Protective Effects of Palm Tocotrienol Against Glucocorticoid Induced Osteoporosis via Regulation of Gene Expressions

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ABSTRAK

Pengambilan glukokortikoid jangka panjang mengaruh stres oksidatif yang mengakibatkan perubahan struktur dan kekuatan tulang. Minyak sawit kaya dengan tokotrienol yang merupakan sejenis antioksidan. Ia boleh digunakan bagi mencegah penyakit yang berkaitan dengan stres oksidatif. Kajian ini bertujuan untuk menentukan mekanisme kesan perlindungan tokotrienol sawit terhadap osteoporosis aruhan glukokortikoid. Sebanyak 32 ekor tikus Sprague-Dawley jantan telah digunakan bagi kajian ini. 16 ekor telah menjalani adrenalektomi dan diberi suntikan deksametason 120 g/kg/hari secara intramukular. Sebanyak lapan ekor tikus diberi suplementasi tokotrienol sawit 60 mg/kg/hari manakala 8 ekor lagi diberi makan vehikel minyak sawit olein 0.1 ml/kg/hari secara gavaj oral. Lapan ekor tikus menjalani pembedahan sham dan diberi vehikel minyak sawit olein 0.05 ml/kg/hari melalui suntikan intramuskular diberi makan vehikel minyak sawit olein 0.1 ml/kg/hari secara gavaj oral. Lapan ekor tikus dijadikan kumpulan kawalan asas yang dikorbankan tanpa diberikan apa-apa rawatan. Tikus-tikus tersebut dikorbankan selepas dua bulan menerima rawatan. Tulang femur kanan digunakan untuk menganalisa kekuatan biomekanikal dan histomorfometri tulang, manakala tulang femur kiri digunakan untuk menganalisa ekspresi gen-gen dan aktiviti enzim-enzim stress oksidatif. Hasil kajian menunjukkan rawatan glukokortikoid jangka panjang telah secara signifikan meningkatkan petanda resorpsi dan mengurangkan kekuatan dan struktur tulang. Ekspresi gen-gen yang berkaitan dengan osteoblast dan osteoklas menunjukkan peningatan kadar tukar ganti tulang. Suplementasi tokotrienol sawit telah dapat mengekalkan petanda serap semula tulang dan memelihara struktur dan kekuatan tulang. Ekspresi gen-gen juga telah menunjukkan pengurangan kadar tukar ganti tulang. Melalui hasil

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kajian ini dapat disimpulkan bahawa tokotrienol sawit berpotensi digunakan sebagai rawatan profilaksis terhadap osteoporosis aruhan glukokortikoid melalui mekanisme pengawal seliaan ekspresi gen-gen yang berkaitan dengan osteoblas dan osteoklas.

Kata kunci: antioksidan, biomekanikal, histomorfometri, tulang, trabekular

ABSTRACT

Long term glucocorticoids administration induces oxidative stress which leads to alteration of bone structure and strength. Palm oil is rich in tocotrienol, an antioxidant. It can be used for the prevention of oxidative stress related diseases. The main objective of this study was to determine the mechanism of palm tocotrienol in maintaining the bone structure and strength in glucocorticoid-induced osteoporosis. Thirty two adult male Sprague-Dawley rats, aged 3 months, weighing 300-320 g rats were used in this study. Sixteen rats undergone adrenalectomy and were administered with 120 g/kg/day intramuscular injection of dexamethasone. Eight rats were supplemented with oral palm tocotrienol 60 mg/kg/day (Adrx+Dex+PTT) and the other eight rats were given oral vehicle palm olein 0.1 ml/kg/day (Adrx+Dex). Eight rats underwent sham procedure and were given vehicle palm olein 0.05 ml/kg/day by intramuscularly and oral 0.1 ml/kg/day (Sham). The rats were euthanized after two months of treatments. Eight rats were euthanized after acclimatic action without receiving any treatment (Baseline). The right femurs were used for bone biomechanical strength and histomorphometry analysis while the left for gene expression and oxidative stress enzymes activities. The results indicated that long-term glucocorticoid treatment significantly increased bone resorption marker, CTX (6060.7 ± 410 pg/ml) and decreased bone structure and strength. Osteoblast and osteoclast related genes expressions indicated an increase in bone turnover. Supplementation of palm tocotrienol had maintained serum resorption (2619.4 ± 209 pg/ml) marker level and preserved bone structure and strength. Gene expression analysis showed decrease in bone resorption. The findings suggested that palm tocotrienol has potential benefits against glucocorticoid-induced osteoporosis by regulating osteoblast and osteoclast related gene expressions.

Keywords: antioxidant, biomechanical, bone, histomorphometry, trabecular

INTRODUCTION

Long term glucocorticoids treatment is one of the causes of osteoporosis. Glucocorticoids decrease bone formation and increase bone resorption (O'Brien et al. 2004; Kim et al. 2006). These result in the rapid loss of trabecular bone followed by slower loss of cortical bone. Bone

loss and fragility fractures are the most devastating adverse effects of glucocorticoid excess (Lukert 2001; Weinstein 2007; Canalis et al. 2007).

Reactive oxygen species (ROS) comprises hydrogen peroxide, hydroxyl radicals and superoxide. They are the derivatives of biological aerobic metabolism. ROS destroy and oxidize proteins, lipids and DNA, leading to alteration of cell function. ROS contributes to the pathogenesis of bone loss in osteoporosis (Arai et al. 2007; Schröder 2015; Filaire et al. 2012; Wauquier et al. 2009).Dexamethasone (DEX) induces the overproduction of ROS, which enhance osteoblast and osteocyte apoptosis through activation of autophagy and endoplasmic reticulum stress (Liu et al. 2018). It is through the activation of caspase family proteins expression (Chu et al. 2003). DEX inhibits the synthesis of fibronectin and collagen, and to activate collagenase synthesis. The action is mediated by the glucocorticoid receptor (GRs) (Reul et al. 1985).

Glucocorticoids therapy leads loss of osteocyte viability that causes osteonecrosis of the femoral neck or head of humerus (Angeli et al. 2006; Calder et al. 2004; Weinstein & Manolagas 2000). Apoptosis of the osteocyte causes cumulative and unrepairable defects and disruptions to the mechanosensory function of the osteocyte–canalicular network. These decrease the bone volume leading to collapse of the femoral head (Mankin 1992; Felson& Anderson 1987).

Glucocorticoids stabilize collagenase III mRNA and cause inhibition of the synthesis of type 1 collagen and β1 intergrin (Delany et al. 1995). Osteoblast functions are inhibited by glucocorticoids by decreasing its proliferation and terminal differentiation. Apart from promoting osteoblasts and mature osteocytesapoptosis, glucocorticoids alsomodify osteoblast specific gene, osteocalcin (O'Brien et al. 2004; Weinstein et al. 1998). Glucocorticoid reduces the total amount of bone replaced in each remodelling cycle due to it's negative effects on bone formation (Delany et al. 1995).

Bone resorption is determined by the balance between two cytokines, Receptor Activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG). (RANKL) is a cell surface receptor produced by osteoblast which plays an important role in the differentiation of osteoclasts. RANKL is inhibited by OPG, a protein which is also produced by osteoblasts (Shiotani et al 2002). Glucocorticoids influence the RANKL/OPG ratio by reducing OPG and increasing RANKL expression (Sivagurunathan et al. 2005). This increases osteoclastogenesis which causes rapid early phase of bone resorption. Glucocorticoids also enhance the priming of monocytes/ monocytes towards differentiation to mature osteoclasts. Osteoclasts are protected against the pro-apoptotic effect of glucocorticoids (Shiotani et al. 2002; Takuma et al. 2003). The Wnt signaling pathway modulation is also involved in the inhibition of skeletal growth by glucocorticoids (Ohnaka et al. 2005). Differentiation and functions of osteoblasts also involve the Wnt

signalling pathways (Raisz 2005).

Vitamin E is an antioxidant that plays a vital role in the endogenous defence against peroxidation of membrane lipids (Packer et al. 2001). Tocotrienol and tocopherol are the two types of vitamin E. They are further divided into isomers of α, β, δ and γ. Tocopherol has saturated bonds whereas tocotrienol has unsaturated bonds in the phytyl side chain. Tocotrienol has gained more scientific interest because of its antioxidative activities. Study by Musalmah et al. 2009 showed that tocotrienol rich fraction (TRF) was able to prevent DNA damage and cell apoptosis induced by oxidative stress. Palm tocotrienol was found to have protective effects on bone of ovariectomised rats by suppressing lipid peroxidation and inducing the superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities (Nazrun et al. 2008). Apart from that, it also exhibits anticancer, anticholesterol and antiplatelet properties. Its beneficial effects on bone metabolism has been reported (Naina Mohamed et al. 2012; Nazrun et al. 2012).

Differentiation and proliferation of osteoblast and osteoclast are regulated by cascades of genes (Bruzzaniti et al. 2006). Abukhadir et al. 2012 showed that expression of Runx2, Osterix, and bone morphogenetic protein-2 were significantly enhanced by palm tocotrienol supplementation in nicotine cessation osteopenia model. Annatto tocotrienol, a pure tocotrienol was found to enhance the expression of genes related to bone formation osteoblast activity which also inhibit the expression of genes related to bone resorption (Chin & Ima Nirwana 2014).

Earlier studies found that palm tocotrienol increased femur length, bone calcium content and bone mineral density (BMD) of rats with long term dexamethasone treatment (Ima Nirwana & Fakhrurazi 2002; Ima Nirwana & Suhainiza 2004). Bone with high BMD is assumed to be strong. However, this may not necessarily be true for glucocorticoid induced osteoporosis as the bone may be weak although it is dense and changes in the BMD could only be detected at a later stage. This leads to inaccuracy in the evaluation of any intervention.

These positive effects led us to further investigate the role of palm tococtrienol and its cellular mechanism in maintaining the structure and strength of bone treated with glucocorticoid. The antioxidant effect of palm tocotrienol is able to reduce the oxidative stress level induced by dexamethasone. Through the reduction of the ROS we hypothesized that the toxic effect to the osteoblasts and osteoclast could be reversed and bone formation would be maintained. The effect of ROS on osteoclast also was expected to be reversed by palm tocotrienol, whereby bone resorption would be reduced. With all these effects, the bone structure and strength could be maintained. Hence, the present study focussed on quantifying the structural histomorphometric parameters, biomechanical strength and the genes that are related to osteoblast and osteoclast activities.

MATERIALS AND METHODS

ANIMALS AND TREATMENT

All procedures were carried following the guidelines and ethical clearance was obtained from the Universiti Kebangsaan Malaysia UKM Research and Animal Ethics Committee (UKMAEC) with the number PP/ANAT/2011/FAIRUS & ELVY/19-MAY/375-JUNE-2011-JUNE- 2013

This study used 32 Sprague-Dawley rats (3 month-old) weighing 280-300 g. The rats were supplied by the Universiti Kebangsaan Malaysia (UKM) Animal Breeding Centre. Adrenalectomy was performed to 16 rats while the other 8 rats underwent sham procedure. Remaining 8 rats were euthanized after acclamitization without receiving any treatment. Prior to all surgical procedures, the rats were anaesthetized with a mixture of Ketapex and Xylazil (Troy Laboratories, Australia) at 1:1 ratio of 0.1 ml/kg dose. The adrenals were visualised via the dorsal midline skin and bilateral flank muscle incisions. The vessels were ligated to secure the bleeding before removing the glands. The incisions were sutured with absorbable suture and the wounds were cleaned with normal saline. The wounds were cleaned daily to to aid wound healing. Intramuscular injection of Baytril 5% was given (Bayer Health Care, Thailand) for 5 days as to prevent infection. Similar procedures were applied to the sham-operated rats except that the adrenal glands were left *in-situ.*

The adrenalectomized rats were divided randomly into two groups which consisted of 8 rats. The treatment groups were divided as follows: SHAM: sham operated group, consisting of 8 rats. They were given intramuscular (IM) injection of vehicle palm olein 0.05 ml/100 g and 0.1 ml/100 g orally. The other group was Adrx+Dex: 8 adrenalectomized rats which were administered with IM dexamethasone 120 g/kg/day and oral palm tocotrienol (Sime Darby, Malaysia) 0.1 ml/100 g. Another group was Adrx+Dex+TT: 8 adrenalectomized rats which were administered with IM dexamethasone 120 g/kg/day and palm tocotrienol 60 mg/kg/day by orally and the baseline group in which rats were euthanized after acclamitization without receiving any treatment. The dose and duration of treatment of Dexamethasone to cause osteoporosis was determined by a previous study (Elvy Suhana et al. 2011). Palm olein (Sime Darby, Malaysia) was used as the vehicle for dexamethasone (Sigma, USA) and palm tocotrienol. All the treatment were given for 6 days a week. Calcein was injected at the dose of 20 mg/kg nine days and two days before the rats were euthanised. The rats were euthanized after completion of 2 months of treatment.

The animals were kept in clean cages in a room with temperature maintained at 25°C under natural sunlight and darkness at night. They were fed with rat pellets (Gold Coin, Malaysia) *ad libitium.* In order to replace the salt loss due to mineralocorticoid deficiency,the adrenalectomized animals were given normal saline to drink *ad libitium* post-adrenalectomy while the sham rats were given tap water. After completion of 2 months

of treatment, the animals rats were euthanized under anaesthesia, mixture of Ketapex and Xylazil which were given in a high dose.Palm tocotrienol used was Gold-Tri E™ 50 consisted of alpha-tocopherol 124 mg/g, alpha tocotrienol 147.5 mg/g, beta-tocotrienol 22.2 mg/g, gamma tocotrienol 173 mg/g and delta tocotrienol 89.9 mg/g.

At the end of two months of treatment, serum CTX and osteocalcin, bone biomechanical strength, gene expression analysis, bone histomorphometry and oxidative stress enzymes activities were measured and analyzed.

SAMPLE COLLECTION

Blood was taken prior to the treatment and before the animals were euthanized. The serum was collected by centrifuging the blood at 3000 rpm at 4℃ for 15 minutes. The serum was kept at -80℃ until it was used for measuments of osteocalcin and Cross Linked C-Telopeptide of Type 1 (CTX).

Both femurs were harvested from each rat and the right femurs were wrapped in gauze soaked with phosphate buffer saline (PBS) and frozen at -80℃. The left femurs were cut at the mid shaft with a rotary blade (Black & Decker). The distal parts of the femurs were cut longitudinally into two halves. The medial halves were used for quantification of gene expressions and the lateral halves for the measuring lipid peroxidation and the oxidative stress enzymes activities.

MEASUREMENT OF SERUM BONE BIOCHEMICAL MARKERS

ELISA technique was used for measuring serum bone biochemical markers measured using Cross Linked C-Telopeptide of Type 1 Collagen Kit (Uscn Lifescience Inc. Wuhan, China) was used to measure serum CTX and ELISA kit (Immunodiagnostic Systems Limited, UK) for serum osteocalcin.

BONE BIOMECHANICAL TEST

Instron Universal Testing Machine (model 5560, Instron, Canton, MA, USA) equipped with Bluehill 2 software (Instron, Canton, MA, USA) was used to measure the biomechanical properties of the femurs. Each femurs were placed on two lower supports that are 5 mm apart in the three-point bending configuration (Haffa et al. 2000). The force was applied at the mid-diaphysis on the anterior surface of the femur so that the anterior surfaces were in compression and posterior surfaces were in tension until it fractured. The load, displacement stress and strain parameters were recorded by the software. Graphs of load against displacement and stress against strain were plotted. The modulus of elasticity of the femur was represented by the slope-value of the load-displacement curve. Bone biomechanical test main parameters were divided into extrinsic (load, energy and extension) which measured the properties of whole bone and the intrinsic parameters (stress, strain and Young's modulus) which measured material of the bone (Turner 2002).

BONE HISTOMORPHOMETRY ANALYSIS

Undecalcified bone samples were used for analysis of the structural parameters of bone histomorphometry. This consists of trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). The medial half of the left femurs were embedded in a mixture of Osteo Bed Resin Solution A (Polysciences Inc., PA, Germany) with Benzoyl Peroxide Plasticized (Catalyst) (Polysciences Inc., PA, Germany) in the ratio of 100 ml of Osteo Bed Resin Solution A to 3.5g of Benzoyl Peroxide Plasticized (Catalyst). The samples were sectioned at 7m thickness using a microtome (Leica RM2155, Nussloch, Germany). The histological sections were stained with von Kossa method and analyzed using an image analyzer (Leica DMRXA2, Wetzlar, Germany) equipped with VideoTest-Master software (VT, St. Petersburg, Russia).

Decalcified bone samples were used for the analysis static parameters. The right medial halves of the femurs were decalcified using ethylene diamine tetraacetic acid (EDTA) for 10 weeks and embedded in paraffin wax. The bone samples were sectioned with a microtome (Leica, Wetzlar, Germany) at 5 m thickness and were stained with haematoxylin and eosin staining method. Photomicrographs of the histological sections were taken at the using a microscope (Nikon Eclipse 80i, Chiyoda, Japan) which was connected to an image analyzer (Media Cybernetics Image Pro-Plus, Rockville, MD, USA) at 200 times magnification. Calculations of the osteoblast, osteoclast number, bone volume and

bone surfaces were performed by a blinded examiner using Weibel Grid technique. The parameters measured were osteoblast surface (Ob.S/BS) and osteoclast surface (Oc.S/BS).

Dynamic parameters were measured using the technique of double fluorescent labeling. The rats were injected with calcein (20 mg/kg body weight) at 9 and 2 days before the rats were euthanized to obtain the fluorescent labelling on the trabecular surfaces. This was done for the assessment of bone formation rate (BFR), mineralized surface (MS/BS) and mineral appositional rat (MAR) within a 7 day interval from undecalcified femurs. Micrographs were taken using fluorescent camera (Nikon, Tokyo, Japan) which was connected to an image analyzer (Eclipse 80i, Nikon) equipped with Pro Plus 5.0 software (Media Cybernatics, Silver Spring,MD). The parameters were derived from single labeled surface (sLS/BS) and double labeled surface (dLS/BS) which was also analyzed using Weibel Grid technique.

Histomorphometric analysis was performed randomly at the secondary spongiosa area, rich in trabecular bone. It is located 3-7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex, excluding the endocortical region. The parameters were measured following to the guidelines set by the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee (1987) (Parfitt 1987).

GENE EXPRESSION MEASUREMENTS

Trabecular bone tissue was taken from the distal part of left femurs. The tissue used was about the size of a rice grain (~5 mg). Tissue from each bone was placed into a vial containing magnatic beads. One vial was used for each sample. An amount of 300 µl of Homogenising Solution (provided in the QG Sample Preparation Kit for Tissue) + 3 µl of Proteinase K (provided in the QG Sample Preparation Kit for Tissue) were added into vial. The vials were placed into Cell Rupture equipment for 15-30 seconds, 2 cycles followed by a quick spin for 5 seconds. The vials were then incubated in a water bath at 65°C for 30-60 minutes followed by centrifuging them for 10 minutes at room temperatureat 13,000 rpm. The homogenate/supernatant was transferred into a microcentrifuge tube and stored at -80°C until used. The kits used for this procedure were supplied by Affymatrix Thermo Fisher Scientific, USA.

Genes expression were quantified using the multiplex Quantiene assays for quantification of DNA or RNA targets directly from a variety of sample types. The procedure was done following the manual given by Affimatrix Quantigene 2.0 Plex Assay. The QuantiGene Plex assay combines branched DNA (bDNA) signal amplification and multi-analyteprofiling beads (xMAP®) technologies to enable the detection and quantitation of multiple RNA targets simultaneously. The bDNA assay is a hybridization-based method of target-specific RNA quantitation that amplifies signal rather than target RNA, using labeled DNA probes.

The QuantiGene Plex assay utilizes fluorescent microspheres (Capture Beads) as a support to capture. Oligonucleotide probe sets used were designed by the manufacturer. Luminex® instrument (Bio-Rad, Hercules, CA, USA) was used to measure the luminescence, and the mean fluorescence intensity specific for each gene (proportional to the mRNA captured by the bead) was generated. Expression of each gene was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase.

LIPID PEROXIDATION AND OXIDATIVE STRESS ENZYMES

Measurements of the oxidative stress enzymes activities and lipid peroxidation were accomplished using bone homogenate which was obtained from the trabecular bone of the distal part of the femurs. The procedures were done following the instructions provided by the manufacturers of the kits. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities were measured by using ELISA technique using the kits supplied by Cayman Chemical Company, USA. Lipid peroxidation activity was quantified by measuring malondialdehyde (MDA) level using (BioVision Incorporated USA) ELISA kit.

STATISTICAL ANALYSIS

Statitical analysis was accomplish using the Statistical Package for Social Sciences (SPSS) version 20.1.2.

Distribution of the data was analyzed using the Kolmogrov-Smirnov test. All the data was normally distributed and parametric statistic was used for comparison between each group; i.e. the ANOVA test followed by the Tukey pos-hoc test. P values < 0.05 were taken as significant. Data were presented as mean ± standard error of the mean (SEM).

RESULTS

SERUM BIOCHEMICAL MARKERS

There were no significant differences in the osteocalcin level in the Adrx+Dex group compared to the Sham group (p>0.05). There was no significant changes to the osteocalcin level seen in the Adrx+Dex+TT group. Osteocalcin levels of Adrx+Dex+TT and Sham group were not significantly different (p>0.05) (Figure 1a).

Dexamethasone treatment (Adrx+Dex) showed a significantly higher CTX level compared to the Sham group (p<0.05). Supplementation of palm tocotrienol (Adrx+Dex+TT) had significantly lowered the CTX level (p<0.05). CTX level of Adrx+Dex+TT and Sham group were not significantly different (Figure 1b).

BONE HISTOMORPHOMETRY

Figure 1: Serum osteocalcin and Cross Linked C-Telopeptide of Type 1 Collagen (CTX). Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. (Baseline=baseline control; SHAM=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 μg/kg/day (Dex); ADRX+Dex+TT=adrx given intramuscular DEX 120 μg/kg/day and oral palm tocotrienol 60 mg/kg/day)
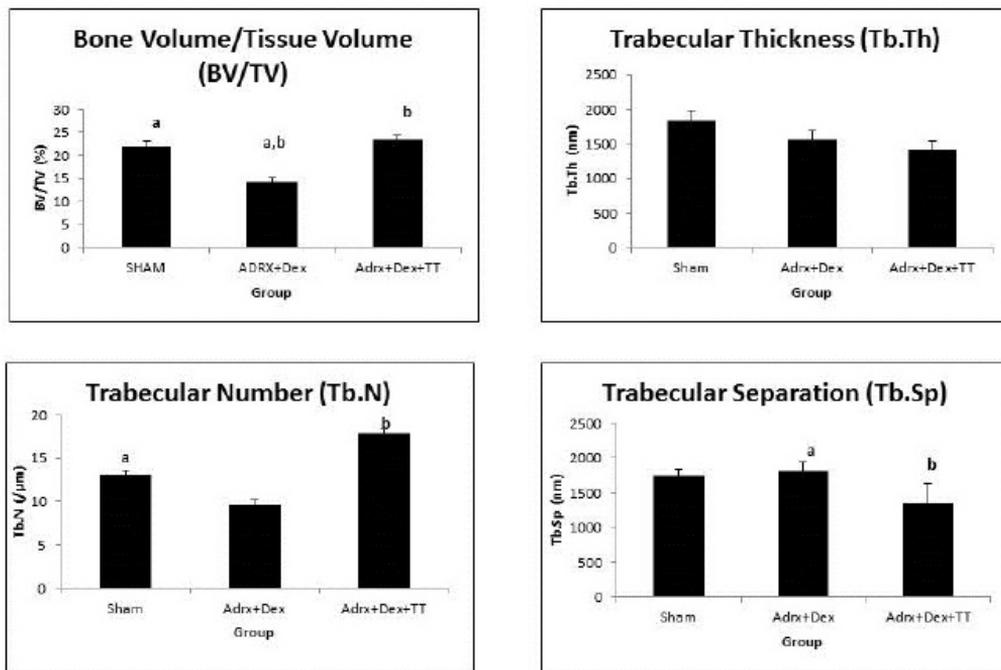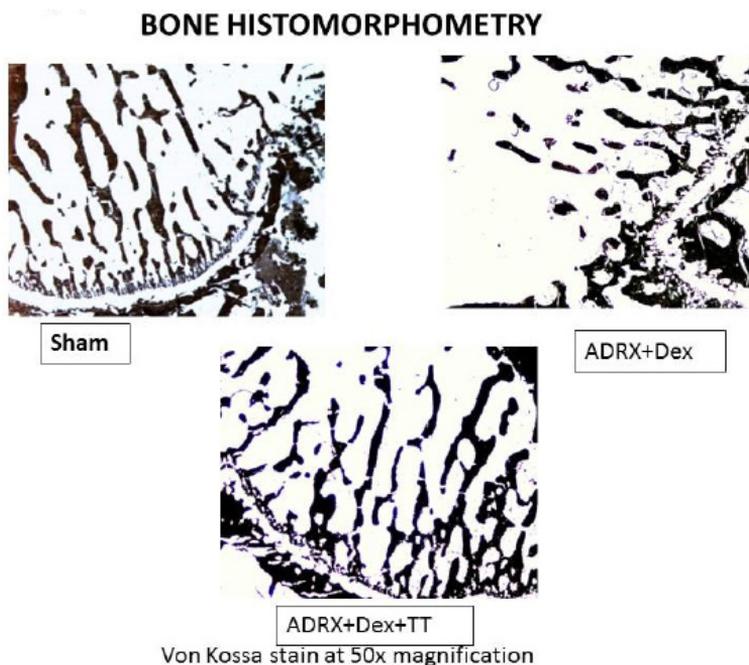
Figure 2: Structural histomorphometric parameters. Data presented as mean + SEM. Same alphabets indicate significant difference between treatmet groups at p<0.05. SHAM=sham operated control; Adrx+Dex=adrenalectomized (Adrx) and given intramuscular dexamethasone 120 µg/kg/day (Dex); Adrx+Dex+TT=adrx and given intramuscular DEX 120 µg/kg/day and oral palm tocotrienols 60 mg/kg/day.

The Adrx+Dex rats had signicantly lower Bone Volume/Tissue Volume (BV/TV) (Figure 2a), and Trabecular Number (Tb.N) (Figure 2b) (p<0.05), compared to the Sham rats but the Trabecular Thickness (Tb.Th) (Figure 2c) and the Trabecular Separation (Tb.Sp) did not change significantly (p>0.05) (Figure 2d). The rats supplemented with palm tocotrienol 60 mg/kg/day (Adrx+Dex+TT) had significantly higher BV/TV and Tb.N and the Tb.Sp was significantly lower compared to the Adrx+Dex group (p<0.05). The BV/TV, Tb.N and Tb.Sp of the Adrx+Dex+TT group were not significantly different compared to the Sham group. However, supplementation of palm

tocotrienol n did not significantly change the Tb.Th. The changes are illustrated in the Photomicrograph 1.

Cellular bone histomorphometric analysis showed that the Adrx+Dex rats had significantly lower osteoblasts surface (Ob.S/BS) (p<0.05) compared to the Sham group with osteoclast surface (Oc.S/BS) did not change significantly (p>0.05). Ob.S/BS of the Adrx+Dex+TT rats was significantly higher compared to the Adrx+Dex rats (p<0.05). Palm tocotrienol supplementation had also resulted in a significant decrease to the Oc.S/BS (p<0.05). The Ob.S/BS and the Oc.S/BS of the Adrx+Dex+TT group and the Sham group were not significanly

BONE HISTOMORPHOMETRY

Photomicrograph 1: Von Kossa stain of undecalcified bone at 25x magnification. SHAM=sham operated control; Adrx+Dex=adrenalectomized and given intramuscular dexamethasone 120 µg/kg/day; Adrx+Dex+TT=adrx and given intramuscular Dexamethasone 120 µg/kg/day and oral palm tocotrienols 60 mg/kg/day.

different ($p>0.05$) (Figure 3a and 3b). The changes are illustrated in the in Photomicrograph 2.

Two months dexamethasone treatment also caused significant changes to dynamic parameters of bone histomorphometry. Bone mineralized surface (MS/BS), mineral apposition rate (MAR) and bone formation rate (BFR) of the Adrx+Dex rats were significantly lower compared to the Sham group ($p<0.05$). Supplementation of palm tocotrienol resulted in higher MS/BS, MAR and BFR in the Adrx+Dex+TT rats compared to the Adrx+Dex group ($p<0.05$). There were no significant difference in the MS/BS, MAR and BFR of the Adrx+Dex+TT group compared to the Sham group (Fig. 4a, 4b and

4c). The changes are illustrated in Photomicrograph 3.

BONE BIOMECHANICAL STRENGTH

Both the intrinsic (Young's modulus, stress and strain) (Figure 5a, 5b, 5c), and extrinsic properties (energy, load, and flexure extension) (Figure 6a, 6b, 6c) of bone biomechanical strength of the Adrx+Dex group were significantly reduced compared to the Sham ($p<0.05$). The rats supplemented with palm tocotrienol (Adrx+Dex+TT) had significantly higher values of intrinsic (Young's modulus, stress and strain) and extrinsic parameters (energy and maximum load) compared to the
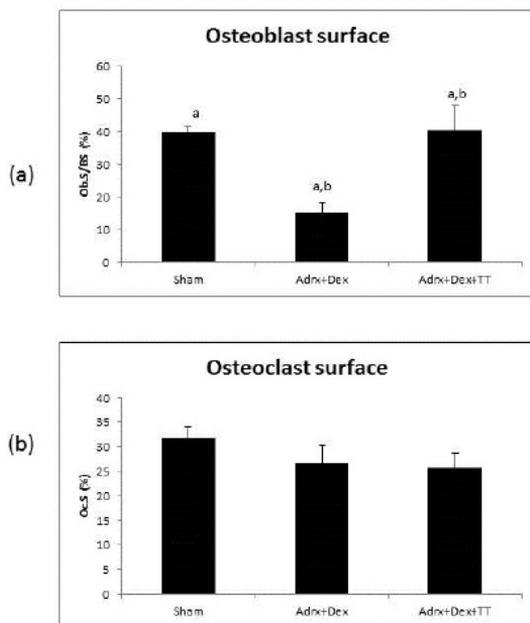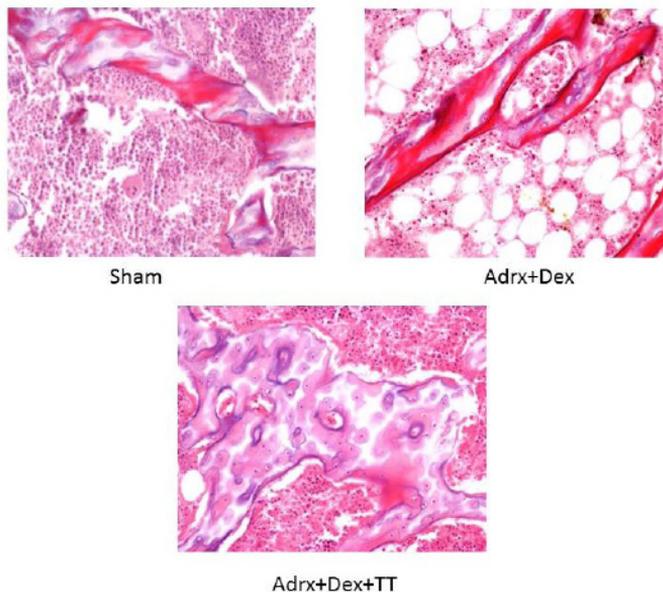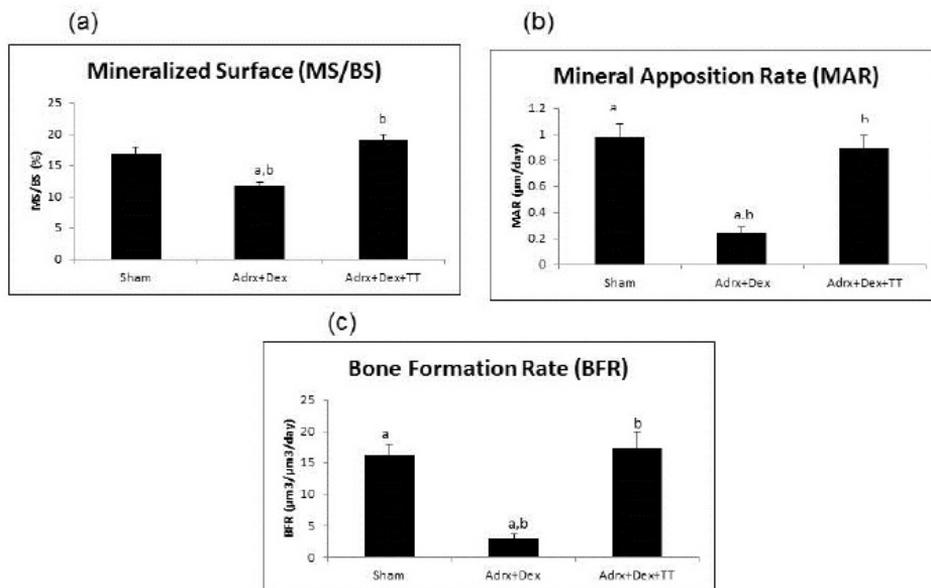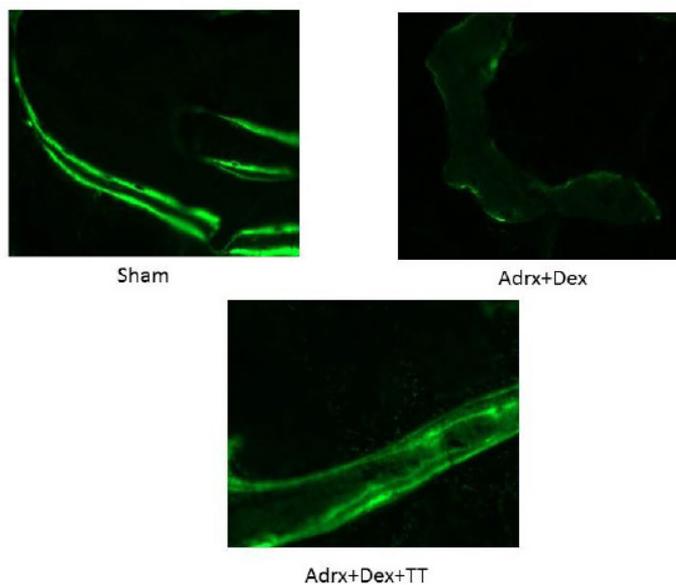
Figure 3: Static histomorphometry parameters. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. SHAM=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 μg/kg/day; Adrx+Dex+TT=adrx and given intramuscular DEX 120 μg/kg/day and oral palm tocotrienols 60 mg/ kg/day

Photomicrograph 2: Haematoxylin & eosin stain of decalcified bone at 200x magnification. Baseline=no intervention; SHAM=sham operated control; Adrx+Dex=adrenalectomized and given intramuscular dexamethasone 120 μg/kg/day; Adrx+Dex+TT=adrx and given intramuscular Dexamethasone 120 μg/kg/day and oral palm tocotrienols 60 mg/kg/day

Figure 4: Dynamic histomorphometry parameters. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. SHAM=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 μg/kg/day; Adrx+Dex+TT=adrx and given intramuscular DEX 120 μg/kg/day and oral palm tocotrienols 60 mg/kg/day.

Photomicrograph 3: Photomicrographs shows calcein labels along trabecular bone, demonstrated using fluorescence microscopy in undecalcified bone sections without staining at 200x magnification. SHAM=sham operated control; Adrx+Dex=adrenalectomized and given intramuscular dexamethasone 120 μg/kg/day; Adrx +Dex+TT=adrx and given intramuscular Dexamethasone 120 μg/kg/day and oral palm tocotrienols 60 mg/kg/day.
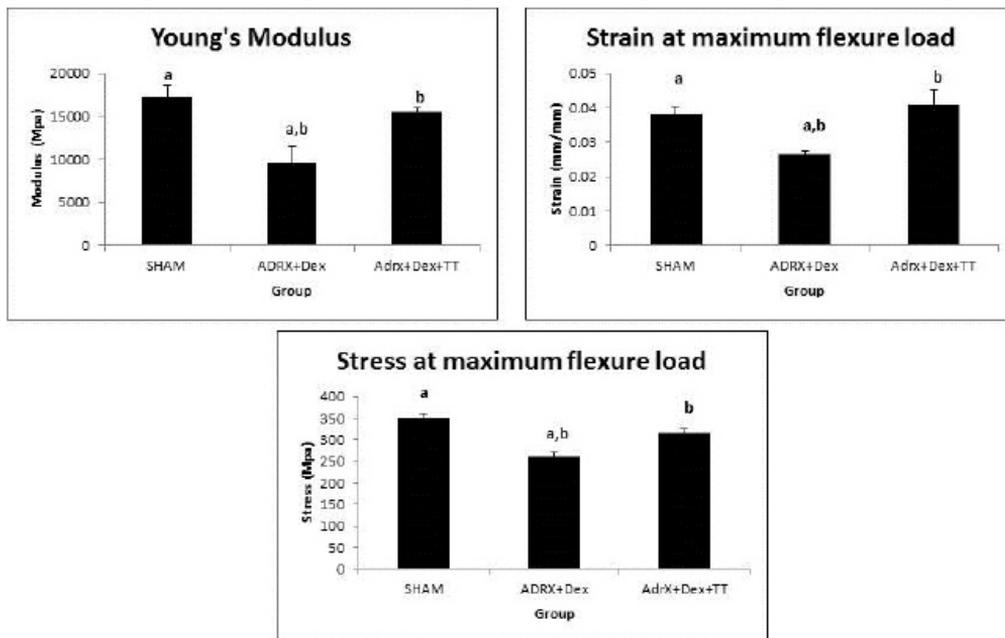
Figure 5: Intrinsic parameters of bone biomechanical strength. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. SHAM=sham operated control; Adrx+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); Adrx+Dex+TT=adrx and given intramuscular DEX 120 µg/kg/day and oral palm tocotrienols 60 mg/kg/day.

Adrx+Dex (p<0.05). However, palm tocotrienol supplementation did not give significant effect to themaximum flexure extension (p>0.05) (Figure 6a, 6b and 6c).

BONE FORMATION RELATED GENE EXPRESSIONS

The osteocalcin (bglap) and collagen 1 alpha 1 (col1a1) gene expressions were increased and the expression of integrin binding sialoprotein (ibsp) and osterix (Sp7) were decreased significantly in the Adrx+Dex group compared to the sham group (p<0.05). Supplementing the dexamethasone treated rats with palm tocotrienol (Adrx+Dex+TT) had maintained the osteocalcin and

col1a1 gene expressions at the similar level with the sham group and it was significantly lower compared the Adrx+Dex group (p<0.05). Palm tocotrienol supplementation (Adrx+Dex+TT) also caused a significant increase in the osterix gene expression compared to the Adrx+Dex group (p<0.05). However there were no significant effect to the expression of ibsp gene (Figure 7a, 7b,7c and 7d).

BONE RESORPTION RELATED GENES EXPRESSIONS

The bone resorption related genes expression; cathepsin K (ctsk), osteopontin (spp1), integrin alpha 5 beta 3 (itgb3) and RANKL/OPG
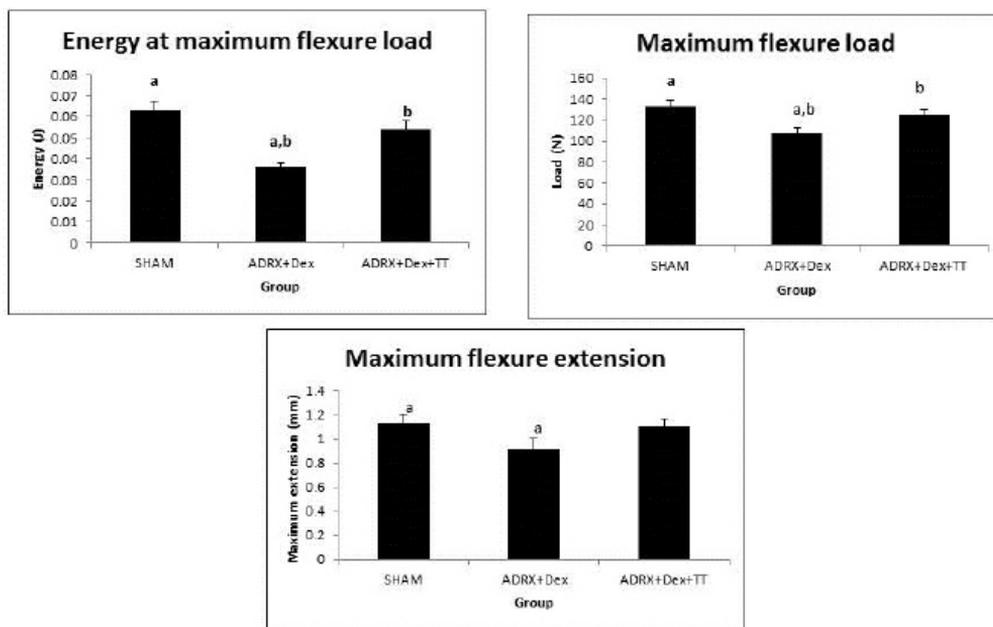
Figure 6: Extrinsic parameters of bone biomechanical strength. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. SHAM=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); Adrx+Dex+TT=adrx and given intramuscular DEX 120 µg/kg/day and oral palm tocotrienols 60 mg/kg/day.

(tnsfs11/tnrsf11b) ratio of the rats treated with dexamethasone were significantly increased in the Adrx+Dex group compared to the Sham group. Palm tocotrienol supplementation (Adrx+Dex+TT) had significantly maintained the expression of cathepsin K, osteopontin and integrin alpha 5 beta 3 (p<0.05). There was no significant changes to the RANKL/OPG ratio caused by the supplementation of palm tocotrienol (p>0.05) (Figure 8a, 8b, 8c and 8d).

OXIDATIVE STRESS

Dexamethasone treated rats (Adrx+Dex) had significantly lower superoxide dismutase (SOD) and higher glutathione peroxidase (GPX) activities (p<0.05). However, catalase (CAT) activity was not significantly different compared to the Sham group. The higher malondialdehyde (MDA) level in the Adrx+Dex group indicated an increase in the lipid peroxidation caused by dexamethasone administration. Palm tocotrienol (Adrx+Dex+TT) had significantly increased the SOD activity and decreased the GPX activities compared to the ADRX+Dex group (p<0.05). The SOD and GPX level was not significantly different compared to the Sham group. CAT activity was not significantly different in rats supplemented with palm tocotrienol compared to Adrx+Dex group. Palm tocotrienol had also significantly reduced the MDA level compared to the Adrx+Dex group and it was not
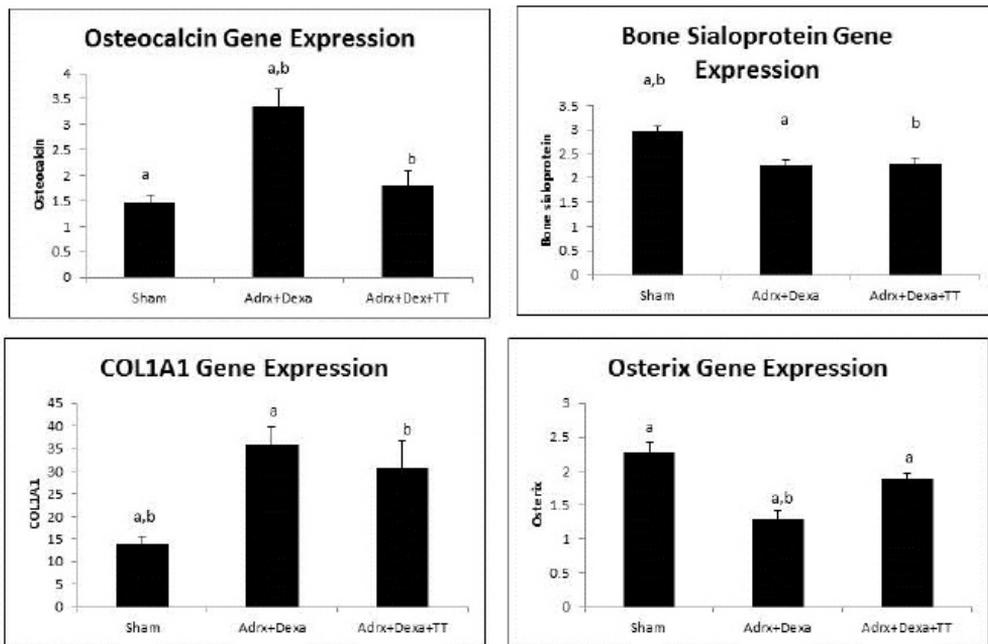
Figure 7: Expression of genes related to bone formation. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. SHAM=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); Adrx+Dex+TT=adrx and given intramuscular DEX 120 µg/kg/day and oral palm tocotrienols 60 mg/kg/day

significantly different compared to the Sham group (Figure 9a, 9b, 9c and 9d).

DISCUSSION

The present study showed that two months dexamethasone treatment had significantly increased the serum resorption marker, *carboxy-terminal collagen cross links* (CTX) but not the formation marker, osteocalcin. This may have caused an imbalance to the bone remodelling process as the total amount of bone replaced in each remodelling cycle was not sufficient. This led to the disruption of bone structure and strength (Delany et al. 1995). Dexamethasone treatment for two months resulted in bones with

reduced BV/TV and Tb.N, that were thinner with decrease in Tb.Th. Loss of bone structure was due the resorption cavities that were not fully restored due to inadequate bone formation. Decreased in bone formation due to long term dexamethasone treatment in this study was evidenced by the significant decrease in the Ob.S/BS, MS/BS, MAR and BFR even though the decrease in serum osteocalcin was not significant. There was significant increase in the bone resorption as indicated by the CTX level as there was also increase in the Oc.S/BS even though the value did not reach significant level. Osteoporosis due to glucocorticoid therapy reflects the failure to fully restore the resorbed
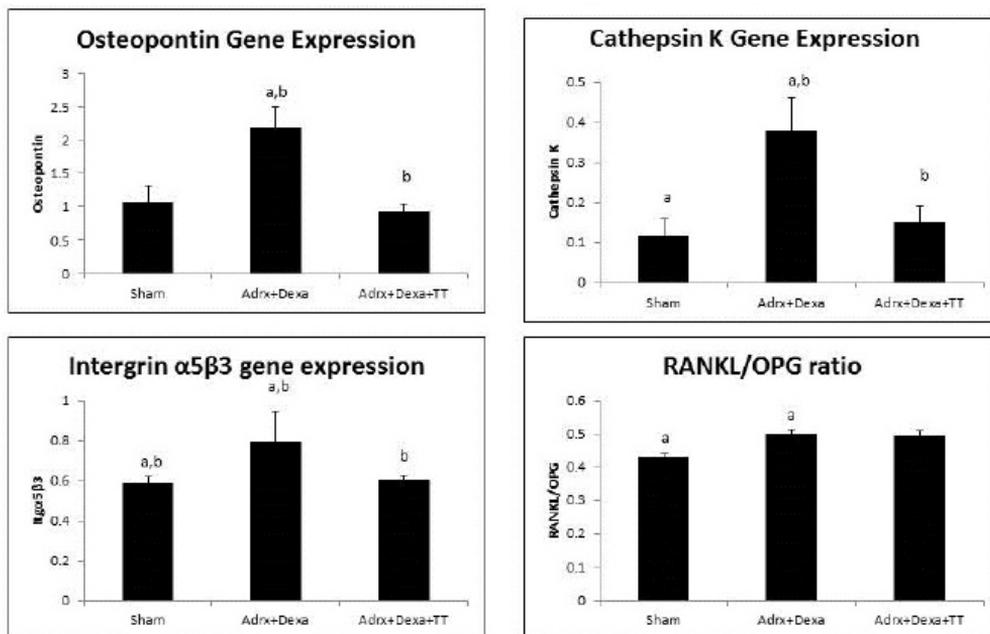
Figure 8: Expression of genes related to bone resorption. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. SHAM=sham operated control; Adrx+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); Adrx+Dex+TT=adrx and given intramuscular DEX 120 µg/kg/day and oral palm tocotrienols 60 mg/kg/day

bone in the remodelling sites (Delany et al. 1995).

The results of this study showed that dexamethasone administration had increased the lipid peroxidation and decreased the SOD enzyme activity in the bone. This may have exposed the bones to high oxidative stress level. Oxidative stress has inhibitory effect and are toxic to osteoblast and this explained the significant reduction in the Ob.S/BS. Oxidative stress has protective effects to osteoclast and this was proven in this study where the Oc.S/BS was not affected with dexamethasone treatment. Osteoclast number did not fall because of their life span was prolonged due to the attenuation of osteoclast apoptosis Weinstein et al. 2002).

Superoxide generated by osteoclasts also contributes directly to bone degradation (Ries et al. 1992).

Our results showed that dexamethasone had also caused reduction in the osteoblast related gene expressions, IBSP and osterix (sp7). However, osteocalcin (bglap) and Col1 1 gene expressions were significantly increased. The decrease in the osteoblast related gene expressions may be due to the toxic effect of oxidative stress causing the apoptosis of osteoblasts and osteocytes which had decreased the number of mature osteoblasts (Canalis et al. 2004; Weinstein 2010). Dexamethasone treated patients also showed a decrease in serum osteocalcin and carboxyterminal propeptide of type
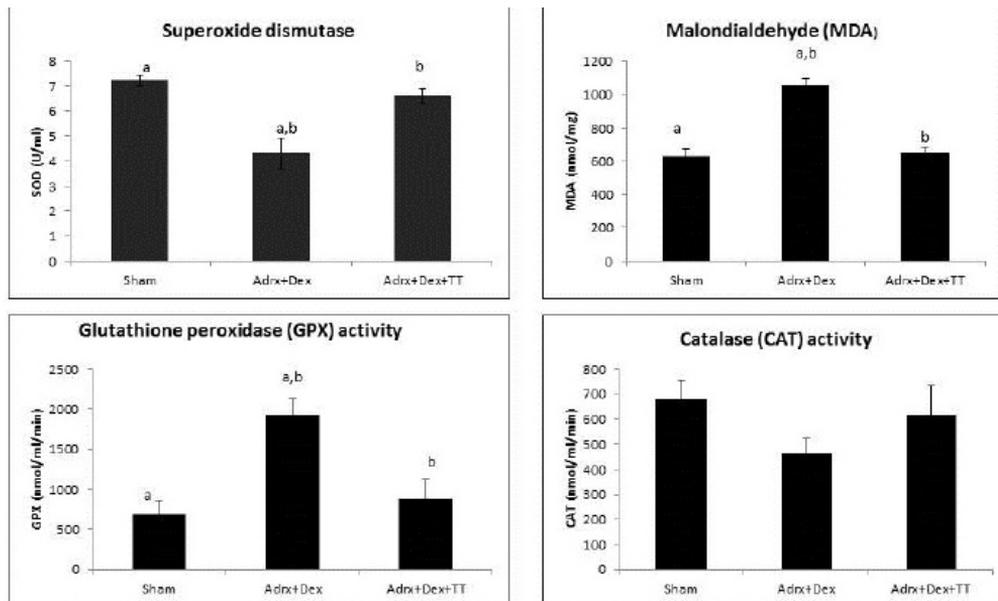
Figure 9: Lipid proxidation, superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) activities in the bone. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05. SHAM=sham operated control; ADRX+Dex =adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+TT=adrx and given intramuscular DEX 120 µg/kg/day and oral palm tocotrienols 60 mg/kg/day

1 procollagen (Lems et al. 1998). Osteocalcin and COL1 1 genes expression were stimulated due to the response to the increase in bone resorption. Kim et al. 2006 stated that osteoclastic bone resorption in some manner promotes bone formation.

Expression of the osteoclast related genes, osteopontin (spp1), alpha(V) beta (3) integrin (itgb3) and cathepsin K (ctsk) were significantly increased. This may indicate the increase in the osteoclasts proliferation induced by high glucocorticoids level through the induction in the oxidative stress production although the increase in osteoclast did not reach significant value. Study by Jia et al. 2011 found that glucocorticoids induced ctsk gene expression and high ctsk expression

will reduce the collagen in the resorption cavity and this contributes to reduction of BV/TV (Søe & Delaissé 2010). Faralli et al. 2013 also found that dexamethasone increased itgb3 expression and affinity. Osteopontin plays a role in anchoring osteoclasts to the mineral matrix of bones and it initiates the begining process of bone resorption (Reinholt et al. 1990). Increased osteopontin expression was found to have negative correlation with the BMD and positive correlation with bone turnover (Qiu et al. 2016). Expression of the OPG gene in this study showed a significant reduction while the RANKL gene expression remained unchanged which had increased the RANKL/OPG ratio. This may have contibuted to the increase

in bone resorption. RANKL/OPG ratio is an essential determinant of bone resorption and it is increased by glucocorticoids treatment (Hofbauer et al. 2004). Glucocorticoids, dose dependently reduce OPG and increase RANKL expression (Sivagurunathan et al. 2005; Hofbauer et al. 1999). It was also reported that OPG stimulate apoptosis of the osteoclasts (Shiotani et al. 2002).

Palm tocotrienol supplementation was found to inhibit lipid peroxidation in the bone as indicated by the decrease in MDA level. SOD enzyme activity was also induced by palm tocotrienol. These effects may have reduced the peroxidised lipid and oxidative stress level in the bones and reversed its toxic effects to the osteoblasts. It was shown in this study where palm tocotrienol had increased the Ob.S/BS and decreased the Oc.S/BS. Decrease in the Oc.S/BS resulted in the inhibition of bone resorption which had preserved the bone structure and strength. Tocotrienol supplementation had resulted in the bones with higher BV/TV, Tb.N that were less separated with decreased Tb.Sp compared to the non-supplemented rats. The effects of palm tocotrienol on osteoclasts were supported by the reduction in the expression of osteoclast related genes, osteopontin, itgb3 and ctsk. These supported the increase in bone resorption. However, the RANKL/OPG ratio did not show significant response to palm tocotrienol supplementation. Palm tocotrienol supplementation had also inhibited the production of oxidative stress induced by dexamethasone. Low oxidative stress level may have protected and preserved the osteoblasts population. This was proven by the significant increase in the Ob.S/BS and enhancement of the osteoblast related gene expressions, osterix (sp7). Palm tocotrienol had also decreased the osteocalcin and Cola1a gene expressions which were similar to the expressions of the sham group.

Data regarding the effects of palm tocotrienol on the osteoblast and osteoclast related gene expressions are still limited. Our results showed that palm tocotrienol caused a decrease in the expression of osteocalcin and Cola1a genes as well as an increase in the osterix (sp7) gene expression. Along with that, it also decreased the expression of genes related to osteoclast activity ctsk, itgb3 and spp1. However, palm tocotrienol supplementation did not exhibit any influence on the RANKL/OPG ratio. These results had demonstrated the antiosteoclastogenic effect of palm tocotrienol.

Cathepsin K inhibitor is a potential treatment for osteoporosis where it acts by inhibiting bone resorption (Bossard et al. 1999). Dresner-pollak & Rossenblatt 1994 demonstrated that by blocking alpha (v) beta (3) integrin receptor, bone resorption was inhibited without changing the number of osteoclasts on the bone surface, suggesting inhibition of osteoclast activity. Shapses et al. 2003 suggested that osteopontin has both a physicochemical effect (inhibiting crystal growth and crystal proliferation) and a role in osteoclast recruitment (Shapses et al. 2003). The ability of palm tocotrienol to inhibit cathepsin K, alpha (v) beta (3) integrin and

osteopontin expression strengthen the fact that it is a potential treatment for osteoporosis through its antiresorptive properties.

OSX is required for osteoblasts differentiation through activation of cola1a promoter activity (Koga et al. 2005). The increase in OSX expression suggested that palm tocotrienol supplementation may had promoted bone formation by increasing osteoblast differentiation. In the other hand, the decrease in the expression of osteocalcin and Cola1a genes along with the reduction of osteoclast related gene expressions indicated that palm tocotrienol had decreased the osteoclastic activities and bone resorption, which attenuated bone formation activities by osteoblasts.

The antioxidant effect of palm tocotrienol had been widely tested and proven (Maniam et al. 2008). Since glucocorticoid induced osteoporosis is a free radical associated condition, the protective effect of palm tocotrienol could at least be partly explained by its antioxidant properties.

From the results, we could postulate that antioxidant effect of palm tocotrienol may have protected the osteoblasts from the toxic effect of the free radicals generated by dexamethasone. Palm tocotrienol supplementation inhibited lipid peroxidation and maintained the SOD activity in the bone. The antioxidant effects of palm tocotrienol may have contributed to the decrease in the oxidative stress level in the bone which prevented the increase in osteoclasts number and activities. The decrease in the oxidative stress may have protected the osteoblasts from its toxic effect, thus maintaining the osteoblast number. Apart from that, tocotrienol through its antioxidant effect may have also prevented the increase in osteoclastogenesis which was induced by the free radicals. These explained the reduction in bone resorption.

This study was limitted by few factors. The groups of rats were not compared with the current gold standard treatment for osteoporosis. The genes tested were also not confirmed with protein expressions. Structural histomorphometry should be better analysed by using microCT technique with less errors.

CONCLUSION

Palm tocotrienol at the dose of 60 mg/kg/day showed antiosteoporotic effects in rats with osteoporosis induced by long term glucocorticoid treatment. The protective effects are attributed to antiosteoclastogenic effect as well as pro-osteoblastic effect, most likely due to its antioxidant properties. Palm tocotrienol may be considered to be used as a supplement to patients who need to be on long term glucocorticoid treatment. This is to protect the bones from osteoporosis and prevent fragility fractures. More extensive studies need to be done to explore the mechanisms of the protective effects of palm tocotrienol on glucocorticoid induced osteoporosis.

ACKNOWLEDGEMENT

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