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Potential Benefits of Annatto Tocotrienol in Glucocorticoid Induced Osteoporosis: An Animal Study

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Abstract

Long-term glucocorticoid treatment induces oxidative stress that cause osteoporosis is an antioxidant and has protective effects against free radical associated diseases. Annatto tocotrienol is a tocopherol free tocotrienol mixture. The purpose of this study was to determine the effects of annatto tocotrienol against glucocorticoid-induced osteoporosis. 32 adult male Sprague-Dawley rats were used in this study. 16 rats were adrenalectomized and divided into two groups; Adrx+Dexa and Adrx+Dexa+ATT and were administered with intramuscular injection of dexamethasone 120 µg/kg/day. Eight rats underwent sham procedure and the other 8 serves as baseline group. The Adrx+Dexa group was given vehicle palm olein 0.1 ml/kg/day orally while Adrx+Dexa+ATT group was supplemented with annatto tocotrienol 60 mg/kg/day. The sham operated rats were given vehicle palm olein 0.05 ml/kg/day by intramuscular injection and 0.1 ml/kg/day orally. The treatments were given for two months before the rats were euthanized. The femurs were tested for biomechanical strength and analyzed for bone histomorphometry. The results showed that long-term glucocorticoid treatment increased, bone resorption marker (CTX), lipid peroxidation; and decreased superoxide dismutase (SOD) activity with no significant changes to serum osteocalcin. Bone biomechanical strength was compromised with reduction in structural, static and dynamic parameters of bone histomorphometry. Annatto tocotrienol supplementation had maintained lipid peroxidation, CTX level, SOD activity and protected bone histomorphometric parameters and biomechanical strength. The results of this study suggested that annatto tocotrienol may have protective effects against osteoporosis induced by glucocorticoids and may be used as prophylaxis for patients on long term glucocorticoid therapy.

Keywords: Annatto tocotrienol; Biomechanical strength; Bone histomorphometry; Glucocorticoids; Osteoporosis

Introduction

Rapid-onset of bone loss and fragility fractures is frequent and severe complications of systemic glucocorticoid therapy [1]. The negative effects of glucocorticoid excess on bone may be mediated by the direct action on bone cells, extraskeletal tissues or both [2]. The initial rapid phase of bone loss is due to glucocorticoid action on osteoclasts, followed by a slower phase in which the bone loss is due to inadequate bone formation. Glucocorticoids extend the life span of osteoclasts and stimulate apoptosis of osteoblasts and osteocytes [3,4]. The negative effects of glucocorticoids on bone formation lead to reduction in the total amount of bone replaced in each cycle [5].

Some human studies showed that glucocorticoids increased resorption parameters [6]. Osteoclastic bone resorption is prompted by the formation of new osteoclasts as well as activation of quiescent osteoclasts. Markers of bone formation and resorption may be used as early indicators of response to therapy. Bone resorption markers are more sensitive than bone formation markers in response to different modalities of intervention [7]. This may be due to reduction in bone resorption markers occur more rapidly than reduction in bone formation markers [8,9].

Dexamethasone was found to stimulate osteoclast-like cell formation [10]. Glucocorticoids increase the expression of receptor activator of NF-kappa B ligand (RANKL) and decrease the expression of its soluble decoy receptor, osteoprotegerin (OPG) in stromal and osteoblastic cells which contribute to the increase in bone resorption [11]. All histomorphometric studies of glucocorticoid treated patients confirmed reduction in bone mass [12]. Based on histomorphometric studies, decreased bone formation is the most significant event leading to chronic glucocorticoid-induced bone loss due to decreased of osteoblastic lineage [6]. Prolonged high secretion of glucocorticoid has been hypothesized to elevate oxidative stress [13]. Oxygen-derived free radicals and lipid peroxidation are involved in bone remodeling [14,15].

Vitamin E is a potent antioxidant that inhibits lipid peroxidation. It is a group of lipid-soluble, structurally related compounds which occur naturally as α, β, γ and δ tocopherols (T) and four corresponding tocotrienols (T3). Tocopherol and tocotrienol isomers have antioxidant properties with tocotrienols also exhibit anti-cancer, neuroprotective, antiplatelet and cholesterol lowering activities [16-18]. A-T3 possesses 40-60 times higher antioxidant activity against lipid peroxidation than α-T [19]. Tocopherols and tocotrienols were found to be able to maintain bone density and prevent further bone loss and stimulate trabecular bone formation in various animal models of osteoporosis [20,21]. Studies have also found that tocotrienol mixture was more potent than α-tocopherol in preventing the deleterious effects of free radicals on trabecular bone structure [22].

Annatto bean is a major source of tocotrienols. Annatto tree is found in the tropical region of South America. It has spinose capsules with seeds and cordate leaves that yield annatto beans. Annatto tocotrienols contain delta tocotrienol (90%) and gamma tocotrienol (10%). It is the only available source of tocopherol free tococtrienol mixture. Its

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cholesterol and lipid lowering properties have already been proven. A recent study using an oestrogen deficient rat model found that daily supplementation of annatto tocotrienol caused a significant increase in the bone formation marker, ostecalcin and reduced the bone resorption marker, cross linked C-terminal (CTX), in an oestrogen deficient rat model. Cellular histomorphometric parameters were also improved in the ovariectomized rats. In combination with lovastatin, annatto tocotrienols showed bone anabolic activity in the oestrogen deficient rats [23].

The availability of safe and effective medication to prevent steroid induced osteoporosis is mandatory to preserve the skeleton. In this way, the incidence of fractures in patients exposed to long term glucocorticoid treatment and those with Cushing's syndrome will be substantially reduced. Current available treatments for glucocorticoid-induced osteoporosis have been associated with increase in Bone Mineral Density (BMD) but do not correlate to a larger decrease in fracture risk [24]. The positive effects of annatto tocotrienols on bone health have been studied [23,25]. The aim of this study was to determine the effects of annatto tocotrienols in maintaining the bone structure and strength against glucocorticoid induced osteoporosis.

Materials and Methods

Animals and treatment

All procedures were carried out in accordance with the institutional guidelines for animal research and approved by the Universiti Kebangsaan Malaysia UKM Research and Animal Ethics Committee (UKMAEC).

Sprague-Dawley rats at the age of 3-9 months are categorized as young adult rats where at this age; most indices of bone mass are at a plateau [26]. Adrenalectomy was done to remove the main source of endogenous steroid so that a constant level of high glucocorticoids in the body can be maintained. The endogenous glucocorticoids were replaced by a constant high dose of synthetic glucocorticoids, dexamethasone. The dose and duration of treatment was determined by a previous study [27]. No replacement for mineralocorticoids was administered since it does not have any effect on bone metabolism [28]. The animals were given normal saline to drink to maintain normal sodium homeostasis. The dose of annatto tocotrienol used in this study was based on the study done by Abdul Majeed et al. [23].

Thirty two, three month-old male Sprague-Dawley rats weighing 280-300 g were obtained from the Universiti Kebangsaan Malaysia (UKM) Animal Breeding Centre. Sixteen rats were adrenalectomized while the other 8 rats underwent sham procedure. Eight rats served as the baseline control group where they were euthanized without given any intervention. The rats were anaesthetized with a mixture of Ketapex and Xylazil (Troy Laboratories, Australia) at 1:1 ratio of 0.1 ml/kg dose. Dorsal midline skin and bilateral flank muscle incisions were made to visualize the adrenal glands. The adrenal glands were identified and vessels were ligated to secure the bleeding before both glands were removed. The incisions were sutured and cleaned with normal saline. Poviderm Cream (Hoe Pharmaceuticals, Malaysia) was applied to the wound daily for five days to prevent infection. The rats were also given intramuscular injection of Baytril 5% (Bayer Health Care, Thailand) for 5 days as a prophylaxis against infection. The sham-operated rats underwent a similar procedure except that the adrenal glands were left in-situ. The glands were examined histologically for confirmation.

The adrenalectomized rats were divided randomly into two groups of 8 and respective treatments were started 2 weeks after adrenalectomy. The rats were given the following treatments: SHAM: sham operated group, given vehicle palm olein 0.05 ml/100 g by intramuscular (IM) injection and 0.1 ml/100 g by oral gavage, ADRX+Dex: adrenalectomized (adrx) control group and given IM dexamethasone 120 µg/kg/day and 0.1 ml/100 g of palm olein by oral gavage, ADRX+Dex+ATT: Annatto tocotrienol group was administered with IM dexamethasone 120 µg/kg/day and annatto tocotrienol (American River Nutrition) 60 mg/kg/day by oral gavage. Dexamethasone (Sigma, USA) was dissolved in palm olein (Sime Darby, Malaysia) and administered intramuscularly (120 µg/kg/day) for 6 days a week. The dose and duration of treatment was determined by a previous study [26]. 600 mg of annatto tocotrienol was dissolved in 10 ml palm olein which made the concentration 60 mg/ml. The rats were given 0.1 ml/100g body weight by oral gavage. All the treatments were given for two months before the rats were euthanized.

The animals were placed in clean cages under natural sunlight and darkness at night and fed with rat pellets (Gold Coin, Malaysia) *ad libitum*. The adrenalectomized animals were given normal saline to drink *ad libitum* to replace the salt loss due to mineralocorticoid deficiency while the sham operated animals were given tap water. The animals were sacrificed under anaesthesia after completing 2 months of treatment.

The following parameters were measured: serum CTX, bone biomechanical strength, oxidative stress enzymes, structural, dynamic and static bone histomorphometry parameters.

Sample collection

Blood samples were taken before the commencement of treatments and after completion of the treatment. The blood samples were centrifuged at 3000 rpm at 4°C for 15 min and the serum was kept in aliquots at -80°C until analyzed.

Both femurs were cleared from soft tissues. The right femurs were wrapped in gauze soaked with phosphate buffered saline (PBS) and frozen at -80°C until used for biomechanical testing. The right femurs were cut at the midshaft with a rotary blade (Black & Decker) to separate the distal and proximal parts. The distal part was cut longitudinally to separate the bones into medial and lateral parts. The left femurs were used for analyzing oxidative stress enzymes.

Measurement of serum bone biochemical markers

Bone biochemical markers were analyzed using ELISA technique. The kit used for serum measurement of CTX was the Cross Linked C-Telopeptide of Type 1 Collagen Kit (Uscn Lifescience Inc. Wuhan, China) and osteocalcin was analyzed using the ELISA kit (Immunodiagnostic Systems Limited, UK). Serum used was obtained right before the rats were sacrificed.

Bone biomechanical test

Biomechanical properties of the femurs were assessed using an Instron Universal Testing Machine (model 5560, Instron, Canton, MA, USA) equipped with the Bluehill 2 software. The femurs were placed in three-point bending configuration; each bone was placed on two lower supports that were 5 mm a part [29]. The force was applied at mid-diaphysis on the anterior surface such that the anterior surface was in compression and the posterior surface in tension until the bone 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 slope-value of the load-displacement curve represented the modulus of elasticity of the femurs. The main parameters of the bone mechanical test can be divided into extrinsic

and intrinsic parameters: The extrinsic parameters (load, energy and extension) measure the properties of whole bone and the intrinsic parameters (stress, strain and modulus of elasticity) measure materials of the bone [30].

Bone histomorphometric analysis

Structural histomorphometric parameters include trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp). Undecalcified bone samples were embedded in the 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: 1.4 g of Benzoyl Peroxide Plasticized (Catalyst). The samples were sectioned at 7 µm thicknesses using a microtome (Leica RM2155, Nussloch, Germany) and stained with Von Kossa method. Structural parameters were analyzed by image analyzer (Leica DMRXA2, Wetzlar, Germany) using the VideoTest-Master software (VT, St. Petersburg, Russia).

Histomorphometric parameter measurements were randomly performed at the metaphyseal region, which 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 selected area is the secondary spongiosa area, which is rich in trabecular bone. All parameters were measured according to the guidelines set by the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee (1987) [31].

For cellular parameters the lateral half of the distal part of the femurs were decalcified in ethylene diamine tetraacetic acid (EDTA) 10 weeks. Decalcified bones were embedded in paraffin wax and sectioned at a thickness of 5 µm with a microtome (Leica, Wetzlar, Germany). The sections were stained with haematoxylin and eosin.

Micrographs of the bone sections were taken at a magnification of 200X using a microscope (Nikon Eclipse 80i, Chiyoda, Japan) that was connected to an image analyzer (Media Cybernetics Image Pro-Plus, Rockville, MD, USA). Counting of bone cellular histomorphometric parameters were performed by a blinded examiner using Weibel Grid technique at the region of secondary spongiosa of the metaphysis which located 1 mm from lateral cortex and 3-7 mm below the epiphyseal plate. The static parameters measured included osteoblast surface (ObS/BS) and osteoclast surface (OcS/BS). The procedure of bone histomorphometry had been described elsewhere [32].

Lipid peroxidation and oxidative stress enzymes

Oxidative stress enzymes ware measured using bone homogenates which was taken from the distal part of the femurs. The homogenates was processed according to the procedures given by the manufactures. Oxidative stress enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities were analyzed using the ELISA technique. The kits used for measurements of SOD, CAT and GPX were ELISA kits (Cayman Chemical Company, USA). Lipid peroxidation activity was determined by measuring through the malondialdehyde (MDA). Sample used for the measurement of MDA level was from bone homogenates which was taken from distal part of the femur. MDA level was measured by using the ELISA kit (BioVision Incorporated USA).

Statistical analysis

The statistical software used for data analysis was the Statistical Package for Social Sciences (SPSS) version 20.1.2. The data was tested for normality using the Kolmogrov-Smirnov test. Since the groups were found to be normally distributed, the data was analyzed using parametric statistics, i.e., the ANOVA test followed by the Tukey pos-hoc test for comparison between treatment groups. P values<0.05 were taken as significant. Data was presented as mean ± standard error of the mean (SEM).

Results

Serum bone biochemical markers

Two months dexamethasone treatment did not cause any significant difference to the osteocalcin level and supplementation of palm tocotrienol did not show any significant effect.

After 2 months, the CTX levels were significantly increased in all groups compared to the Baseline group. The CTX level of the ADRX+Dex group was significantly higher compared the Sham group. The CTX level of the group supplemented with annatto tocotrienol (ADRX+Dex+ATT) was significantly lower compared to the ADRX+Dex group and the CTX levels of ADRX+Dex+ATT group and the SHAM were not significantly different (Figures 1a and 1b).

Bone histomorphometric structural parameters

The Bone Volume/Tissue Volume (BV/TV), and Trabecular Number (Tb.N), were significantly reduced after 2 months in ADRX+Dex group compared to SHAM group but Trabecular Thickness (Tb.Th) and Trabecular Separation (Tb.Sp) did not show significant changes. Supplementation of annatto tocotrienol 60 mg/kg/day (ADRX+Dex+ATT) had maintained structural histomorphometric parameters. The BV/TV and the Tb.N were significantly higher and the Tb.Sp was significantly lower in the ADRX+Dex+ATT group compared to ADRX+Dex group. There was no significant difference in the BV/TV, Tb.N and Tb.Sp seen in ADRX+Dex+ATT group compared to the SHAM group. However, supplementation of annatto tocotrienol did not cause significant changes to the Tb.Th. The changes are illustrated in Photomicrograph 1 (Figures 2a-2d).

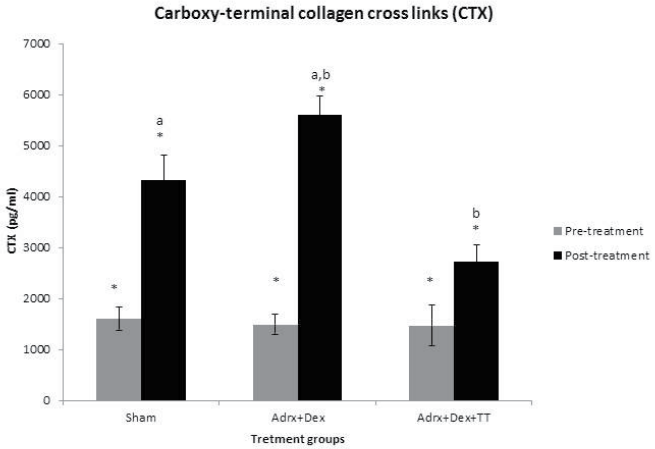

Figure 1: Effects of annatto tocotrienol on serum osteocalcin and cross linked C-telopeptide of type 1 collagen (CTX). Data presented as mean ± SEM.

Same alphabets indicate significant difference between the treatment groups at p<0.05

Baseline=No Intervention; SHAM=Sham Operated Control; ADRX+Dex=Adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day; ADRX+Dex+ATT=Adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienol 60 mg/kg/day

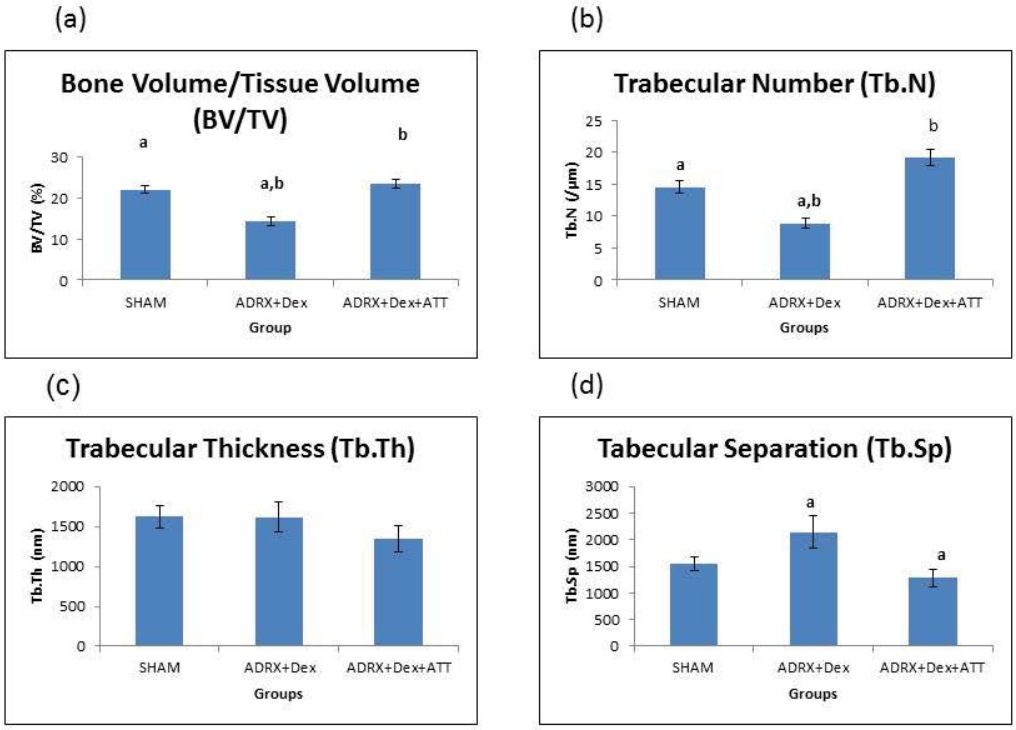

Figure 2: Effects of annatto tocotrienol on structural histomorphometric parameters. Data presented as mean ± SEM.
Same alphabets indicate significant difference between treatment groups at $p<0.05$
Baseline=No intervention; SHAM=Sham Operated Control; ADRX+Dex=Adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day, ADRX+Dex+ATT=Adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienol 60 mg/kg/day
ADRX+Dex group exhibit more separated, less and thinner trabecular bone compared to SHAM. ADRX+Dex+PTT group showed thicker, less separated and more trabecular bone compared to ADRX+Dex group
Photomicrograph 1: Von Kossa stain of undecalcified bone at 25x magnification.
Baseline=No intervention; SHAM=Sham Operated Control; ADRX+Dex=Adrenalectomized and given intramuscular dexamethasone 120 µg/kg/day, ADRX+Dex+ATT=Adrx and given intramuscular Dexamethasone 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day

Bone histomorphometric static parameters

Two months dexamethasone treatment had caused significant decrease in the osteoblasts surface (Ob.S/BS) but did not cause significant changes to the osteoclast surface (Oc.S/BS) as seen in the Adrx+Dex group compared to Sham group. Annatto tocotrienol supplementation had maintained the Ob.S/BS and significantly decreased the Oc.S/BS. There were no significant difference between the Ob.S/BS and the Oc.S/BS of the Adrx+Dex+ATT group compared to the Sham group. The changes are illustrated in Photomicrograph 2 (Figures 3a and 3b).

Bone histomorphometric dynamic parameters

After two months, Adrx+Dex group had significantly lower mineralized surface (MS/BS), mineral apposition rate (MAR) and bone formation rate (BFR) compared to Sham group. Annatto tocotrienol supplementation had reversed the dexamethasone effects by significantly maintained the MS/BS, MAR and BFR in the Adrx+Dex+ATT group which were significantly higher than the Adrx+Dex group. MS/BS, MAR and BFR of the Adrx+Dex+ATT group were not significantly different from the Sham group. The changes are illustrated in Photomicrograph 3 (Figures 4a-4c).

Bone biomechanical strength

Both intrinsic (Young's modulus, stress and strain) and extrinsic properties (energy, load, and flexure extension) of the femur ADRX+Dex group were significantly lower compared to the SHAM group. Femur of the rats supplemented with annatto tocotrienol (ADRX+Dex+ATT) had significantly higher intrinsic (stress) and extrinsic properties (energy) compared to ADRX+Dex. However, annatto tocotrienol was unable to prevent the reduction in the Young's modulus, strain, load and flexure extension (Figures 5 and 6).

Lipid peroxidation and oxidative stress

Two months of dexamethasone treatment had significantly reduced the superoxide dismutase (SOD) activity and increased the glutathione peroxidase (GPX) activity in the femur. However, catalase (CAT) activity remained unchanged. There was also an increase in lipid peroxidation as shown by the significant increase of the malondialdehyde (MDA) level in the bone. The ADRX+Dex+ATT group had significantly higher SOD activity and lower GPX activity compared to the ADRX+Dex group and the level was not significantly different compared to SHAM group. Annatto tocotrienol supplementation did not change CAT activity. MDA level was significantly lower in the ADRX+Dex+ATT group compared to ADRX+Dex group and it was not significantly different compared to SHAM group (Figures 7a-7d).

Discussion

Previous studies had shown that administration of dexamethasone for two months at the dose of 120 µg/kg/day caused osteoporosis based

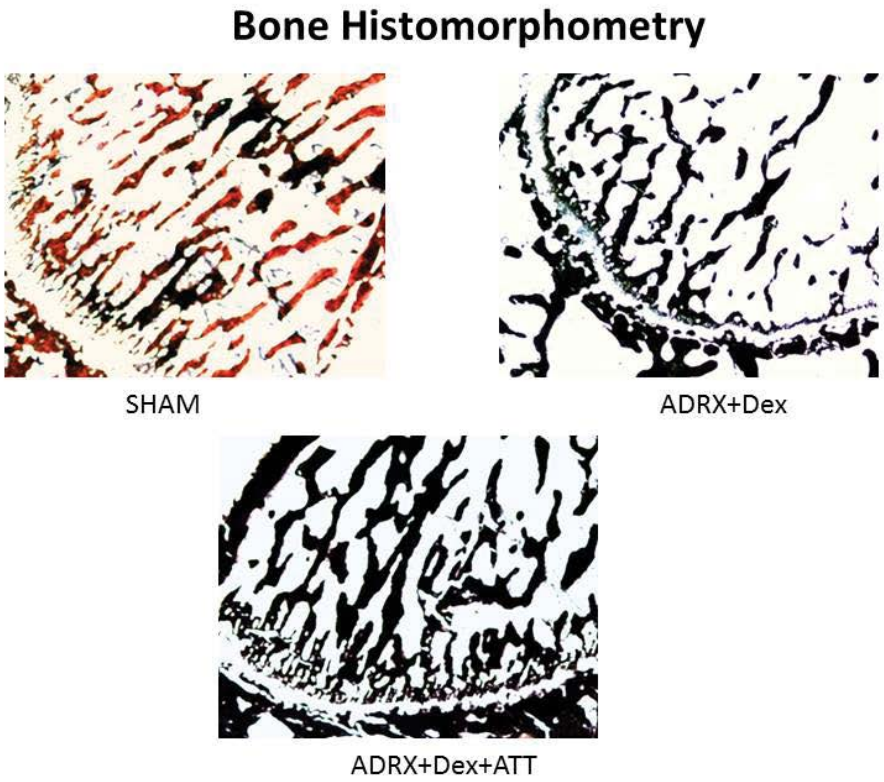

Figure 3: Effects of annatto tocotrienol on static histomorphometry parameters.

Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at p<0.05

Baseline=No Intervention; SHAM=Sham Operated Control; ADRX+Dex=Adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day, ADRX+Dex+ATT=Adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day

Photomicrograph 2: Haematoxylin and 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+ATT=Adrx and given intramuscular Dexamethasone 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day

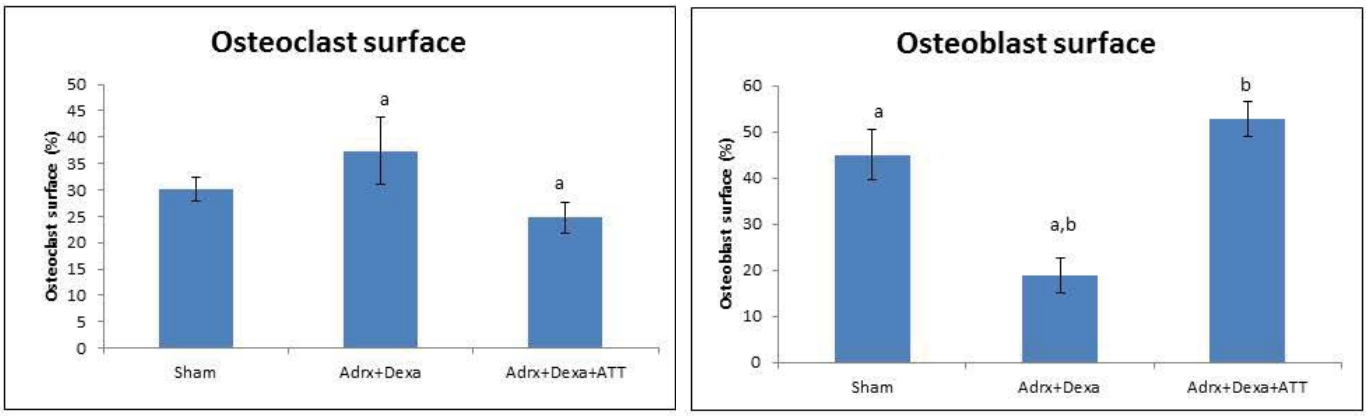

Figure 4: Effects of annatto tocotrienol on dynamic histomorphometry parameters.

Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at p<0.05

Baseline=No intervention; SHAM: Sham Operated Control; ADRX+Dex=Adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day; ADRX+Dex+ATT=Adrx and given intramuscular DEX 120 µg/kg/day and oral annatto 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.

Baseline=No intervention; SHAM=Sham Operated Control; ADRX+Dex=Adrenalectomized and given intramuscular dexamethasone 120 µg/kg/day, ADRX+Dex+ATT=Adrx and given intramuscular Dexamethasone 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day

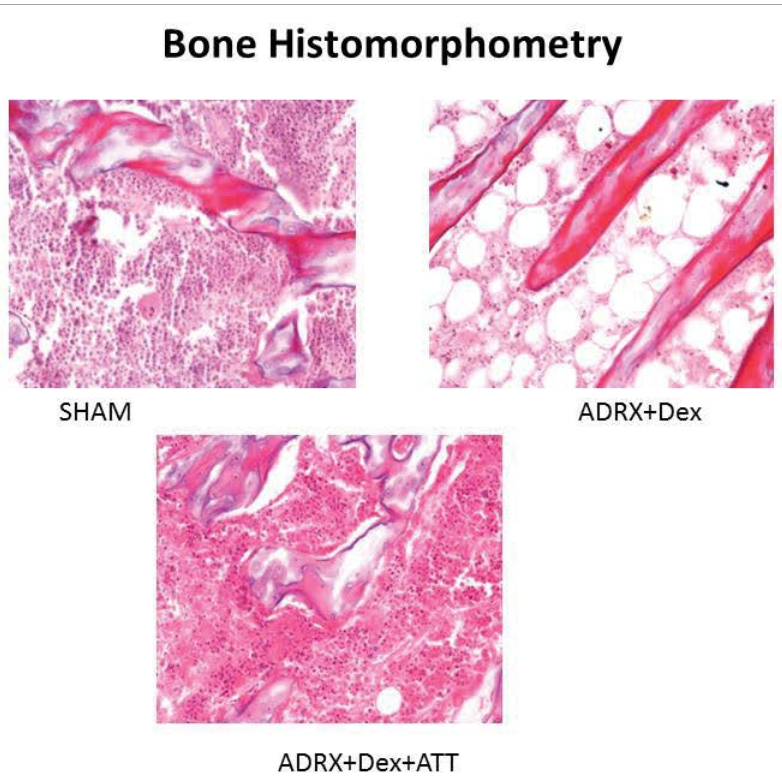

Figure 5: Effects of annatto tocotrienol on the intrinsic parameters of bone biomechanical strength.

Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at $p<0.05$

Baseline=No intervention; SHAM=sham operated control; ADRX+Dex=adrenalectomized and given intramuscular dexamethasone 120 µg/kg/day; ADRX+Dex+PTT=Adrx and given intramuscular DEX 120 µg/kg/day and oral palm tocotrienol 60 mg/kg/day

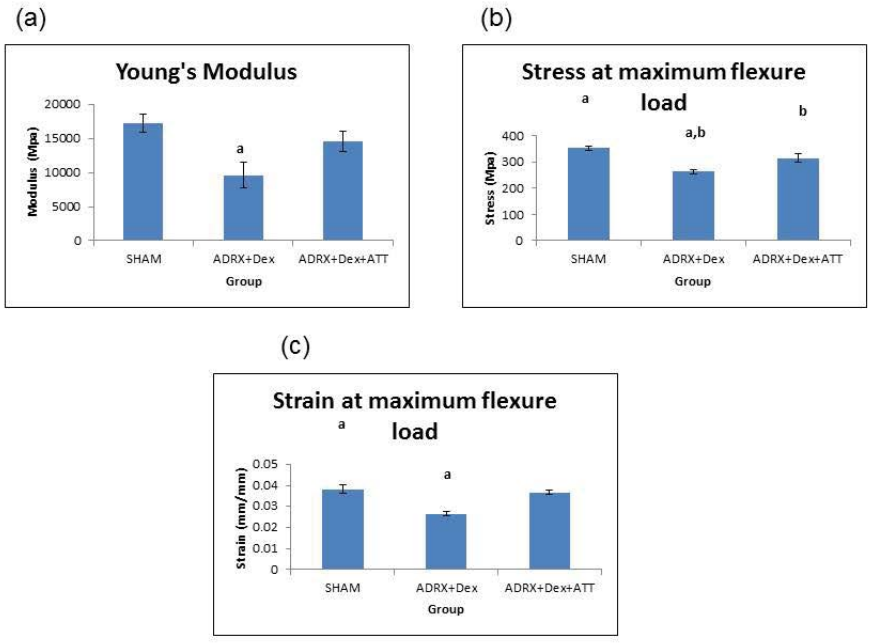

Figure 6: Effects of annatto tocotrienol on the extrinsic parameters of bone biomechanical strength.

Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at $p<0.05$

Baseline=No intervention; SHAM=sham operated control; ADRX+Dex=Adrenalectomized and given intramuscular dexamethasone 120 µg/kg/day; ADRX+Dex+ATT=Adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day

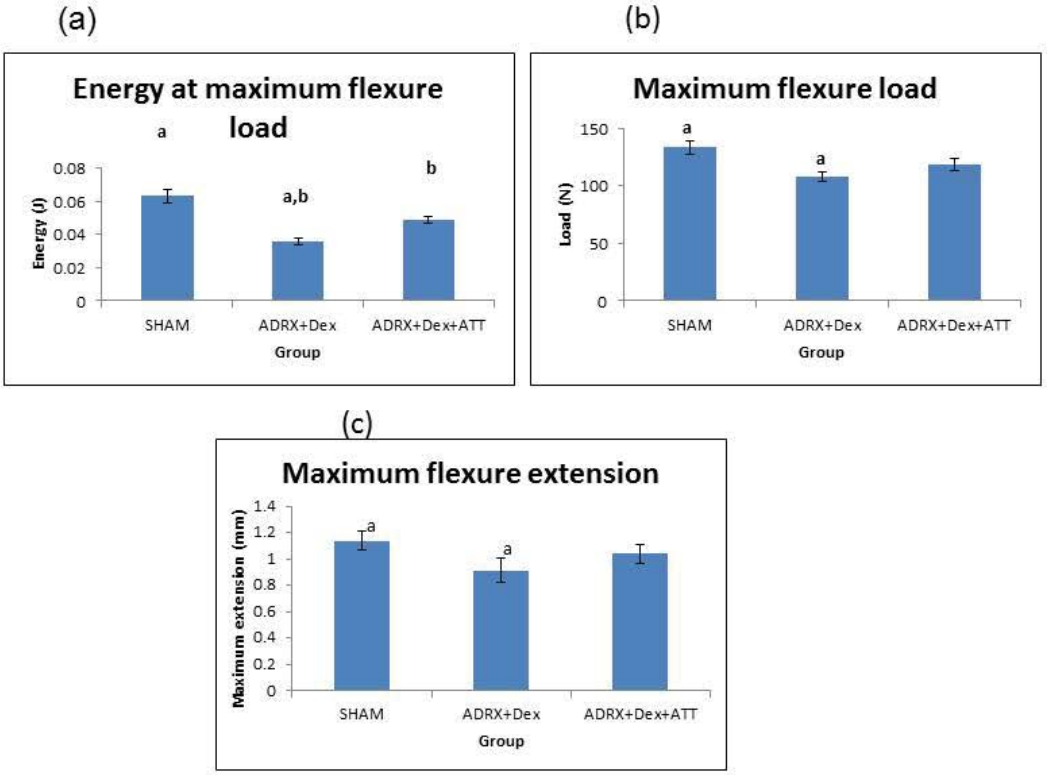

Figure 7: Effects of annatto tocotrienols on the lipid proxidation, superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) activities in the bone. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at $p<0.05$

Baseline=No intervention; SHAM=Sham Operated Control; ADRX+Dex=Adrenalectomized and given intramuscular dexamethasone 120 µg/kg/day; ADRX+Dex+ATT=Adrx and given intramuscular DEX 120 µg/kg/day and oral annatto tocotrienols 60 mg/kg/day

on the decrease in bone mineral density (BMD) and bone calcium content. Our recent study shown that the same dose of dexamethasone caused osteoporosis base on the decrease in BV/TV, Tb.N and increase in Tb.Sp in the femur [27].

Oxidative stress is a condition where there is imbalance between the pro-oxidant compound and the anti-oxidant defense mechanism of biological system due to imbalance between generation of reactive oxygen species (ROS) and detoxification by antioxidants in living tissues [33]. The results of this study showed that, two months dexamethasone treatment stimulated lipid peroxidation which was shown by a significant increase in the MDA level which may have caused oxidative stress in the bone. Long term dexamethasone treatment had also caused a decrease in SOD activity while CAT activity remained. However, the GPX activity in the bone was stimulated. These results indicated that two months dexamethasone treatment had induced oxidative stress in the bone. Increase in lipid peroxidation has been implicated in osteoporosis. Parhami et al. showed that there is accumulation of lipid which will undergo oxidation in the osteoporotic bone [34]. Oxidized lipids promote bone resorption by recruitment and differentiation of osteoclast precursor cells and inhibit osteoblast differentiation [14,15]. Superoxide anion is involved in resorption when osteoclasts are stimulated by factors such as PTH, IL-1 and TNF. Osteoclasts contain superoxide dismutase and produced free radicals in response to hormones [20]. Decrease in SOD activity showed in this study might have caused an increase in the superoxide radical. Increase in superoxide radical together with the increase of lipid peroxidation might have increased oxidative stress level, exposing the bone to oxidative damage. Anti-oxidative enzymes offer protection against oxidative damage. GPX plays a major role in reducing lipid hydro peroxide, and this is necessary to prevent further oxidation of the free radicals [35]. The increase in GPX level caused by long term dexamethasone treatment seen in this study could be due to the response to the increased in superoxide level caused by reduction of SOD activity. The GPX activity may then be stimulated to reduce superoxide and hydrogen peroxide to water. Previous study found that glucocorticoid treatment had caused reduction in GPX level hippocampal culture [36].

Serum CTX is the most sensitive bone resorption marker where it shows a positive response to treatment at 1 or 3 months of intervention. Serum CTX has also been reported to be more specific for bone resorption than other bone resorption markers [9]. In this study, dexamethasone administration did not cause significant changes to the osteoclast number (Oc.S/BS). The increase in lipid peroxidation and decrease in SOD activity shown by this study could have generated free radicals that may have stimulated osteoclastic activities. Increase in the osteoclastic activities stimulated bone resorption and this was indicated by the significant increase in the bone resorption marker, CTX. Although osteoclast number did not increase significantly, increase in the serum resorption marker CTX indicated an increase in the osteoclastic activities [37]. Osteoclast has the ability to release free radicals into the resorption site which might also have contributed to the increase in bone resorption process [38]. Glucocorticoids extend the lifespan of osteoclasts and also promote apoptosis of osteoblasts leading to imbalance of the coupling actions of these cells [3]. Increase in the oxidative stress level in the bone could had reduced osteoblast number

due to its toxic effects on osteoblasts and it was shown by the significant decrease in the osteoblast surface (Ob.S/BS) but serum osteocalcin did not show significant changes. This might indicate an decrease in the osteoblastic differentiation and proliferation while osteoblastic activities continue in response to the increase in bone resorption.

Histomorphometry analysis of this study had also showed deterioration of bone volume and structure after two months dexamethasone treatment. This was shown by the significant reduction in the Bone Volume/Tissue Volume (BV/TV) and Trabecular Number (Tb.N) of the femoral bones after two months of receiving dexamethasone treatment. The bone loss could be due to osteoblastic dysfunction and stimulation of osteoclastic bone resorption due to generation of free radicals. Decrease in osteoblast number and function leads to the reduction in synthesis of bone matrix and consequently in the mean Wall Thickness (W.Th), Trabecular Bone Volume (BV/TV) and Trabecular Thickness (Tb.Th). This presents the end product of osteoblastic activity and any changes will lead to a change in the total amount of bone replaced in each remodeling cycle [39]. These results were consistent with previous histomorphometric studies of glucocorticoid treated patients [12].

Dynamic parameters, MB/BS, MAR and BFR were significantly reduced due to dexamethasone treatment and these results were similar as reported by Chavessieux et al. [40]. Reduction in the Mineral Apposition Rate (MAR) is the evidence of reduced osteoblastic activity [6]. Early rapid loss of bone cause by glucocorticoid excess results primarily from direct action on osteoclasts. Furthermore, prevention of the glucocorticoid induced increase in osteoclast number does not prevent glucocorticoid-induced osteocyte apoptosis or increase in the prevalence of osteoblasts [41]. Even if osteoblasts and bone formation did not decrease with glucocorticoid administration, the glucocorticoid-induced prolongation lifespan of osteoclasts would still result in an early transient increase in the remodeling span and loss of bone mass [42].

In this study, the disruption of bone structure was associated with reduction in the biomechanical strength. Both the intrinsic (Young's Modulus, Stress, Strain) and extrinsic properties (energy, load and maximum flexure extension) were found to be reduced in this study. As reported by previous study, glucocorticoid treatment causes reduction of bone strength by 20% for trabecular thinning, 70% for trabecular loss and 77% for both thinning and loss [43]. High dose glucocorticoid treatment results in substantial changes in bone mass, structure and formation which are more pronounced in the axial skeleton compared to the peripheral sites [44]. This is due to the increase in the percentage of non-viable osteocytes and empty lacunae due to disruption of osteocyte-canalicular network that reduced bone ability to repair the microdamage and ultimately reduce bone strength [4]. Trabecular bone responds more rapidly to either positive or negative changes in bone since bone remodeling takes place only at the bone surfaces [45].

Supplementing the rats with annatto tocotrienols prevented the reduction in the intrinsic parameter (stress) and extrinsic parameters (energy, maximum extension and maximum load) of the femur. However, it was unable to prevent the changes caused by dexamethasone treatment to the other intrinsic parameters (strain and Young's Modulus) of bone biomechanical properties. Annatto tocotrienol had prevented the increase in bone resorption activity as indicated by the decrease in the CTX level. This subsequently maintained the bone structure as showed by the histomorphometric analysis. The BV/TV, Tb.N and Tb.Sp were preserved and this might have contributed to the stronger bones in rats supplemented with annatto tocotrienol. Previous study found that annatto tocotrienol which was administrated to testosterone deficient rat model had preserved the structural histomorphometric indices of the femur [25].

Antioxidants and anti-oxidative enzymes are known to protect against oxidative damage. The antioxidant properties of tocotrienols may have played a role in the endogenous defense against peroxidation of membrane lipids [46]. In this study we used annatto tocotrienol that contains 90% delta tococtrienol and 10% gamma tocotrienol. To date, annatto tocotrienol is the only available source of tocopherol free tocotreinol mixture. This could be a novel approach in the protection against glucocorticoid induced osteoporosis.

Annatto tocotrienol supplementation had also inhibited lipid peroxidation and maintained the SOD activity in the bone. However, GPX and CAT activities remained unchanged. Consistent with our results, lipid proxidation was reported to be decreased in normal male and ovariectomized female rats due to administration of palm oil derived tocotrienol [47,48]. The antioxidant effects of annatto tocotrienol might have contributed to the decrease in the oxidative stress level in the bone which prevented the increase in osteoclasts number and activities and decrease in bone resorption. Annatto tocotrienol supplementation had also maintained the Ob.S/BS and prevented the increase of the Oc.S/BS. The decrease in the Oc.S/BS explained the decrease in serum CTX caused by annatto tocotrienol.

Similar findings were reported by a recent study where annatto tocotrienol suppress free-radical induced osteoclast formation and prevented the increase in resorption activity in testosterone deficient rat model [49]. Decrease in the oxidative stress level may have protected the osteoblasts against the toxic effects of free radicals. It was shown in this study where annatto tocotrienol preserved the Ob.S/BS. By maintaining the osteoblast number and activity, annatto tocotrienol may have maintained bone formation which subsequently contributed to the maintenance of bone structure and strength. The effects of annatto tocotrienols in protecting the osteoblast and preventing the increase in osteoclast number were proven in a study [50]. Annatto tocotrienol did not cause any significant changes to the GPX and a study using palm tocotrienol had obtained the same outcome [47]. However, another study showed that palm oil derived tocotrienol caused increased GPX activity in ovariectomized rats [48]. This study confirmed the antioxidant properties of annatto tocotrienol which had reduced oxidative stress level in the bone of rats receiving dexamethasone treatment. This may have contributed in protecting the bone against the detrimental effects of glucocorticoids.

A study reported that vitamin E stimulated bone resorption and decreased bone mass by inducing osteoclasts fusion but did not have any influence to osteoblastic differentiation and proliferation [51]. Previous study found negative correlation between alpha tocopherol and bone mass. Other studies had demonstrated protective effects of tocotrienol on various osteoporotic rat models. There were also studies done which compared the effectiveness of tocopherol and tocotrienol on various bone parameters of different osteoporotic models. Most studies found that tocotrienol offered better protection than tocopherol based on its action on bone resorbing cytokines, biomarkers, calcium content and histomorphometric parameters [52-54]. There were several conditions where alpha tocopherol are equally effective as tocotrienol and there were also reports where alpha tocopherol showed better results than tocotrienol [55]. In our study we used annatto tocotrienol, tocopherol free tocotrienol mixture.

The complexity in the pathogenesis of glucocorticoid-induced fracture and the efficacy of treatment should be determined on the basis of fracture prevention. Even though the bone structure showed marked improvements, it did not correlate to a larger increase in bone strength. This might suggest that the increase risk of fracture in glucocorticoid induced osteoporosis does not directly correlate with the improvement in bone structure. The mechanism how annatto tocotrienol counteract the effects of glucocorticoids need to be explored. Annatto tocotrienol reduced bone resorption but glucocorticoid therapy affects bone primarily through reduction in bone formation and osteocyte apoptosis. The General Practice Research Database (GPRD) study reported that the increase in fracture risk in glucocorticoid induced osteoporosis is mostly independent of BMD. Adjustment for BMD did not substantially change the relative rate of fracture in oral glucocorticoid users where glucocorticoid users had considerably higher fracture risk at the same BMD level compared to control [56,57]. The tested dose of annatto tocotrienol was effective in decreasing the bone resorption but might not be sufficient to increase the bone formation. Further intervention with higher doses or longer duration of treatments should be evaluated in future studies.

There are several limitations of this study. Bone formation marker was not included in this study for technical reasons. By including the bone formation marker, the effect of annatto tococtrienol on osteoblast and bone formation activities can be further determined. In this study, only a single dose of annatto tocotrienol (60 mg/kg/day) was tested. Its effects at the higher or lower doses were not determined. Radiological examination, such as microCT which can provide better bone structural evaluation was not performed in this study which will be considered in the future.

Conclusion

The results of this study confirmed the antioxidant effects of annatto tocotrienol and its ability to protect the bone against damages caused by free radicals. This might open up a new possibility for preventing glucocorticoid-induced osteoporosis and the resulting fractures. Based on its positive effects and safety profile annatto derived tocotrienol is a potential effective natural supplement in the prevention of glucocorticoid induced osteoporosis. However the mechanisms involved need further exploration.

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