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Bone Proteome Study in Ovariectomised Rats Supplemented with Palm Vitamin E

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***Abstract*—**Supplementation of palm vitamin E has been reported to prevent loss of bone density in ovariectomised female rats. The mechanism by which palm vitamin E exerts these effects is still unknown. We hypothesized that palm vitamin E may act by preventing the protein expression changes. Two dimensional poly acyrilamide gel electrophoresis (2-D PAGE) and PD Quest software genomic solutions Investigator (proteomics) was used to analyze the differential protein expression profile in femoral and humeri bones harvested from three groups of rats; sham-operated rats (SO), ovariectomised rats (Ovx) and ovariectomised rats supplemented for 2 months with palm vitamin E. The results showed that there were over 300 valued spot on each of the groups PVE and OVX as compared to about 200 in SO. Comparison between the differential protein expression between OVX and PVE groups showed that ten spots were down –regulated in OVX but up-regulated in PVE. The ten differential spots were separately named P1-P10. The identification and understanding of the pathway of the differential protein expression among the groups is ongoing and may account for the molecular mechanism through which palm vitamin E exert its anti-osteoporotic effect.

***Keywords*—**Palm vitamin E**,** ovariectomised**,** osteoporosis protein expression, 2-d-page.

I. Introduction

OSTEOPOROSIS, a disease characterized by high bone fragility and increase risk of fractures with high mortality and morbidity rates, has become an expensive health menace worldwide. Depletion of ovarian hormone following menopause, imbalance between bone resorption and bone formation during remodeling process is believed to be a major cause of brittle bones. The etiology of osteoporosis is complex and not completely known. Various cells, cytokines, mediators and numerous signaling pathways as well as genetic and environmental factors play role in bone remodeling process [1-3]. Activated T cells ,macrophages and stromal bone cells produce a variety of inflammatory cytokines, including IL-1 and TNF-α [4] IL-6 [5] interferone –γ (IFN-γ) [6], M-CSF and GM-CSF [7] , IL-12 [8], IL-11,IL-15 and IL-17 [9]. Animal studies have proposed a crucial role for T cells in bone metabolism after ovariectomy which produces TNF-α and RANKL resulting in up - regulation of osteoclastogenesis [10, 11].

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 phytyl side chain [12]. As an antioxidant, vitamin E would help the endogenous defense system to protect bone from oxidative stress. For the past decade, research has been focused on the bone protective effects of tocotrienol against various stressors of osteoporosis. It was found that vitamin E deficiency induced a state of calcium deficiency [13] that could be related to increased free radicals activity [14].

Supplementation of palm vitamin E has been reported to reverse the negative effects of estrogen deficiency on bone mineral density and bone calcium. It has also been shown to prevent the increase in IL-1 and IL-6 seen in ovariectomised controlled rats and increasing osteoblastic bone formation relative to osteoclastic bone resorption, resulting in positive bone remodeling [15]. In addition, it lowered the serum alkaline phosphatase activity and serum tartrate-resistant acid phospatase activity, the markers for bone formation and resorption respectively [16]. The mechanism of protection of vitamin E against osteoporosis is not clearly understood although it has been suggested that vitamin E may protect bone by interrupting the free radical chain reaction and therefore preventing damage due to lipid peroxidation [17-19]. In another study, it was shown that ovariectomy-induced osteoporosis in rats caused protein expression changes compared to the control animals [20]. Thus, we hypothesized that palm vitamin E may act by preventing the protein expression changes.

II. Materials and Methods

A. Experimental Animals

Thirty adult female Sprague Dawley rats weighing 350-400g, (3-4months old) were obtained from the Univesity's

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animal house and were allowed free access to tap water and normal standard chow diet. The rats were housed in a plastic cage and were kept under controlled conditions of 25 ± 2^0C, 55± 5% relative humidity, and 12h light –dark cycles throughout the experiment. Rats were maintained in these facilities for 2 weeks before the experiment. The animals were randomly divided into three groups of 10 rats each; Sham – operated (SO), Ovariectomy (OVX) and Ovariectomy + palm vitamin E (PVE). This experiment has been performed according to the animal's ethics rule of Universiti Kebangsaan Malaysia with the approval number FP/FAR/2008/NORAZLINA/13-FEB/211-FEB-2008-AUG-2009.

B. Treatment

Bilateral ovariectomy in rats was performed as previously described [21]. The rats were anesthetized with Ketapex: Xylazil (1:1) to allow exposure of the ovaries. The gonads were removed in the OVX and PVE groups but were left intact in SO group. After operation, a few drops of antibiotic were applied daily for a week to prevent against infection. The PVE group was supplemented with 0.4ml (60mg/kg) of palm vitamin E solution. The palm vitamin E solution was prepared by mixing 3 mg of palm vitamin E (Golden Hope, Malaysia) with 50ml olive oil. The OVX and SO groups received 0.4ml of olive oil. All treatment was given to the respective group 6 days a week for 2 months by oral gavage.

C. Tissue Preparation

After two months, the rats were sacrificed via intravenous injection of pentobarbital sodium (25 mg/kg of body weight). Both femora and humeri (a total of four bones) were taken. The bones were cleansed from cartilage and bloodstain was rinsed in deionized water. The fresh bone specimen was stored at -80 ^{0}C for future use.

D. Preparation of Protein Samples

Prepared bone (1g) was placed in an earthen bowl and ground into powder in liquid nitrogen. Then 3 ml ice-cold buffer (1.44 g urea, 0.46 g sulfocarbamide, 0.12 g CHAPS, 0.047 g DTT, 0.015 g Tris-base, 30 Al 100 mM PMSF) was added. After storage at 4^0C overnight, the sample was centrifuged at 12,000g for 30 minutes. The protein content in the supernatant was concentrated using vivispin 15R and protein quantification of sample was done with Nano drop at 280nm. The samples were then stored in -80 ^{0}C for future use.

E. Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2–DE) was performed essentially as reported with a few modifications [12, 15, 16]. The first – dimension known as isoelectric focusing (IEF) was done using ready IPG stripe 17cm (ph 3-10, Biorad ,USA) with focusing condition start voltage and end voltage 0 and 10,000 V respectively, 40-60,000 V-hr, rapid and 20^{o}C. Prior to the second-dimensional gel separation, the gel strips were equilibrated for 15 minutes each, with gentle shaking in 4 ml of equilibration buffer 1 and equilibration buffer 2 (Biorad, USA). To prepare gel for the second dimension, 0.05g SDS (Biorad USA) was dissolved in 0.5 ml deionized water, mixed with 20 ml of pre-mixed bis-acryamide (Biorad, USA) and 12.5ml of 1.5M tris-HCl in 22 ml of ddH_2O. To start polymerization, 0.5ml of ammonium persulfate (Biorad, USA) and 100 µl of TEMED (Biorad, USA) were added to the mixture. The second-dimensional SDS-PAGE with a 12% resolving gel was carried out in Protean II XL, 16mA/p gel 30 minutes, then 24 mA/gel for 6 hours. After placing the gel strip on the top of the second-dimensional polyacrylamide gel, electrophoresis was performed at a constant power of 120 v/gel until tracking dye reached the bottom of the gel. The experiment was repeated fifty times.

F. Gel Staining, Scanning and Image Analysis

Coomassie Bio- Safety Blue stain (Biorad, USA) was used to stain the gel. The image was scanned with GS-800 calibrated densitometer (Chemopharm, USA). 2-DE map analysis was performed using PD Quest software genomic solutions Investigator.

III. Result

Protein fractions obtained from the bones were analyzed by 2-DE. The 2-DE maps of the groups are shown in Fig. 1. There were over 300 valued spot on each of the PVE and OVX groups, as compared to about 200 in SO. Comparison of the differential expression of protein spots between SO/PVE groups and SO/OVX groups yielded 87 protein spots (Fig. 1). Comparison of the differential protein expression between OVX and PVE groups showed that ten spots were down – regulated in OVX but up-regulated in PVE. The ten differential spots were separately named P1-P10 (Fig. 2). The individual intensity of each of the protein spots in the 2-D gel was shown in Fig. 3.

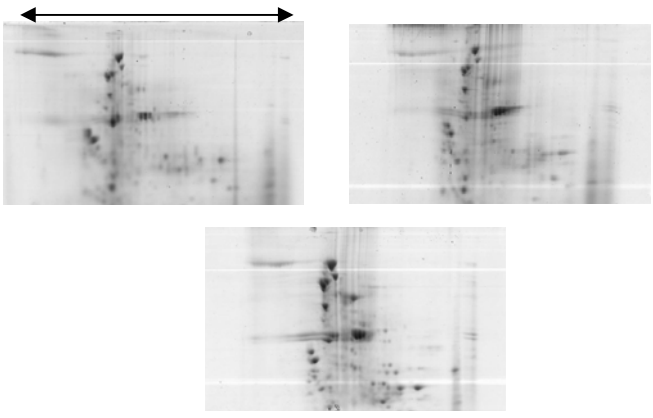

Fig. 1 2-DE gel map derived from the bone of the experimental rat; A, Sham-operated (SO), B, ovariectomized rats (OVX); C, ovariectomized rats supplemented with palm vitamin E (PVE)

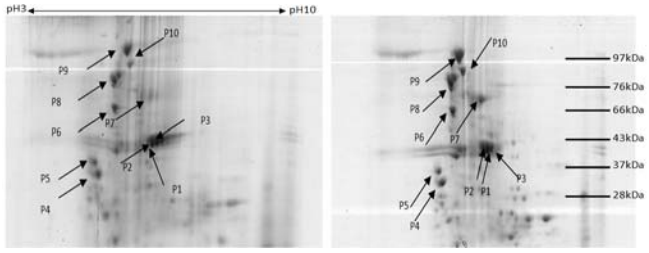

Fig. 2 2-DE gel maps derived from the bone of the experimental rat A, ovariectomized rats supplemented with olive (OVX); B, ovariectomized rats supplemented with palm vitamin E (PVE)

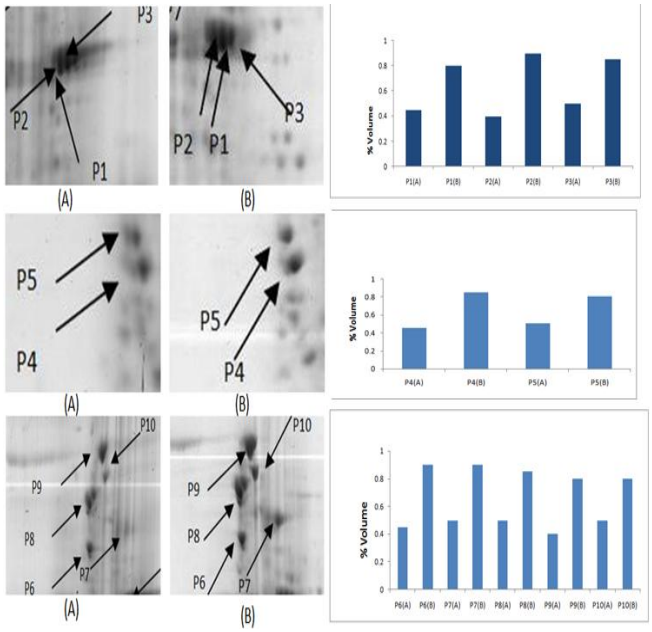

Fig. 3 Segments of the 2-DE gel map derived from OVX (A) and PVE (B).Zoom-in map of the proteins with conspicuous variance in 2-D maps. Enlarged regions of the respective for ten (10) differential proteins (P1-P10) indicated by arrows in this figure show significant differences in abundance between ovariectomized rats (OVX) and ovariectomized rats supplemented with tocotrienol (PVE) groups. The graphs show the relative abundance of the altered proteins between the two gels. The scales of the graphs are in arbitrary units. A: OVX; B: PVE

IV. Discussion

Postmenopausal women undergo changes due to low estrogen levels and are thus exposed to osteoporosis. Ovariectomy in rats induced changes similar to those seen in women with menopause [22]. Bone loss in rats and human share many similar characteristics such as greater loss of cancellous than cortical bone. Since bone changes in ovariectomised rats are similar to those in post-menopausal women, the ovariectomised rat is a suitable model for postmenopausal bone loss [23]. This was demonstrated in a study in which the rats began to show reduction in bone mineral density 2 months after ovariectomy in lumbar vertebrae and distal femur, which are rich in cancellous bone [24]. Physiological levels of 17α-estradiol have been shown to protect human low density lipoprotein against oxidation [25], while low estrogen levels in postmenopausal women has been associated with oxidative stress [26,27]. Estrogen can also be considered an antioxidant as it was found to exhibit antioxidant protection of lipoprotein in aqueous system [28] and was also shown to increase the expression of glutathione peroxidase in osteoclasts [29]. Glutathione peroxidase is an enzyme which is responsible for degradation of hydrogen peroxide. Estrogen deficiency reduces the enzymes and renders the bone susceptible to hydrogen peroxide attacks.

This explained why vitamin E, an antioxidant, was able to offer protection against osteoporosis in ovariectomised-induced osteoporotic model [15]. It has been reported that vitamin E may protect bone by interrupting the free radicals chain reaction and preventing damage due to lipid peroxidation [17-19]. Free radicals can activate transcription factor NF-kB to produce bone resorbing cytokines IL-1 and IL-6. Other studies demonstrated a reduction in the levels of bone resorbing cytokines when supplemented with vitamin E [30, 31]. It was suggested that 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 [32]. Vitamin E was found to increase the activities of antioxidant enzymes glutathione peroxidase, superoxide dismutase and suppressed MDA level in the bone [33]. These studies suggested that tocotrienol protects the bone against osteoporosis based on its antioxidant properties.

Molecular mechanism underlying the anti-osteoporosis effect of tocotrienol could provide new therapeutic strategies to the treatment of osteoporosis. A study found that there were changes in bone proteins expression in ovariectomised rats. The proteins were overexpressed in the ovariectomised rats as opposed to the control rats. The researchers suggested that overexpression of the proteins might be the molecular mechanism of osteoporosis in response to loss of estrogen [20]. In this present study, proteomics methodologies were used to characterize differential expressed proteins between the sham-operated rats (SO), ovariectomised rats (OVX) and ovariectomised treated with palm vitamin E rats (PVE). Proteomics, a new post – genomics field that encompasses protein expression and function in a cell or tissue, is a promising approach for better understanding the biology of organisms including human. Since an organism's proteome depends dynamically on a particular state of that organism, proteomic studies may reveal pathologic state of disease, including the amounts of proteins present and sub-cellular localization of the proteins associations. The proteomic approach is therefore conceptually attractive because it directly elucidates protein expression, accumulation, and degradation profiles in tissue [34].

The results of this study confirmed that there are indeed changes in bone proteins expression. Proteins were over-expressed in the ovariectomised rats as opposed to control sham-operated rats. These protein expression changes might be the molecular mechanism of osteoporosis in response to loss of estrogen. This report is similar to findings by others

[20, 35]. According to previous study, the protein expression change in osteoporosis is due to estrogen deficiency which increases the level of osteoclastogenic cytokines such as IL-1, IL-6 and TNF-α. This will stimulates COX-II expression which is followed by production of PGE2. TNF-α in cooperation with PGE2 increases RANKL expression which is in favour of bone loss.

In bone, both IL-6 and IL-11 are under influence of systemic bone metabolism regulating hormones such as estrogen, parathyroid hormones (PTH), vitamin D and thyroxine [9]. IL-6 is produced by osteoblasts, monocytes, adipocytes, endothelial cells, fibroblasts and T cells and acts as a promoter and activator of osteoclast differentiation, thus accelerating bone remodeling [36, 37, 5] IL-6 can also be considered as predictor of bone loss in postmenopausal women [38]. IL-6 through costimulation controls the production of monocytes, chemotactic proteins, and adhesion molecules. All these substances finally cause the release of inflammatory cytokines such as TNF-α and IL-1β [39].

Estrogen inhibits IL-6 synthesis by osteoblast and antagonizes the IL-6 receptors [40]. The overexpression of proteins in the estrogen deficient ovariectomised rats as observed in this study , may be due to the activities of released inflammatory cytokines such as TNF-α and IL-1β , since there is no estrogen to antagonize their effects. Ten protein spots were obviously down-regulated in the OVX, but up-regulated in PVE group. The down-regulated protein spots in the OVX may be due to the release of the inflammatory mediators such as TNF-α, IL-1β, IL-6, IL-1, COX-II, PGE2 and RANKL which favors bone loss [41, 42, 43]. The up-regulation of protein spots in PVE might be due to the inhibition of these mediators through its antioxidant activity and revival of the antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and suppressed MDA level in the bone [17, 18, 19, 30, 31, 32, 33]. Tocotrienol suppresses COX-II expression in RAW 264.7 cells (exposed to LPS) [44] and inhibits HMG CoA -reductase activity [45], the rate limiting enzyme in the mevalonate pathway which is essential for osteoclasts differentiation. The suppression of these inflammatory mediators and stimulation of these antioxidant enzymes might have enhanced the up-regulation of the enzymes that was down-regulated in the OVX. Identification of these proteins spot (P1-P10) of interest will help to unravel the molecular mechanism of the anti-osteoporotic effect of palm vitamin E. More research is ongoing to identify the protein spot P1-P10.

V. Conclusion

Palm vitamin E may exert its bone protective effects in ovariectomized rats via changes in protein expression.

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