They did not display any signs of discomfort. Weight-bearing on the leg with fractured femur began within seven to ten days after the fracture procedure. The fractures of the osteoporotic femora of ovariectomised rats represented pathological fractures (due to osteoporosis) while the fracture of normal femora of sham-operated rats represented traumatic fracture. X-rays performed immediately after the fracture procedure confirmed that all the fractures had occurred at mid-diaphysis of the femora and the K wires were in the correct position (Fig. 1).

Bone lipid peroxidation (TBARS): After 14 days of fracture, the femoral TBARS level of the OVC group was not different from the SHAM group. The TBARS level of the TT group was

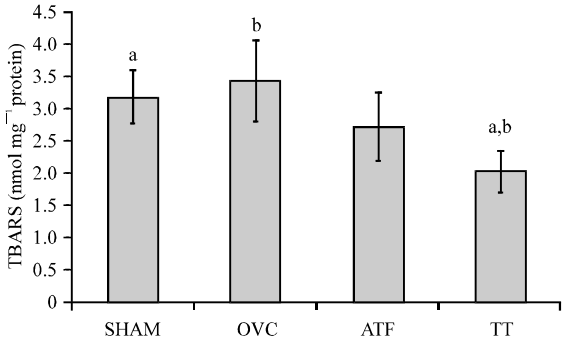

Fig. 2: Impact of α-tocopherol (ATF) and tocotrienol (TT) supplementations on thiobarbituric acid reactive substances (TBARS) in the fractured femur of ovariectomised rats during the early phase of fracture healing. Values marked by the same letters indicate significant difference between groups at $p<0.05$. SHAM: Sham-operated, OVC: Ovariectomised-control, ATF: Ovariectomised +60 mg kg^{-1} α-tocopherol acetate, TT: Ovariectomised+60 mg kg^{-1} palm tocotrienol mixture

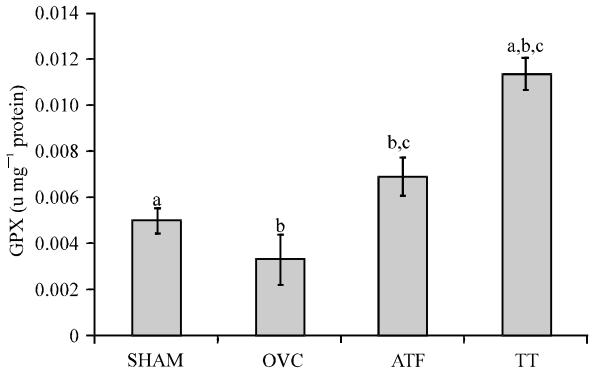

Fig. 3: Impact of α-tocopherol (ATF) and tocotrienol (TT) supplementations on the specific activity of glutathione peroxidase (GPX) in the fractured femur of ovariectomised rats during the early phase of fracture healing. Values marked by the same letters indicate significant difference between groups at $p<0.05$. SHAM :Sham-operated, OVC: Ovariectomised-control, ATF: Ovariectomised +60 mg kg^{-1} α-tocopherol acetate, TT: Ovariectomised+60 mg kg^{-1} palm tocotrienol mixture

significantly lower than the OVC and SHAM groups. There was a non-significant decrease in the TBARS level of the ATF group compared to the SHAM and OVC groups (Fig. 2).

GPX activity: There was a non-significant decrease in the femoral GPX activity of the OVC group compared to the SHAM group. The ATF group had significantly higher femoral GPX activities than the OVC group only. The femoral GPX activity of TT group was significantly higher than other groups (Fig. 3).

SOD activity: The femoral SOD activity of the OVC group was significantly lower than the SHAM group. The ATF group had significantly higher femoral SOD activity than the OVC group. The

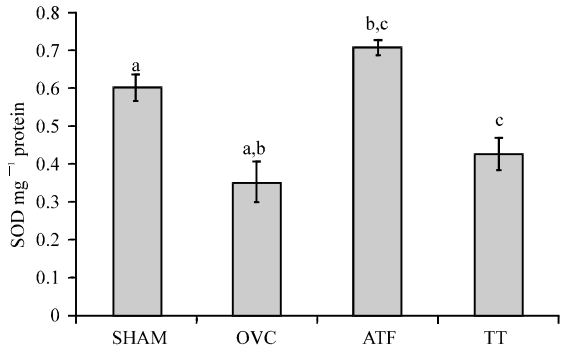

Fig. 4: Impact of α-tocopherol (ATF) and tocotrienol (TT) supplementations on the specific activity of superoxide dismutase (SOD) in the fractured femur of ovariectomised rats during the early phase of fracture healing. Values marked by the same letters indicate significant difference between groups at $p<0.05$. SHAM: Sham-operated, OVC: Ovariectomised-control, ATF: Ovariectomised +60 mg kg^{-1} α-tocopherol acetate, TT: Ovariectomised + 60 mg kg^{-1} palm tocotrienol mixture

SOD activity of the TT group was higher than the OVC group but was not statistically significant (Fig. 4)

DISCUSSION

Fractures were induced under anesthesia according to the guillotine method by Vialle *et al.* (2004), which produces a consistent fracture of the femur with minimal trauma to the surrounding tissues to mimic osteoporotic fracture. In the present study, lipid peroxidation and anti-oxidant enzymes were measured in the bone which would reflect accurately the oxidative status that may influence bone fracture healing.

Vitamin E especially tocotrienol has been shown to be effective in preventing osteoporosis (Shuid *et al.*, 2010). While, there were mixed results on the effects of tocopherol on normal fracture healing. Turk *et al.* (2004) found that α-tocopherol could improve fracture healing while Durak *et al.* (2003) found that α-tocopherol resulted in better fracture healing after 21 days. These studies suggested that α-tocopherol may be responsible for better fracture healing. However, Sarisozen *et al.* (2002) could not find any changes in the callous indices after 2 weeks of tibial fracture in normal rats supplemented with α-tocopherol.

The current belief is that the anti-oxidant properties of vitamin E may be responsible for protecting the bone against the deleterious effects of free radicals (Ahmad *et al.*, 2005). During the early phase of fracture healing, excess free radicals are being released on top of the oxidative stress induced by estrogen deficiency and these free-radicals may impair fracture healing (Foschi *et al.*, 1988). The early phase of fracture healing is very important as most of the biological insufficiencies appear at the first week after fracture (Cornell and Lane, 1992; Frost, 1989). Thus, it is important to control these excess free-radicals during the early phase to ensure proper healing. Sheweita and Khoshhal (2007) recommended administration of antioxidants to accelerate the healing of fractured bones. Potent antioxidants, such as vitamin E may be useful in this phase to overcome oxidative stress and promote osteoporotic fracture healing. Vitamin E at the dose of 60 mg kg^{-1} was shown to be effective in preventing osteoporosis in various rat models (Norazlina *et al.*, 2001;

Ima-Nirwana *et al.*, 1993; Khalid *et al.*, 2005). A toxicity study on palm vitamin E has shown that the effective dose used to prevent or treat osteoporosis was safe as only very large doses were found to cause bleeding tendencies and liver impairment but there was no liver toxicity (Ima-Nirwana *et al.*, 2011).

There are conflicting evidences as to whether ovariectomy would give additional stresses to fracture healing. Some animal studies have shown impaired healing, especially in the early phase (Xu *et al.*, 2003; Durak *et al.*, 2003; Meyer *et al.*, 2001), while some did not (Melhus *et al.*, 2007). Other studies suggested that ovariectomy affected the late phase of healing only (Hill *et al.*, 1995; Kubo *et al.*, 1999). The differences in the timing of ovariectomy, age of the animals and dietary factors further complicate the issues. The present study showed that the fractured femoral TBARS level and GPX activity of ovariectomised rats were similar to sham-operated rats, while the SOD activity was lowered. Studies have shown that postmenopausal osteoporosis is associated with oxidative stress (Maggio *et al.*, 2003; Sontakke and Tare, 2002; Ozgocmen *et al.*, 2007). In these postmenopausal women, the MDA levels were unchanged or elevated, while the SOD activities were unchanged or lowered. Only the GPX activities were consistently lowered in all the studies. These oxidative parameters were measured in blood samples since bone samples were not available in human study. Therefore, it may not be a direct representation of the bone oxidative environment. It may not be fair to compare an animal study with these human studies, but there seems to be a different pattern in the antioxidant enzymes activities during osteoporotic fracture. Even though there was reduction in SOD activity, the GPX activity was unchanged during fracture healing. This pattern may be contributed by the high productions of superoxide radicals during the early phase of fracture healing (Reilly *et al.*, 1991). SOD is the direct antioxidant enzyme responsible to catalyze the dismutation of superoxide radicals to hydrogen peroxide before they could cause oxidative stress and lipid peroxidation. The high levels of superoxide radicals may have overwhelmed SOD, thus causing a drop in its activities. The reduction of hydrogen peroxide is in turn catalysed by two antioxidant enzymes, GPX and catalase. The catalase activity was not measured in the present study but their cooperation in the removal of hydrogen peroxide may have preserved the GPX activity.

Tocotrienol supplementation was able to significantly lower the TBARS level during early fracture healing of osteoporotic bone compared to SHAM and OVC groups, while α-tocopherol failed to do so. Vitamin E especially tocotrienols can react directly with peroxyl radicals to convert them to hydroperoxides. This lipid peroxidation-lowering effect of tocotrienol was also demonstrated in normal rats at the dose of 100 mg kg^{-1} b.wt. (Maniam *et al.*, 2008).

In the early phase of osteoporotic fracture, both the ATF and TT groups had significantly higher GPX activity than OVC group. GPX is the primary antioxidant enzyme which catalyses the reduction of hydrogen peroxide and peroxyl radicals. Vitamin E supplemented to the rats may have assisted GPX in reducing peroxyl radicals, therefore keeping the GSH levels high. Tocotrienol was more superior to α-tocopherol in keeping the GSH activity high as seen by Maniam *et al.* (2008). Interestingly, vitamin E can be recycled to its active form by GPX (Kagan *et al.*, 1992). In other studies, vitamin E has been shown to work together or closely linked to glutathione in alleviating oxidative stress (Gokkusu *et al.*, 2004; Gupta *et al.*, 2011). The synergistic actions of vitamin E and GPX may have hampered lipid peroxidation and increased the GPX activity.

As for the SOD activity, during the early phase of osteoporotic fracture healing, α-tocopherol supplementation was able to increase the SOD activity compared to OVC and TT groups. The present study found that both α-tocopherol and tocotrienol have influenced the antioxidant

enzymes activities during osteoporotic fracture healing. More importantly, tocotrienol has managed to not only suppress but further reduce lipid peroxidation during fracture healing of osteoporotic bone. It has dominantly increased the activities of GPX, which deals directly with peroxyl radicals and hydrogen peroxide. Whereas, α-tocopherol managed to significantly raise the GPX and SOD activities but only maintained the lipid peroxidation level. In normal rats, the SOD activity was found to be similar whether they were supplemented with 60 mg kg^{-1} α-tocopherol or tocotrienol-rich fraction. Maniam *et al.* (2008) have suggested that the high GPX activity and lower lipid peroxidation level in bones induced by tocotrienol were achieved by over-expression of GPX. The superiority of tocotrienol was also demonstrated in a study comparing the effects of the two forms of vitamin E on gastric lesions in rats (Kamsiah-Jaarin *et al.*, 1999). Tocotrienol may be more superior to tocopherol as it has better interaction with lipoproteins in the membrane lipid and are uniformly distributed in the membrane layer (Serbinova *et al.*, 1991).

CONCLUSION

Tocotrienol was able to reduce lipid peroxidation during early fracture healing of osteoporotic bone probably by enhancing its GPX activity. The α-tocopherol have raised the GPX and SOD activities but failed to lower lipid peroxidation. Antioxidants may be beneficial in promoting fracture healing of osteoporotic bone. Further studies are required to confirm this.

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