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Beneficial Effects of Annatto (*Bixa Orellana*) Tocotrienol on Bone Histomorphometry and Expression of Genes Related to Bone Formation and Resorption in Osteoporosis Induced by Dexamethasone

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ABSTRACT

Prolonged use of dexamethasone increases reactive oxygen species (ROS) which contributes to osteoporosis. Treatment with the antioxidant abolished the effect of DEX and protects the bone against osteoporosis. This study was done to compare the effects of pure tocotrienol and α-tocopherol in dexamethasone induced osteoporosis. In this study, adult male Sprague-Dawley rats were used (n=32, aged 3 months, weighing 280-300 grams). Adrenalectomy was performed on 24 rats and they were replaced with dexamethasone 120 µg/kg/day intramuscularly. Adrx+Dex group (n=8) were given oral vehicle palm olein 0.1 ml/kg/day. The Adrx+Dex+ATT group (n=8) was supplemented with oral annatto tocotrienol 60 mg/kg/day and the Adrx+Dex+ATF group (n=8) received alpha tocopherol 60 mg/kg/day. Sham group (n=8) underwent the sham procedure and received an intramuscular injection of palm olein 0.05 ml/kg/day and 0.1 ml/kg/day orally. The rats were euthanized after 2-months. The right femurs were tested for bone biomechanical strength test and bone histomorphometry analyses. The left was used to quantify the gene expression and oxidative stress enzymes activities. The tibias were used for microCT imaging. The 2-months exposure to dexamethasone had caused significant deterioration of the bone strength and structure. Gene expressions analysis showed an increase in bone resorption and the decrease in bone formation. Supplementation of annatto tocotrienol had significantly improved the bone structure and strength. There was also a decrease in the bone resorption with an increase in some of the bone formation related genes. However, alpha tocopherol exhibit less significant effects on the bone. This study showed that annatto tocotrienol provides better protective effects compared to alpha tocopherol in the osteoporosis induced by glucocorticoid excess through down regulation of gene expression.

Keywords: Annatto tocotrienol, Alpha tocopherol, Gene expressions, Bone histomorphometry, Osteoporosis, Antioxidant

INTRODUCTION

Prolonged high glucocorticoid level and an increase of lipid peroxidation increase the oxidative stress level [1]. Bone resorption is promoted by the oxidized lipids by the recruitment and differentiation of osteoclast precursor cells [2,3]. Superoxide dismutase is found in osteoclast and free radicals are generated by osteoclast in response to hormones [4]. Superoxide anion promotes bone resorption when the osteoclasts are stimulated by factors such as PTH, IL-1, and TNF. Reactive oxygen species (ROS) mediates the pro-apoptotic effect of glucocorticoid in the osteoblast cells [5].

Bone loss occurs gradually without showing any symptom until a fracture occurs [6,7]. The risk for bone fracture in Glucocorticoid-Induced Osteoporosis (GIO) is not proportionate to the BMD [8,9]. In GIO, differentiation of osteoblast precursor cells and proliferation of the matrix synthesis by mature osteoblasts are also reduced. It is also complicated with the induction of apoptosis of the osteoblasts and osteocytes, which suppresses bone formation [10,11]. Shifting of differentiation of osteoblastic lineage towards adipocytic lineage causes further impairment of bone formation [12]. Reduction in osteoclast number in GIO is not as much as the osteoblast and glucocorticoid it also has the ability to prolong the osteoclast lifespan [13,14].

Receptor Activator of Nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) which is produced by osteoblast regulates osteoclast activities [15]. Bone resorption is stimulated by the binding of RANKL to RANK receptors which are located on the osteoclast precursors. This promotes differentiation into mature osteoclasts [16]. Glucocorticoids increases in bone resorption by decreasing the expression of RANKL soluble decoy receptor, osteoprotegerin (OPG) in the stromal and osteoblastic cells [17].

The use of high resolution micro-computed tomography (μCT) imaging for the assessment of trabecular and cortical bone morphology is growing immensely. μCT has become the gold standard for the evaluation of bone microarchitecture in small animal models *ex vivo* [18]. Structural parameters through histologic analysis are derived from the stereologic analysis of 2D sections while μCT directly measure bone microarchitecture [19]. Studies show a high correlation of 2D and 3D morphology measurements by μCT with 2D histomorphometry analysis [20].

Currently, bisphosphonates are the first line therapy for the treatment of GIO. Bisphosphonates reduce the incidence of vertebral fractures by increasing the bone mineral density of the spine and hip [21]. Parathyroid hormone (PTH 1-34) and zoledronic acid are the new options for the treatment of GIO, increasing the bone mineral density of the spine and hip [22]. However, these treatments are facing problems regarding patients' compliance due to the side effects and route of administration.

Vitamin E is composed of 2 compounds, tocotrienol and tocopherol which are further divided into alpha (α), beta (β), gamma (γ) and delta (δ) isomers. It is a lipid soluble vitamin with potent antioxidant properties. Studies had found that tocotrienol exhibits higher antioxidant activities compared to tocopherol [23]. Its benefits against oxidative stress related diseases have been proven [24]. Tocotrienol was found to be effective in preventing the loss of bone mineral density in testosterone-deficient rats as well as in ovariectomized female rats [25,26]. It also exhibits anabolic properties in adult male rats [27].

Annatto bean is a major source of tocotrienol and the only available tocopherol free tocotrienol. It contains 90% delta tocotrienol and 10% gamma tocotrienol. Zou, et al., found that annatto tocotrienol (ATT) was more effective than alpha tocopherol and palm tocotrienol in inhibiting lipid peroxidation [28]. A recent study found that ATT supplementation increased to the bone formation marker (osteocalcin) and decreased the bone resorption marker (CTX) in oestrogen deficient rat model. ATT was also found to exhibit bone anabolic activity in the oestrogen deficient rats in combination with lovastatin [29].

The potential of α-tocopherol (ATF) as an antiosteoporotic agent has been tested in animal studies. Studies have found conflicting results on the antioxidant properties of ATF and its effects on bone health. Johnson, et al., indicated that osteoporosis was prevented by ATF in ovariectomized female rats [30]. Muhammad, et al., found that 4-weeks supplementation of ATF at 60 mg/kg body weight prevented degenerative changes to the trabecular bone structural parameters (bone volume, trabecular separation, and number) and also maintained the osteoclast surface [31]. This study also found that the efficacy of α-TF was found to be similar to palm tocotrienol mixture. A study by Nazrun, et al., showed that α-TF was found to have no positive effects on bone biomechanical strength and lipid peroxidation [27]. However, a study was done by Norazlina, et al., showed that the bone calcium content in ovariectomized rats was preserved by ATF supplementation [26].

In this study, we evaluated the effects of ATT, a pure tocotrienol and ATF on bones of rats receiving high dose glucocorticoids treatment. We would like to compare the antioxidant between the pure tocotrienol and tocopherol to bone histomorphometry, biomechanical strength and gene expressions. Microcomputed tomography (μCT) was used in this study for more accurate 3-dimensional estimation of bone structural parameters. Bone formation and resorption relate gene expressions were quantified to evaluate the effects of ATT and ATF at the molecular level. The mechanism of action at the molecular level revealed by this study could strengthen the results of previous studies on the properties of annatto tocotrienol in osteoporosis caused by prolonging glucocorticoid treatment. It also could strengthen the basis for the use of annatto tocotrienol in clinical trials in human.

PATIENTS AND METHODS

Animals and Treatment

Approval from the University of Kebangsaan Malaysia, UKM, Research, and Animal Ethics Committee was obtained before the commencement of the procedures (UKMAEC).

Male Sprague-Dawley rats (n=32) weighing 280-300 g were used in this study. They were obtained from the University of Kebangsaan Malaysia (UKM) animal breeding center. The rats were divided into 4 groups. Three groups were adrenalectomized and replaced with dexamethasone 120 µg/kg/day intramuscularly while the other one group underwent the sham procedure. Adrx+Dex group (n=8) were given oral vehicle palm olein 0.1 ml/kg/day. The Adrx+Dex+ATT group (n=8) was supplemented with oral ATT (American River Nutrition, USA) 60 mg/kg/day and the Adrx+Dex+ATF group (n=8) received ATF (Sigma, USA) 60 mg/kg/day. Sham group (n=8) underwent the sham procedure and was given an intramuscular injection of palm olein 0.05 ml/kg/day and 0.1 ml/kg/day orally. The treatments were given for 2-months before the rats were euthanized (Figure 1).

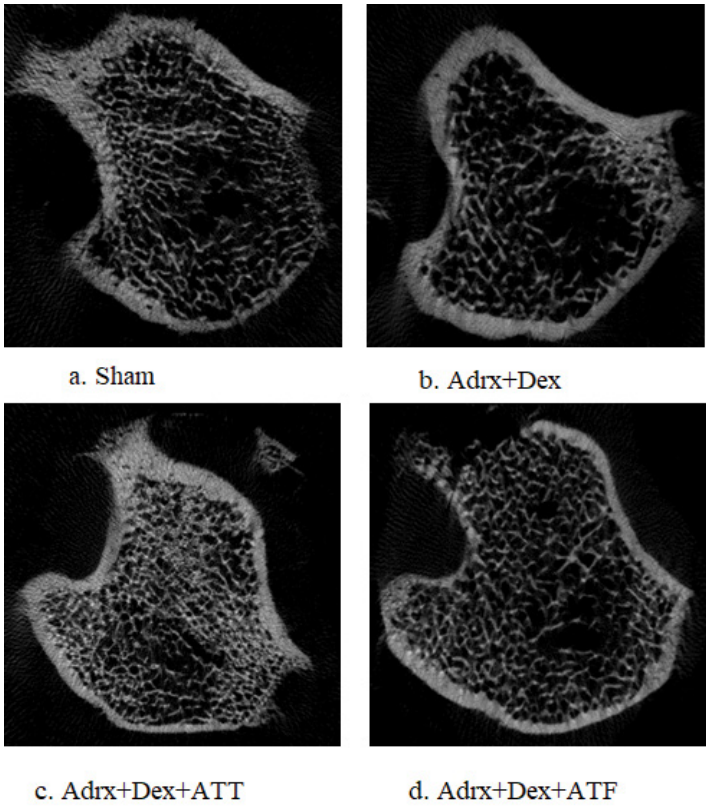

Figure 1 3-dimensional micro CT images of proximal tibia

A mixture of Ketapex and Xylazil (Troy Laboratories, Australia) were administered prior to the adrenalectomy procedure. The adrenal glands were visualized and identified via a dorsal midline skin and bilateral flank muscle incisions. Adrenal glands were removed after ligating the vessels together with the gland to secure the bleeding. The incisions were sutured with absorbable suture. The wound was cleaned daily, Poviderm cream (Hoe Pharmaceuticals) was applied to prevent the infection. The sham rats underwent a similar surgical procedure but the adrenal glands were not resected.

The rats were housed in a plastic cage under natural sunlight in an air conditioned room. They were allowed to free access of rat pellets (Gold Coin, Malaysia) ad libitum and normal saline for the adrenalectomized rats and distilled water for the sham operated rats.

Palm olein (Sime Darby, Malaysia) was used as the vehicle for dissolving dexamethasone (Sigma, USA). Dexamethasone was administered intramuscularly (120 µg/kg/day) for 6 days a week. ATT (Sime Darby, Malaysia) and ATF (Sigma, USA) were also dissolved in palm olein and given at the dose of 60 mg/kg/day orally. The rats were euthanized at the end of 2-months.

The effectiveness of the treatments was evaluated through the analysis of bone histomorphometry, bone biomechanical strength, gene expression analysis, and oxidative stress enzymes activities were measured.

Sample Collection

Blood was collected from the orbital sinus prior to the treatments and right before the rats were euthanized. The serum was obtained by centrifuging the blood at 3000 rpm at 4°C for 15 at -80°C until they were used. Carboxy-terminal of type 1 collagen crosslinks was measured using an Enzyme-linked Immunosorbent Assay (ELISA) kit and osteocalcin was measured using (Immunodiagnostic Systems, Tyne and Wear UK) ELISA kit. The bones were harvested wrapped individually in gauze soaked with phosphate buffer saline (PBS) and kept at -80°C until used.

Lipid Peroxidation and Oxidative Stress Enzymes

The lateral halves of the left femur were used for measuring the oxidative stress enzyme activities and lipid peroxidation. The homogenates were prepared following the procedures and guidelines provided by the manufactures. The SOD, CAT, and GPX were quantified using the kits manufactured by Cayman Chemical Company, USA. Malondialdehyde (MDA) level was measured using the kit manufactured by Bio Vision Incorporated USA.

Bone Micro CT

Structural parameters of the proximal part of the left tibia were done using high resolution cone-beam microCT scanner, Skyscan 1076 (Burker microCT, Belgium) equipped with 11 MPix camera model. Images of the left tibia were scanned at high resolution, the voltage of 50 kV and a voxel size of 9 µm. Each tibia was scanned from the proximal to mid-diaphysis with the length of 0.18 µm (165 slices; each slice=9 µm). Noise ring was removed using aluminium. Skyscan NRecon (Burker microCT, Belgium) version 1.7.0.4 software was used for reconstructing the 3D microstructural image data.

Skyscan CT analyzer (CTAn) (Burker microCT, Belgium) version 1.10.1.0 software was used to calculate the structural indices. This software separated the cortical and trabecular bone manually a few voxels away from the endocortical surface with an irregular region of interest (ROI) tool. The volume of interest for trabecular microarchitectural variables, started at the point of 1.9 mm from the proximal tibial condyles, just distal to the growth plate. It was directed to the metaphysis extending for 100 slices (0.6 mm) distally. An upper threshold of 255 and a lower threshold of 80 were used to delineate each pixel as “bone” or non-bone”. Contouring was performed for the selection of the trabecular region using a semi-automated method. Scanco software (Scanco Medical) was used to measure the 3-dimensional reconstruction and evaluation which were performed using the following bone parameters: trabecular bone volume, trabecular number (BV/TV), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), trabecular number (Tb.N).

Bone Histomorphometric Analysis

The right medial halves of the femurs were decalcified using a mixture of ethylenediaminetetraacetic acid (EDTA) and formalin for 2 months before they were embedded in paraffin wax. The bone samples were sectioned at 5 µm thickness with a microtome (Leica, Wetzlar, Germany) and stained using haematoxylin and eosin (Figure 2).

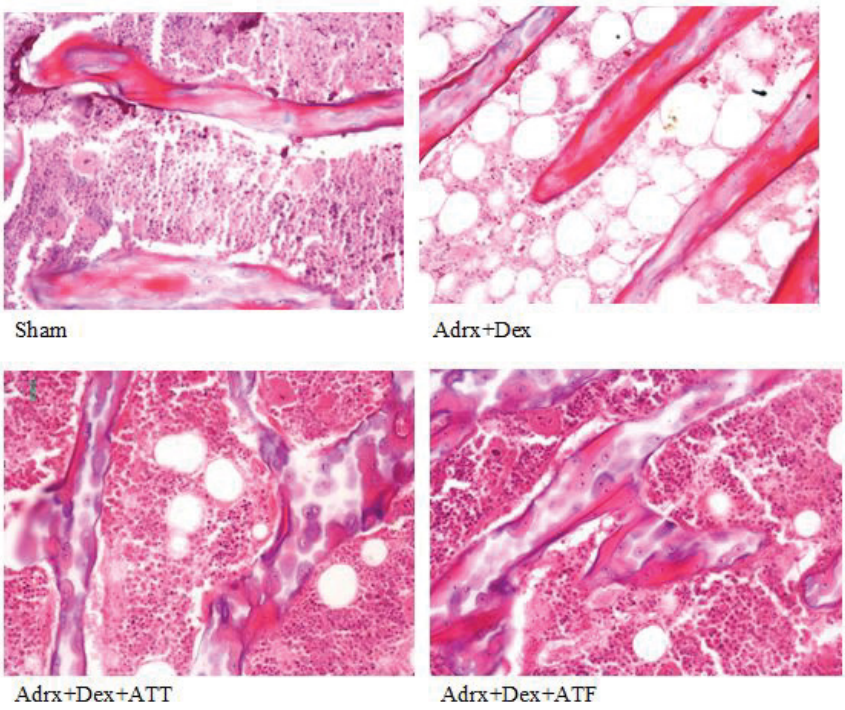

Figure 2 Haematoxylin and eosin stain of the decalcified bone of the distal femur at 200x magnification showing trabecular bone with osteoblast and osteoclast

A microscope (Nikon Eclipse 80i, Chiyoda, Japan) which was connected to an image analyzer (Media Cybernetics Image Pro-Plus, Rockville, MD, USA) was used to take the photomicrographs of the bone sections at 200x magnifications. Osteoblast surface (Ob.S/BS) and osteoclast surface (Oc.S/BS) were calculated by blinded examiners using Weibel Grid technique.

The rats were administered with 20 mg/kg body weight calcein intraperitoneally 9 days and 2 days before the rats were euthanized. This procedure was performed in order to label the bone with fluorescent for measuring the dynamic parameters. Micrographs from undecalcified bone samples were taken using a fluorescent microscope (Nikon, Tokyo, Japan). The microscope was connected to an image analyzer (Eclipse 80i, Nikon), equipped with Pro Plus 5.0 software (Media Cybernatics, Silver Spring, MD). Single labeled surface (sLS/BS) and double labeled surface (dLS/BS) were measured using Weibel Grid technique. Bone formation rate (BFR), mineralized surface (MS/BS) and mineral appositional rat (MAR) were generated from these 2 measurements (Figure 3).

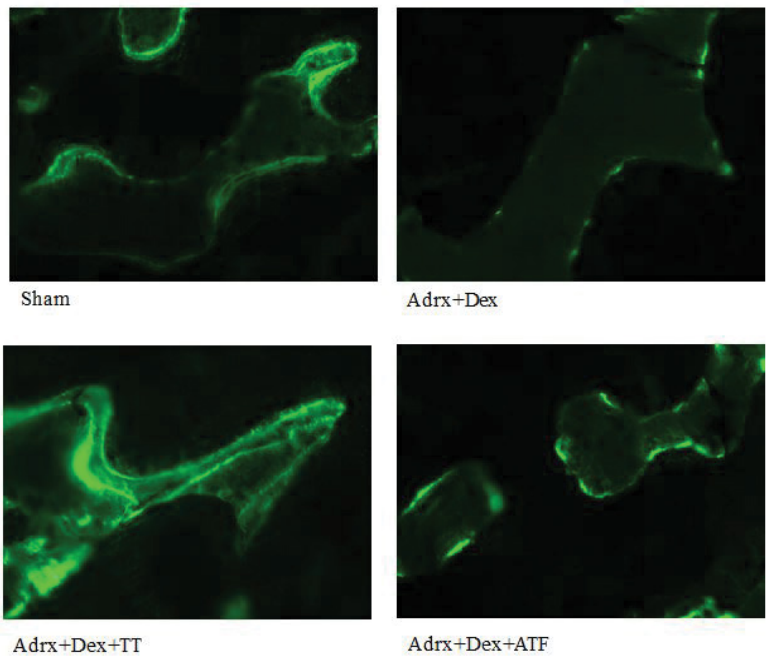

Figure 3 Photomicrograph undecalcified sections of calcein labeled trabecular bone of the distal femur (200X magnification)

All histomorphometric parameters were measured according to the guidelines set by the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee (1987) [19].

Gene Expression Analysis

Bone homogenates were obtained from trabecular bone samples taken from the distal part of the right femur. Each sample was placed into a tube containing steel beads which were homogenized in the buffer provided by the manufacturer using a high-speed homogenizer (Bead Ruptor 24, Omni, Kennesaw, GA, USA) at 4°C. The tissue lysate was incubated at 65°C and centrifuged to precipitate the debris. The QuantiGene Plex 2.0® technique (Panomics/Affymetrix Inc, Santa Clara, CA, USA) was used to quantify the mRNAs expression. All the procedures were performed following the manufacturer's instructions. Tissue lysates were pipetted into a 96-well plate preloaded with capture reagent and a probe set and incubated overnight at 54°C. After the incubation, the plate was put for hybridization with the preamplifier, amplifier, and biotinylated label. 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.

Bone Biomechanical Test

Biomechanical properties of the femurs were measured using the Instron Universal Testing Machine (model 5560, Instron, Canton, MA, USA). The machine was equipped with the Bluehill 2 software. The femurs were placed in a 3-point bending configuration; each bone was placed on 2 lower supports which were 5 mm apart [32]. The force was applied at the mid-diaphysis on the anterior surface of the bone where the anterior surface was in compression and the posterior surface in tension until the bone fractured. Readings of the parameters were recorded by the software and graphs load against displacement and stress against strain were plotted. The slope-value of the load-displacement curve represented the modulus of elasticity of the femurs. The main parameters of the bone mechanical test are divided into extrinsic and intrinsic parameters: The extrinsic parameters (load, energy, and extension) measure the properties of whole bone while the intrinsic parameters (stress, strain, and modulus of elasticity) measure materials of the bone [33].

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 22 software. Data were tested for normality using the Kolmogrov-Smirnov test. The data were analysed using parametric statistics, ANOVA test followed by the post-hoc Tukey test for multiple comparisons between the treatment groups. The $p<0.05$ was taken as significant. Data were presented as mean+standard error of the mean (SEM).

RESULTS

Oxidative Stress

Adrx+Dex rats showed significantly lower superoxide dismutase (SOD) activity and higher glutathione peroxidase (GPX) activity compared to the Sham group. However, no significant difference was seen in the catalase (CAT) activity. Malondialdehyde (MDA) level was significantly increased in the Adrx+Dex group indicating an increase in lipid peroxidation. The Adrx+Dex+ATT rats showed significantly higher SOD and lower GPX activities compared to the Adrx+Dex group, and the level was not significantly different compared to the Sham group. However, the change to CAT activity compared to the Adrx+Dex group did not reach significant value. The Adrx+Dex+ATT rats had significantly lower MDA level compared to the Adrx+Dex group and the level was not significantly different compared to the Sham group. Rats supplemented with ATF showed a significant higher GPX and CAT activities compared to the Adrx+Dex group. However, there were no significant changes seen in the SOD activity. ATF supplementation had also caused a significant reduction to the lipid peroxidation (Figure 4).

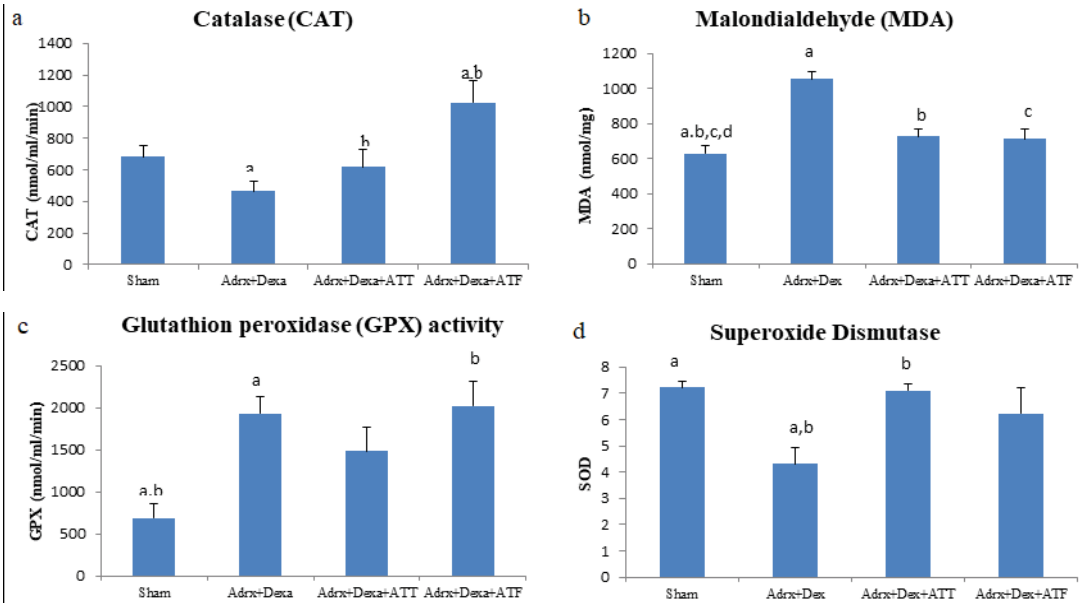

Figure 4 Lipid proxidation, superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) activities in the bone. Data presented as mean+SEM. Same alphabets indicate significant difference between treatment groups at $p<0.05$, Sham=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

Bone Histomorphometry

Bone quality of the proximal tibia was reduced after dexamethasone treatment (Adrx+Dex). Structural parameters measured by the microCT showed significant reductions in the total bone volume (TBV) and trabecular number (Tb.N), as well as a significant increase in the trabecular separation (Tb.Sp), compared to the sham-operated group (Sham). The cortical bone volume (CBV), trabecular bone volume (BV/TV) and trabecular thickness (Tb.Th) did not show significnt changes. The Adrx+Dex+ATT had significantly higher TBV, CBV, BV/TV, and Tb.Th compared to the Adrx+Dex group ($p<0.05$) and were not significantly different from the Sham group. Tb.N and Tb.Sp also showed improvements but the values did not reach statistical significance. Treatment with ATF (Adrx+Dex+ATF) also showed patterns of improvements to all the structural indices but all the values did not reach statistical significance

except for Tb.Sp (Figure 5).

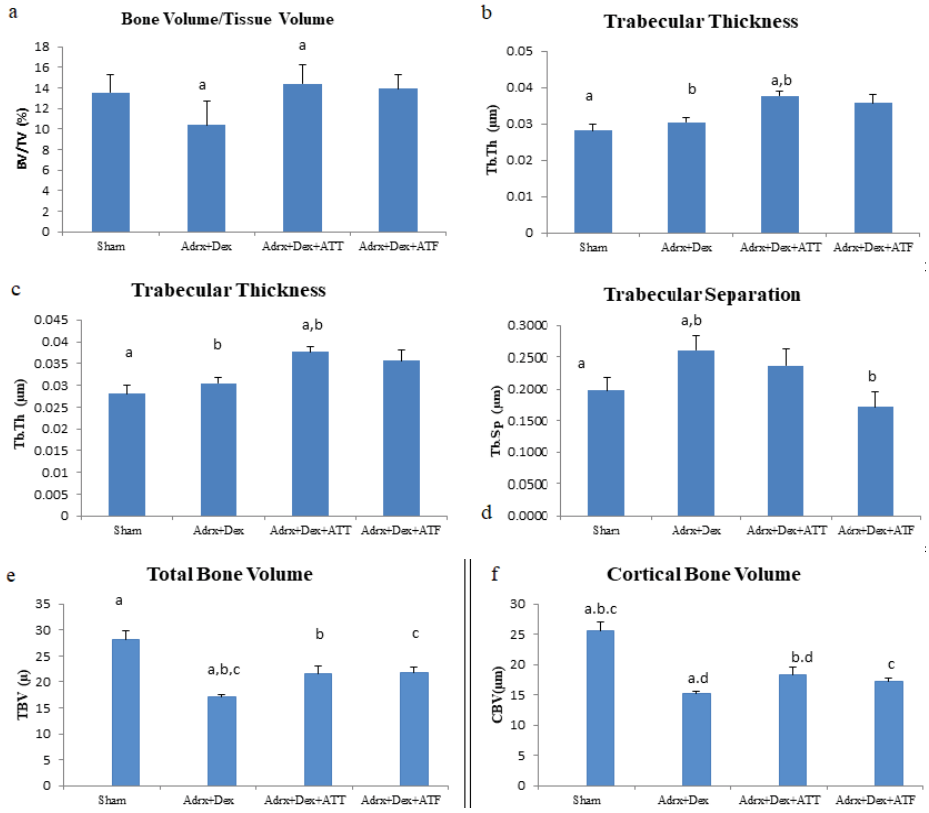

Figure 5 Structural histomorphometric parameters evaluated by microCT imaging. Data presented as mean + SEM. Same alphabets indicate significant difference between treatmet groups at p<0.05, Sham=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

No significant difference was seen in the Oc.S/BS caused by dexamethasone treatment but the Ob.S/BS was significantly reduced in the dexamethasone treated rats. Annatto tocotrienol supplementation significantly reduced the Oc.S/BS and prevented the decrease in Ob.S/BS. ATF supplementation also significantly maintained the Ob.S/BS but did not provide any significant effect on the OcS/BS (Figure 6).

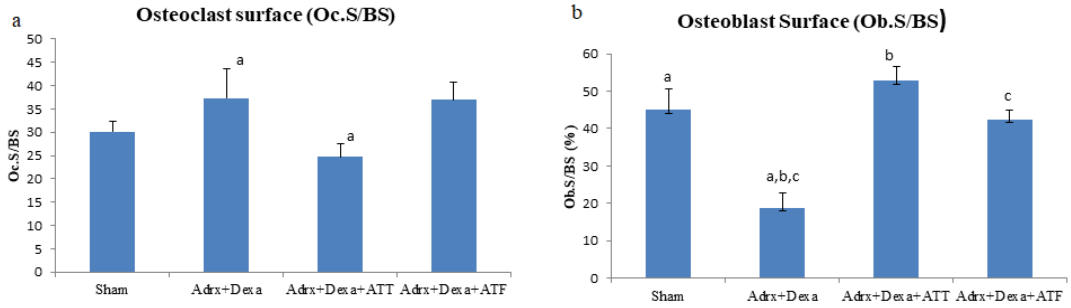

Figure 6 Results of bone static histomorphometric parameters. Data presented as mean + SEM. Same alphabets indicate significant difference between treatmet groups at p<0.05, Sham=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

Dynamic histomorphometric variables (MS/BS MAR, and BFR) were significantly reduced after 2-months of

dexamethasone treatment compared to sham group ($p>0.05$). ATT supplementation significantly increased all the dynamic parameters compared to the Adrx+Dex group. However, treatment with ATF did not result in any significant changes to the dynamic parameters (Figure 7).

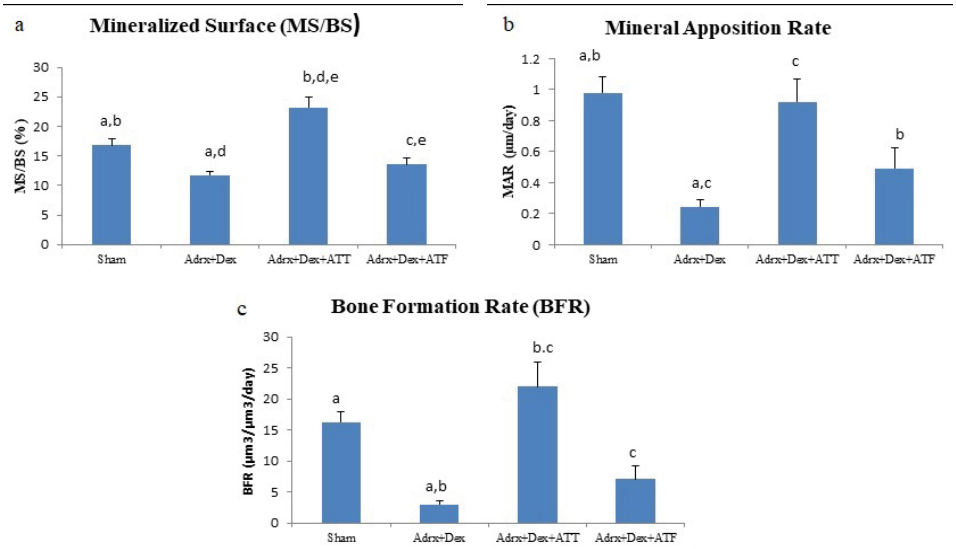

Figure 7 Results of bone dynamic histomorphometric parameters. Data presented as mean + SEM. Same alphabets indicate significant difference between treatmet groups at $p<0.05$, Sham=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

Gene Expressions

Expression of the genes related to the bone formation of the Adrx+Dex rats showed a significant difference compared to the Sham rats. The expression of the osteocalcin and COL1α1 genes increased significantly in the Adrx+Dex group, while the expression of the osterix (SP7) and integrin binding sialoprotein genes were significantly decreased ($p>0.05$). There was no significant difference in the RANKL gene expression in the Adrx+Dex group compared to the Sham group but the expression of the osteoprotegrin gene was significantly reduced, leading to a significant increase to the RANKL/OPG ratio. There was also a significant increase in the expression of other bone resorption related genes including the cathepsin K, osteopontin and MMP-9. Treatment with tocotrienol and tocopherol significantly reversed the effect of dexamethasone expression to bone formation related genes, osteocalcin and COL1α1 were decreased and the expression of osterix gene was increased. However, the expression of IBSP gene remained unchanged compared to the Adrx+Dex group (Figure 8).

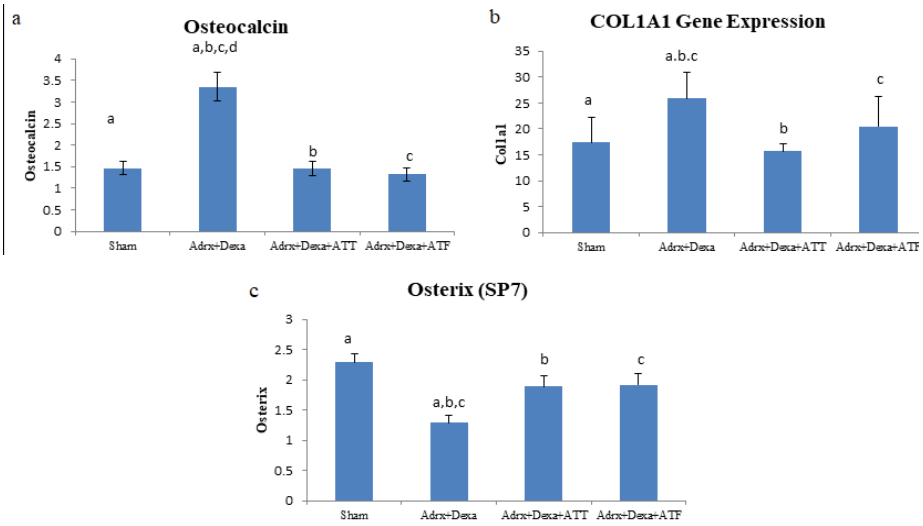

Figure 8 Expression of genes related to bone formation. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05, Sham= sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

Supplementation with tocotrienol and tocopherol had caused significant suppression to the expression of cathepsin K and osteopontin genes. However, tocotrienol and tocopherol supplementation showed no significant effect to the RANKL, OPG and the RANKL/OPG ($p>0.05$) (Figure 9).

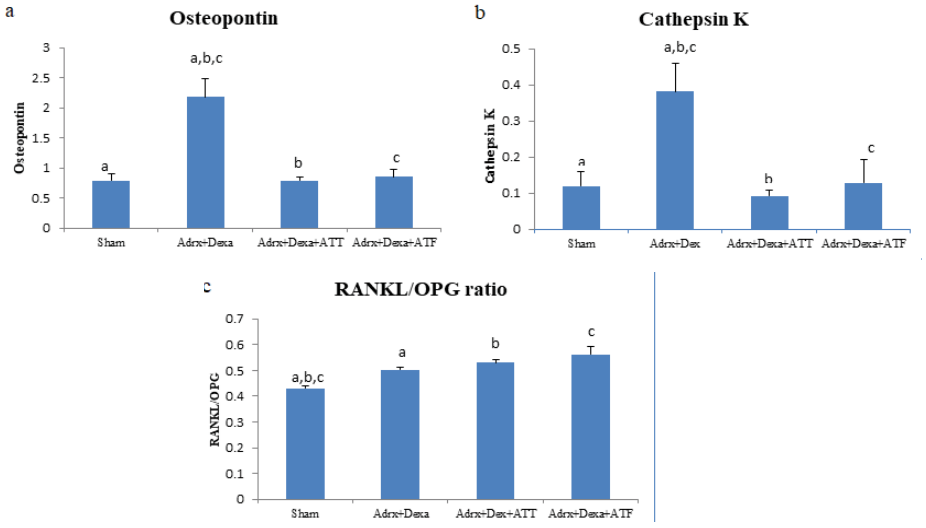

Figure 9 Expression of genes related to bone formation. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05, Sham=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

Bone Biomechanical Strength

There were significant reductions in both the intrinsic (stress), and extrinsic (energy, load, and flexure extension) biomechanical properties of the femurs after 2-months of dexamethasone treatment (Figures 10 and 11).

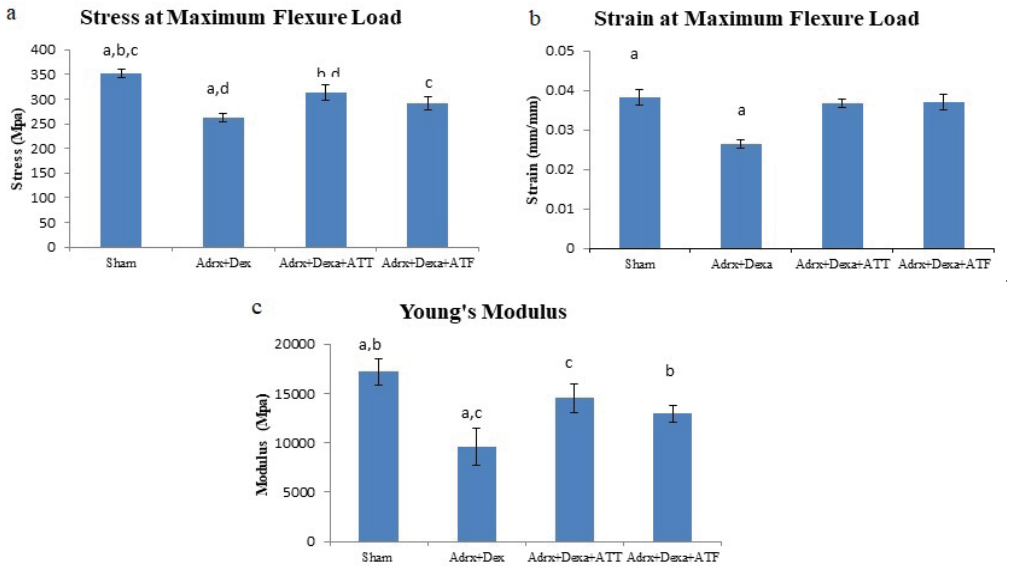

Figure 10 Intrinsic parameters of bone biomechanical strength. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05, Sham=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

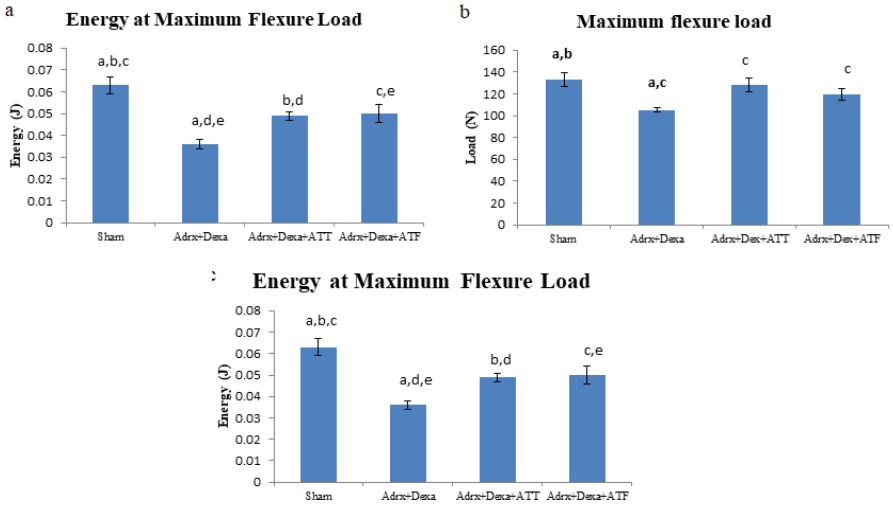

Figure 11 Extrinsic parameters of bone biomechanical strength. Data presented as mean + SEM. Same alphabets indicate significant difference between treatment groups at p<0.05, Sham=sham operated control; ADRX+Dex=adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); ADRX+Dex+ATT=adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day; ADRX+Dex+ATF=adrx and given intramuscular DEX 120 µg/kg/day and oral alpha tocopherol 60 mg/kg/day

Tocotrienol supplementation (Adrx+Dex+ATT) had maintained the intrinsic (stress) and extrinsic properties (energy, maximum extesion, and load) of the bone. However, ATF suplementation was able to significantly prevent the reduction only to one of the extrinsic parameter (energy) (Figure 9).

DISCUSSION

The antioxidant and antioxidative enzyme is known to have protective effects against oxidative damage. The antioxidant properties in tocotrienol play a role in providing protection against peroxidation of membrane lipid [34]. Dexamethasone is found to induce lipid peroxidation which increases the oxidative stress [35]. ROS cause

alteration of cell function through the destruction and oxidation of proteins, lipids, and DNA. It was found in this study that dexamethasone induced lipid peroxidation suppressed the SOD enzyme activities. However, the GPX activity was induced where else the reduction of CAT activity was not statistically significant. The decrease in SOD activity and an increase in lipid peroxidation and activity might have increased the ROS level and induced oxidative stress in bone. DEX is a synthetic glucocorticoid hormone which inhibits the synthesis of fibronectin and collagen as well as activates collagenase synthesis. There is evidence which indicated that DEX activates the expression of caspase family proteins and promotes osteoblast and osteocyte apoptosis [35]. This might have caused a reduction in osteoblast proliferation and differentiation and reduced the osteoblast population, leading to a reduction in bone formation. Increase in the ROS triggers the binding of RANKL to RANKL which initiates osteoclast differentiation that promotes bone resorption. This explains the increase in bone CTX in this study.

Bone histomorphometry analysis provides direct information on the changes in bone microarchitecture, remodeling/modeling, and cellular properties to provide an accurate depiction of the skeletal changes due to osteoporosis and drug interventions [36,37]. The use of high-resolution micro-computed tomography (microCT) imaging to assess trabecular and cortical bone morphology has grown immensely. The micro-CT scanner has many advantages where specimens can be evaluated 3-dimensionally and does not cause destruction and less time is consumed. Another great advantage of μCT is that the high number of slices can be obtained so that spatial differences within the areas of the specimen become visible.

In this study, dexamethasone administration had caused a reduction to the Ob.S/Bs, while the Oc.S/Bs was maintained. The decrease in the osteoblast number and osteoblast dysfunction leads to a reduction in the synthesis of bone matrix and consequently in the mean trabecular bone volume, trabecular number, and thickness. In this current study, it was evidenced that administration of DEX had caused destruction to the bone sturucture that significantly reduced the total bone volume (TBV), cortical bone volume (CBV) and also the trabecular bone (BV/TV). The trabecular separation (Tb.Sp) also showed a significant increase. The trabecular were also thinner (Tb.Th) with a less trabecular number (Tb.N). The resorption cavities were not fully restored due to the reduced bone formation with the maintenance of the resorption activities causing damage to the bone structure. Glucocorticoids also extend the lifespan of differentiated osteoclast leading to the imbalance between the bone forming and resorbing cells [14]. Dexamethasone treatment in this study caused a significant decrease in the MS/BS, MAR, and BFR which contributed to the decrease in the bone formation. This resulted in the bones that were more porous, fragile and were more susceptible to fragility fracture. The fagility of DEX treated bone was proven to be the reduction of the biomechanical strength of the femurs through the biomechanical test. The intrinsic (Young modulus, stress, and strain) and extrinsic (energy, load, and maximum extension) parameters in the bone treated with DEX were significantly decreased.

It was also found in this study that DEX administration had caused downregulation of the bone formation related gene expression, IBSP, and osterix. This could be due to suppression of the osteoblast function by DEX. Suppression of the osteoblast function which could also be due to the antagonization of the BMP and the Wnt signalling pathway [38]. However, osteocalcin and Col1α1 genes expression was significantly increased in the DEX treated rats although most studies reported that the expression of both genes was down regulated by glucocorticoid therapy [39,40]. Upregulation in the expression of genes related to bone formation, osteocalcin and Col1α1 might indicate the induction bone formation in response to the increase in bone resorption. Kim, et al., found that bone resorption promotes bone formation in order to refill the resorbed cavities [41]. Induction of osteoclast proliferation by DEX was evidencedby the upregulation of the osteoclast related genes, osteopontin, alpha (V) beta (3) integrin and cathepsin K-gene expressions. OPG gene expression in this study was significantly reduced, as the RANKL gene expression remained unchanged. This led to an increase in the RANKL/OPG ratio, which might have induced bone resorption. This could explain the rise of the resorption marker in the DEX treated rats.

Antioxidant properties of tocotrienol were proven by the results of this study. Supplementation of ATT which is a pure tocotrienol had significantly reduced the MDA level, indicating inhibition of lipid peroxidation. ATT was also found to induce the SOD enzyme activity. These might have caused the reduction in the peroxidised lipid and oxidative stress level in the bones. Subsequently, the toxic effects of the oxidative stress to the osteoblasts was reversed. It was proven by the increase of the Ob.S/BS and a decrease of the Oc.S/BS in the ATT treated group. ATT supplementation decreased the Oc.S/BS which caused a reduction in bone resorption. This might have contributed to the preservation of the bone structure and strength. Since glucocorticoid induced osteoporosis is a free radical associated condition, the

protective effect of ATT could be contributed by its antioxidant properties. In this study, we can see the antioxidant effects ATF. Supplementation with ATF had resulted in the increase in catalase and GPX and had also reduced the MDA level. ATF supplementation had also increased the Ob.S/BS but did not change the Oc.S/BS.

Earlier studies found that the femur length, bone calcium content and BMD of rats on long term dexamethasone treatment were preserved by palm oil derived tocotrienol supplementation [42,43]. Bone with high bone mineral density (BMD) is assumed to be strong. However, this might not be necessarily true in the glucocorticoids induced osteoporosis. In GIO, the bone may be weaker and the fracture risk is higher although it has higher BMD compared to the other causes of osteoporosis [44]. Moreover, changes in the BMD could only be detected at the late stage. This lead to the inaccuracy in the evaluation of any intervention and further mechanisms need to be explored. MicroCT evaluation in this study showed that ATT was effective in preserving the bone structure. Bones with higher trabecular volume (BV/TV), with a more trabecular number (Tb.N), that were less separated with lower Tb.Sp was seen in the bones of the ATT supplemented rats. The TBC and CBC were also maintained. However, supplementation with ATF did not show any significant benefits in preserving the bone structures. This result was supported by a previous study where tocotrienol was found to give a better protection against free radical damage induced bone loss [45]. All the dynamic parameters, MS/BS, MAR, and BFR were significanly improved in the ATT treated group but none was affected by the ATF suplementation.

Bones that received ATT supplementation were also proven to be stonger with higher extrinsic parameters (energy, load, and maximum extension) of bone biomechanical strength compared to non supplemented rats. However, no significant effect was seen in the intrinsic parameters due to ATT supplementation. The ATF supplementation also showed some protective effects to the biomechanical strength, where the strain and energy were significantly preserved.

Results of this study also revealed that both ATT and ATF supplementation had downregulated the expression bone resorption related genes, osteopontin, alpha (V) beta (3) integrin, and cathepsin K. ATT had also maintained the osteoblast number and functions as the Ob.S/BS was increased. Upregulation in the osterix gene expression might indicate the induction of osteoblast proliferation. This suggested that ATT supplementation may have increased osteoblast differentiation, and maintained bone formation. ATT had also downregulated the osteocalcin and COL1α1 gene expression in the bone which was similar to the sham group. Reduced expression of osteocalcin and COL1α1 genes together with the genes related to bone resorption, might indicate that ATT had inhibited osteoclastic activity and bone resorption. With this, the needs for new bone replacement was also reduced.

Inhibition of cathepsin K expression shown in this study indicated ATT has a potential to be used as a treatment for osteoporosis where it acts by inhibiting bone resorption. 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. The inhibitory effects of ATT to cathepsin K, alpha (V) beta (3) integrin and osteopontin expressions indicated its antiresorptive properties and have the potential to protect against osteoporosis.

From the results obtained in this study, we concluded that the antioxidant effect of ATT and ATF had protected the osteblasts from the toxic effect of the free radicals induced by DEX and inhibited the osteoclast activities. ATT was found to have better protective effects in the osteoporosis induced by DEX compared to ATF.

CONCLUSION

ATT and ATF at the dose of 60 mg/kg/day showed antiosteoporotic effects in rats with osteoporosis induced by long term glucocorticoid treatment via regulations of the bone formation and resorption related gene expressions. ATT showed better protective effects compared to ATF and may be considered to be given as a supplement to patients who are chronic users of glucocorticoid for treatments. 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 ATT osteoporosis induce by prolonging glucocorticoid administration.

DECLARATIONS

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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