Research Article
Effects of Vitamin E on Bone Oxidative Parameters during Fracture Healing of Postmenopausal Osteoporosis Rat Model

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ABSTRACT
Osteoporosis increases the risk of bone fracture and may interfere fracture healing. Studies have proven that vitamin E promoted fracture healing of osteoporotic bone. The objective of this study is to determine whether the antioxidative actions of tocopherol and tocotrienol promote the late-phase fracture healing of ovariectomised rats. Thirty-two female Sprague-Dawley (200g - 250g) rats were divided among four groups: Sham (SO), Ovariectomised-control (OVXC), Ovariectomised + 60mg/kg alpha-tocopherol (ATF), and ovariectomised + 60mg/kg tocotrienol enrich fraction (TEF). The right femora of the rats were fractured eight weeks after ovariectomy and vitamin E supplementations were given two days post-fracture, by oral gavage, six days per week, for eight weeks. The rats were euthanised and the right femora were harvested for bone oxidative status determination (TBARS, SOD, GPX, and catalase). Result from the study showed no significant changes for all oxidative parameters measured (MDA, SOD, GPX and Catalase). Supplementation of α-tocopherol and tocotrienol had no effects on the bone oxidative status during late phase fracture healing of postmenopausal osteoporosis rat model. These anti-oxidative actions of vitamin E may be important only during the early-phase of fracture healing.

Keywords: osteoporosis, α-tocopherol, tocotrienol, fracture healing, oxidative status.

1. Introduction

Osteoporosis is a skeletal disorder characterized by low bone mass and micro architectural deterioration of bone tissue, resulting in bone fragility and susceptible to fracture [1]. The National Institutes of Health (NIH) Consensus Conference revised this definition to “a skeletal disorder characterized by compromised bone strength, predisposing a person to an increased risk of fracture” [2]. Diagnosis of osteoporosis is based upon the T-score value of 2.5 standard deviation below the mean bone mineral density (BMD) of young adult women [3]. T-score manifestation of bone mineral density is calculated using an imaging technique called bone densitometry. The global incidence of osteoporosis is increasing every year. In Malaysia, the incidence of osteoporosis was reported to be 24.1% in the year 2005, and it mainly caused hip fracture [4].

Bone fracture is the most important and common consequence of osteoporosis, which mainly cause fractures of the vertebrae, hips and forearm. A slight trivial force to an osteoporotic bone may cause fracture, which usually would not cause any bone injuries of healthy individuals. Animal studies have shown that osteoporotic fracture may be more severe and may cause delay of the fracture healing [5, 6]. Asian population showed a higher prevalence of osteoporosis than the western countries since they have lower body mass index and shorter height [7]. Almost 50% of the fragility fractures in the world are estimated to occur in Asia by the next century [8].

Fracture healing is a complex process which recovers fully within 12 weeks after fracture in rats [9]. It has three distinct phases, which are the reactive phase, reparative phase, and remodeling phase [10]. Reactive
phase occurs immediately after fracture with inflammation and formation of granulation tissue. This is followed by the reparative phase, where a callus is formed and lamellar bone deposited at the fracture site. The final phase of bone fracture healing is the remodeling phase where the bone will be remodeled to its original bone contour. Animal osteoporotic models are used widely in osteoporotic fracture healing studies because of the complexities of the healing process, and the ethical issues involved [11]. Rats are commonly used as the animal osteoporotic model with ovariectomised rats widely accepted as the model to simulate bone loss among postmenopausal women [12, 13]. This model resembles the biphasic bone loss in human, with initial rapid phase of bone loss occurring up to 100 days [14].

Vitamin E is a group of naturally-occurring tocopherols and tocotrienols. They both have four different isomers that include α, β, γ, and δ isomers. Tocopherol is abundantly present in staple foods such as nuts and vegetable oils. Tocotrienol on the other hand, is present in cereal grains, rice bran, and is most abundant in palm oil of E. guineensis species [15]. Previous studies have shown that tocotrienol has better antioxidant capacity compared to tocopherol [16, 17]. Vitamin E acts as an antioxidant and it is generally agreed that vitamin E gives protection against lipid peroxidation.

In this study, fractures were induced to ovariectomised rats to mimic osteoporotic fracture in postmenopausal osteoporosis women. The oxidative statuses were then measured during the late phase of fracture healing to determine whether the improvement of fracture healing process with tocotrienol supplementation described earlier [18] was due to its ability to alleviate oxidative stress. The late phase of fracture healing occurs eight weeks after fracture, which is characterized by bone remodeling to original bone contour [19].

2. MATERIALS AND METHODS

Animals and Treatment

A total number of 32 females Sprague-Dawley rats weighing between 250 to 300 grams were divided among four groups. Rats in the first group were sham-operated (SO), while rats in the other three groups were ovariectomised. After the right femora were fractured, the rats were kept one animal in each cage. Rat chow and water were given to the rats ad libitum, and they were maintained in a room with light dark cycle of 12 hours and the room temperature of 29°C. The rats were left untreated for two months after ovariectomy to allow development of osteoporosis before the left femora were fractured. The right femora were fractured using a blunt guillotine-like apparatus according to the method by Vialle et al. [20]. The procedure was performed aseptically under anesthesia with ketamine and xylazine (1:1) at the dose of 0.1ml/100g rat weight. Iodine was first applied to the area of operation (the right knee) before making an incision. The right patella was then dislocated, and a Kirshner wire (K wire) (1.0 mm in diameter) was inserted using a drill into the intramedullary canal of the femur. The K wire acted as an internal fixation to the fractured bone. The patella was then relocated back, and the incision sutured using a non-absorbable suture (nylon 4). The Fracturer device described earlier was used to fracture the right femur of all the rats. This device uses the same principle as the guillotine principle, where a 500g steel blunt bar was dropped on the mid-diaphysis of the femur, which was supported by two metal bases. This formed a three-point system, producing a closed and standardized fracture. The device with has three platforms. The middle platform is 30cm height from the lowest platform and 10cm height to the upper platform. The triggering bar is placed at the upper platform which acted to release the bar. The upper platform acted to stabilize the whole structure of the device. Following the fracture procedure, Baytril was injected intramuscularly for perioperative antibiotic prophylaxis. Buprenorphine (analgesics) was injected subcutanously every 12 hours at the dose of 0.3mg/kg rat weight. X-rays were taken immediately after fracture using x-ray machine (Proteus XR/a, GE UK) to confirm that fractures had occurred at the mid-diaphysis of the right femur, and the K wires were inserted correctly. The rats were then allowed unrestricted weight bearing after their recovery from anesthesia. The ovariectomised rats were then randomly divided into four groups of ovariectomised-control (OVXC), α- tocopherol (ATF), and Tocotrienol Enriched Fraction (TEF) groups. The ATF treated group was given α- tocopherol acetate (Sigma, USA), whereas the TEF group was given Tocotrienol Enriched Fraction (Golden Hope Biorganic Sdn. Bhd., Malaysia) which contains 73.9mg/g α-tocopherol, 167.1mg/g α-tocotrienol, 41.1mg/g β-tocotrienol, 165.2mg/g γ-tocotrienol and 98.5mg/g δ-tocotrienol. Both α- tocopherol and Tocotrienol Enriched Fraction were given at the dose of 60mg/kg rat weight. On the other hand, SO and OVXC groups were only given vehicle, olive oil (Bertoli, Italy). All the treatments were given via oral gavage at 6 days per week for 2 months [10]. After 2 months, all the rats were euthanized and the left femora harvested. The femora were kept wrapped in aluminum foil at -70°C. This study has been approved by the UKM Animal Ethics Committee (FP/FAR/2008/NAZRUN/13-FEB/217-FEB2008-FEB2010).

Determination of bone lipid peroxidation

Lipid peroxidation in the fractured bone was determined by measuring the TBA reacting substance (TBARS) according to a method described by Baltaci et al. 2004 with slight modifications [21]. 0.2g fractured bone samples were grounded and vigorously mixed with 0.15M Potassium Chloride (KCl) solution making it a 10% (w/w) homogenate. The homogenate was then centrifuged at 3000rpm in cold temperature (4°C) for 15 minutes. 0.5ml supernatant was added with 3ml 1% cold orthophosphoric acid, and let to stand for 15 minutes before addition of 1ml 0.675% fresh TBA. The mixture was vortex before sealing the test tube with double layer aluminum foil. This step was important to ensure that the mixture did not spill
while incubated in water bath at 95°C for 45 minutes. The mixture was left to cool before adding 4ml n-butanol and vigorously vortex for 3 minutes. Once again, the mixture was centrifuge at 3000rpm for 10 minutes. The values were read using spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) at 532nm against n-butanol and presented as thiobarbituric reactive substance (TBARS) in nmol-1 mg protein.

**Determination of bone superoxide dismutase (SOD)**

Superoxide dismutase enzyme’s activity in the fractured bone was measured using the method by Marklund & Marklund 1974 [22]. 0.2g fractured bone samples were grounded and vigorously mixed with 0.1M Tris HCl buffer solution with pH 7.4, making it a 10% (w/v) homogenate. The homogenates were vortex for several minutes before they were centrifuged with the speed of 10 000g for one hour at 4°C temperature. Then, 750µl of the supernatant was pipette out to a glass test tube and 2ml Tris HCl buffer (pH 8.2) and 750µl distilled water were added. It was then vortex and left at room temperature for 10 minutes to make sure that it has sufficient oxygen concentration for auto oxidation to occur. 500µl pyrogallol was added to the solution and quickly transferred to a cuvette and measured with a spectrophotometer at 420nm against 0.05M phosphate buffer. It was left for 30 seconds (lag time) and read for 60 seconds. One enzyme’s unit is the amount of enzyme needed to inhibit 50% of pyrogallol auto-oxidation process.

**Determination of bone glutathione peroxidase (GPX)**

Glutathione peroxidase activity in the fractured bone was determined using EnzyChromeTM Glutathione Peroxidase assay kit (EGPX-100) (BioAssay Systems, USA). Enzyme-Linked Immunosorbent Assay (ELISA) method was employed and this assay measures directly the use of NADPH in the enzyme’s reaction. The definition of one unit of GPX activity means total glutathione peroxidase which produced 1µmol GS-SG per minute at pH 7.6 and room temperature.

**Determination of bone catalase enzyme**

Catalase activity in the fractured bone was measured using EnzyChromTM Catalase assay kit (BioAssay Systems, USA). Catalase is an anti-oxidant enzyme that is always available in a human body. It facilitates decomposition of hydrogen peroxide (H2O2) to water and oxygen. This assay measures directly the degradation of H2O2 by catalase using a redox stain. A change in the colour intensity at 570nm wavelength is proportionate to catalase activity in the samples. The unit for catalase activity is unit/l. One unit is considered as the amount of catalase that degrades 1µmol H2O2 per minute at pH 7.0 and room temperature.

**Statistical analysis**

The results were expressed as mean ± standard error mean (SEM). Analysis was done using Statistical Package for Social Sciences software (SPSS version 20). Normality of the data was tested using Kolmogorov-Smirnov test. ANOVA was performed for normally distributed data, followed by Tukey hsd. The level of significance was taken as p<0.05.

### 3. RESULTS

All rats recovered after eight weeks of fracture procedure and weight bearing of the fractured femur began within seven to ten days after the procedure. Confirmation of fracture that was done immediately after procedure showed that all the fractures occurred at the mid-diaphysis of the femora and the Kirshner wires were inserted properly.

The MDA, SOD, GPX, and Catalase levels of the OVXC group were not significantly different from Sham group. Therefore, the bone was not under oxidative stress during the late phase of fracture healing. There were also no significant changes in the MDA, SOD, GPX, and Catalase levels between the rest of the groups (Figure 1, Figure 2, Figure 3, and Figure 4).

**Figure 1** Bar chart shows that there is no significant difference in the lipid peroxidation levels (MDA) between the groups. Results were given in mean ± S.E.M.

**Figure 2** Bar chart shows that there is no significant difference in the SOD between the groups. Results were given in mean ±S.E.M.
4. Discussion

Oxidative stress is known to be the underlying pathology for bone loss during osteoporosis. The association between oxidative stress and osteoporosis was evident in a study by Nazrun et al. [23], which found vitamin E to have the antioxidant capacity to protect against osteoporotic bone loss and promote fracture healing. This was proven by another study where vitamin E managed to inhibit lipid peroxidation and increase glutathione peroxidase activity in bone [24].

In the present study, the levels of MDA, SOD, GPX, and catalase activities were measured in the fractured bone of ovariectomised rats. There were no significant differences in all the oxidative parameters measured during late phase of fracture healing. In the early phase of fracture healing, which takes place within two weeks after fracture, superoxide radical is produced abundantly to scavenge the debris during inflammation. This is the time when there is oxidative stress at the fracture site, which would benefit most from administration of anti-oxidants such as vitamin E [25]. In the present study, the oxidative parameters were measured eight weeks after the induction of fracture, which is the late phase of fracture healing. The fractured bone would have been in the remodeling phase; hence, oxidative stress is no longer significant for vitamin E to produce any anti-oxidant effects.

There are contradictory findings on whether ovariectomy causes extra stress to bone healing process. Several studies on animals have shown that it interfere the healing process especially in the early phase [26-29]. However, several studies suggested that ovariectomy only affect the late phase of the healing process [30, 9]. These contradictory findings may be due to the different timing of ovariectomy, the age of the animals, and the diet given to them during the fracture healing period.

Postmenopausal osteoporosis has been linked with oxidative stress [31-33]. These studies were done on women with postmenopausal osteoporosis, where their levels of MDA were the same or elevated, whereas SOD activity was the same or decreased. Only the level of GPX activity was consistently decreased in all the studies. However, these oxidative parameters were measured indirectly on blood samples as it is impossible to take out the bone for analysis in human study. Therefore, this may not be representative of the oxidative environment for bone, and it would not be fair to compare the animal and human study.

Oxidative stress is said to interfere the early phase of fracture healing. Nazrun et al. (2011) [34], studied the early phase of fracture healing using the same animal model. The results showed that there are differences on the lipid peroxidation, GPX, and SOD levels between the treated and control rats. Lipid peroxidation for rats supplemented with tocotrienol was significantly lower compared to sham-operated and control rats. Tocotrienol-supplemented rats have significantly higher GPX activity compared to sham-operated, control, and α-tocopherol groups. However, tocotrienol-supplemented rats had significantly lower SOD activity compared to α-tocopherol-supplemented rats [34].

In conclusion, supplementation of α-tocopherol and tocotrienol did not have any effects on the bone oxidative status during late phase fracture healing of postmenopausal osteoporosis rat model.

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References


