Effect of 11β-HSD1 dehydrogenase activity on bone histomorphometry of glucocorticoid-induced osteoporotic male Sprague-Dawley rats

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ABSTRACT

Introduction: Glucocorticoids cause osteoporosis by decreasing bone formation and increasing bone resorption activity. Glucocorticoid action in bones depends on the activity of 11-beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme, which plays an important role in regulating corticosteroids. 11β-HSD1 is expressed by human and rat osteoblasts. We aimed to investigate the relationship between 11β-HSD1 dehydrogenase activity and bone histomorphometric changes in glucocorticoid-induced osteoporotic bone in rats.

Methods: A total of 30 male Sprague-Dawley rats (aged three months, weighing 200–250 g) were divided into three groups of ten each. Group 1 rats were the baseline control, which were sacrificed untreated at the beginning of the study. Group 2 rats underwent sham operation and were administered with vehicle olive oil intramuscularly at 0.05 ml/kg. Group 3 rats were adrenalectomised and administered with an intramuscular injection of dexamethasone 120 µg/kg body weight/day. The treatment was started two weeks after the operation, for a duration of two months. Plasma osteocalcin, plasma pyrodinoline, plasma corticosterone and 11β-HSD1 were measured, and bone histomorphometry analysis was performed.

Results: Dexamethasone treatment caused an increase in plasma corticosterone level, together with a significant reduction in 11β-HSD1 dehydrogenase activity of the bone, along with a higher plasma level of the bone resorption marker, pyridinoline. Dexamethasone treatment also caused a reduction in trabecular volume, number and thickness, and an increase in trabecular separation.

Conclusion: Long-term glucocorticoid treatment reduces the 11β-HSD1 dehydrogenase activity in the bone, which can otherwise lead to bone loss due to the increased level of active glucocorticoids.

Keywords: 11-beta-hydroxysteroid dehydrogenase type 1, bone, dexamethasone, glucocorticoids, osteoporosis

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INTRODUCTION

Long-term glucocorticoid therapy has serious clinical implications, as it can induce osteoporosis.[1] Excess glucocorticoid increases the risk of fracture due to its rapid impact on bone mineral density, which is dependent on the dose and duration of glucocorticoid therapy. Osteoporosis induced by glucocorticoid affects the trabecular bone more severely than the cortical bone. Bone microarchitecture, rather than mineral content, is the main target of glucocorticoid action, which leads to fracture.[2,3]

The process of bone remodelling is targeted at multiple levels by glucocorticoids, leading to both reduced formation and enhanced resorption. Osteoblasts are the principal sites of action of glucocorticoids in the skeleton. Cortisol interferes with the metabolism of mature osteoblasts by stabilising collagenase III mRNA and inhibiting the synthesis of type I collagen and β1-intergrin, which are the two major components of the extracellular matrix.[4] Osteocalcin gene expression is tightly regulated during bone development. Glucocorticoids modify the gene expression via the glucocorticoid-responsive elements, which have been identified in the osteocalcin promoter.[5,6] Glucocorticoids inhibit osteoblast functions by decreasing cell proliferation and terminal differentiation. Osteoporosis caused by excessive glucocorticoids can be attributed to the inability of glucocorticoids to balance its apoptotic effect on the osteoblasts and mature osteocytes by inhibiting bone resorption. This is because mature osteoclasts lack

functional glucocorticoid receptors.[7] Glucocorticoids also promote apoptosis of the osteoblasts and mature osteocytes. Moreover, osteoclasts are protected against the pro-apoptotic effect of glucocorticoids.[8,9]

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) has been shown to be important in mediating glucocorticoid hormone action in bone tissue.[10] Sufficient glucocorticoid availability is crucial for normal bone development and maintenance.[11,12] 11β-HSD1 is a low-affinity nicotinamide adenine dinucleotide phosphate (NADP)(H)-dependent enzyme that acts bidirectionally (dehydrogenase/reductase), interconverting inactive cortisone into active cortisol in humans and 11-dehydrocorticosterone to corticosterone in rodents. Both 11β-HSD1 and 11β-HSD2 are present in skeletal tissues; however, 11β-HSD1 is the prominent isoenzyme present in adult bone and is expressed by osteoblasts and osteoclasts.[10,13,14] Both reductase (cortisone to cortisol conversion) and dehydrogenase (cortisol to cortisone conversion) are evident in human bone. Reductase acts as a local activator, regenerating the natural ligand of glucocorticoid receptor through a mechanism known as pre-receptorial regulation, which appears to be the key aspect of tissue responsiveness to glucocorticoids.[15]

Expression of reductase activity provides a mechanism for local generation of active glucocorticoids, and dehydrogenase activity plays an important role in attenuating local availability of active glucocorticoids. Bone-forming cells can generate active glucocorticoids from the inactive form and self-regulate the local concentration of active glucocorticoids by modulating 11β-HSD1 expression and activity. Mature osteoblasts and osteocytes do not require endogenous glucocorticoid action for normal skeletal development, bone peak mass and bone cell number.[8] Osteoblasts provide themselves with the right microenvironment through the regulation of glucocorticoid concentration by the 11β-HSD1 enzyme. Inhibition of 11β-HSD1 activity *in vivo* leads to a significant decrease in bone resorption markers.

Bone histomorphometry involves counting and measuring tissue component, and allows for the measurement of mineralisation rate and the study of bone formation cells, remodelling unit and tissue level. It is a useful tool to explain the pathogenesis and cellular mechanisms of different metabolic bone diseases. Based on histomorphometric studies, decreased bone formation is the main determinant of glucocorticoid-induced bone loss that leads to slower bone turnover, with a disproportionate reduction of bone formation over bone resorption. It is also associated with decrease bone remodelling, which is similar to senile osteoporosis.[16] This is the result of a decreased pool of osteoblasts available for bone formation due to cell genesis and death.[17,18] Histomorphometric studies also showed a decreased rate of mineral apposition and decreased width of trabecular packets.[19]

Since 11β-HSD1 acts as a pivotal determinant of steroid response in the bone, and as its osteoblastic activity is a contributing factor in glucocorticoid-induced osteoporosis, we aimed to determine the relationship between 11β-HSD1 activity and bone histomorphometric changes. To date, many past research studies have defined the relationship between glucocorticoid treatment and 11β-HSD1 to bone metabolism; unfortunately, they were not supplemented with adequate bone histomorphometric analysis. The aim of this study was to determine the relationship between 11β-HSD1 activity and histomorphometric changes in glucocorticoid-induced osteoporotic bones. The results could play an important role to determine whether 11β-HSD1 inhibitors could improve the histomorphometric changes caused by long-term glucocorticoid treatment and whether this agent could be considered to be potentially useful in preventing glucocorticoid-induced osteoporosis.

METHODS

Prior ethical clearance for the study was obtained from Universiti Kebangsaan Malaysia Research and Animal Ethics Committee. A total of 30 three-month-old male Sprague-Dawley rats weighing 220–250 g were obtained from the National University of Malaysia Animal Breeding Centre. The animals were divided into three groups of ten rats and given the following treatment: G1, the baseline control group did not receive any treatment; G2, the sham-operated control was administered with vehicle olive oil intramuscularly at 0.05 ml/100 g; and G3 was adrenalectomised and given intramuscular dexamethasone 120 μg/kg/day (0.05 ml/100 g). Adrenalectomy was performed two days after obtaining the animals. The animals were first anaesthetised with Ketapex and Xylazil (Troy Laboratories Pty Ltd, Glendenning, NSW, Australia). Dorsal midline and bilateral flank muscle incisions were then made and the adrenal glands were identified and removed. The incisions were sutured and Poviderm Cream (Hoe Pharmaceuticals, Petaling Jaya, Malaysia) was applied to the wound daily to prevent infection and aid wound healing. The rats were also given an intramuscular injection of Baytril 5% (Bayer Korea Ltd, Ansan-Shi, Korea) for five days. The sham-operated rats underwent a similar procedure, except that their adrenal glands were left *in situ*.

Two rats were kept per cage under 12-hour natural

sunlight and darkness at night. They were fed rat pellets (Gold Coin, Port Klang, Malaysia) ad libitum. The sham-operated rats were given tap water, while the adrenalectomised rats were given normal saline ad libitum in order to replace the salt lost due to mineralocorticoid deficiency post adrenalectomy. The treatment was started two weeks after the adrenalectomy procedure. Dexamethasone (Sigma Chemical Co, St Louis, MO, USA) 120 µg/kg was prepared by mixing dexamethasone 2,400 µg in 10 ml of olive oil (Bertolli, Secaucus, NJ, USA) and administered intramuscularly at 0.05 ml/100 g per rat for six days in the week. The dosage used was based on previous published studies.[20,21] The sham-operated rats were administered equivalent volumes of vehicle olive oil intramuscularly. Both treatments were given for two months. The following parameters were measured at the end of the two-month treatment: plasma corticosterone level, 11β-HSD1 enzyme activity in the bone, bone biochemical markers (plasma osteocalcin and pyridinoline) and structural parameters of bone histomorphometry.

Blood samples were taken before and after the completion of the treatment period. The blood samples were centrifuged at 3,000 rpm at 4°C for 15 minutes, and the plasma was kept in aliquots at –70°C until analysis. On completion of the treatment, the animals were sacrificed under anaesthesia, and the femoral bones were cleared from the surrounding tissues, wrapped in gauze and aluminium foil and frozen at –70°C until analysis. For the bone histomorphometric study, the distal parts of the left femoral bones were cut longitudinally into two halves and the lateral halves were fixed in 70% ethanol.

Plasma corticosterone measurement was conducted using high-performance liquid chromatography (HPLC) (Waters Corp, Milford, MA, USA) using the mobile phase, which consisted of methanol and deionised water in the ratio of 60:40. 100 µl of dichloromethyl (Merck, Darmstadt, Germany) containing 0.025 mg/L cortexolone (Sigma Chemical Co, St Louis, MO, USA) was added to 100 µl of plasma. The mixture was then shaken vigorously for ten minutes and centrifuged at 1,000 *g* for five minutes. The upper aqueous phase was discarded, and 0.05 M sodium hydroxide (NaOH) was added and centrifuged for five minutes at 1,000 *g*. The aqueous phase was discarded, and this process was repeated. After being washed with NaOH, the organic layer was transferred into another tube and evaporated to dryness in a vacuum concentrator. It was then resuspended in 1 ml of HPLC mobile phase and placed in vials. Steroids were detected at 254 nm using Empower™ 3 Software (Waters Corp, Milford, MA, USA).

Enzyme activity was measured by the method used in previous studies, with some modifications.[22,23] The bones were ground into small pieces using mortar and pestle, and then suspended in Krebs-Ringer bicarbonate buffer. The suspended bone pieces were then homogenised and kept overnight at 4°C. The homogenised bone was centrifuged at 12,100 *g* for 20 minutes at 4°C, and the supernatant was decanted. Total protein content was estimated calorimetrically (Bio-Rad, Hercules, CA, USA) on aliquots of the homogenate. 200 µm nicotinamide adenine dinucleotide phosphate (NADP) (Sigma Chemical Co, St Louis, MO, USA) and 12 nM (1,2,6,7-^{3}H) B (specific activity: 84 Ci/mmol) (Amersham, Buckinghamshire, England) were added to the bone tissue homogenates containing 0.5 mg protein. Co-factor NADP drove the enzymatic oxidation reaction. Krebs-Ringer bicarbonate buffer containing 0.2% glucose and 0.2% bovine serum albumin was added to make up the total assay volume of 250 µl. The required protein concentration and incubation period were determined by the standard curve using various concentrations of the standard.

After incubation in a water bath at 37°C for two hours, the reaction was terminated by the addition of ethyl acetate, and steroids were then extracted. The organic layer was separated by centrifugation at 3,000 rpm at 4°C for 10 minutes. The top layer was then transferred into new test tubes and evaporated to dryness at 55ºC in a vacuum concentrator. Steroid residues were dissolved in ethanol containing non-radioactive carrier 11-dehydrocorticosterone and corticosterone. They were then separated by thin layer chromatography (Whatman, W & R Balston Limited, Kent, UK) in chloroform and 95% ethanol in the ratio of 92:8. The fractions corresponding to the steroid were located by UV lamp absorption at 240 nm, scraped, transferred to scintillation vials and counted in scintillation fluid (Cocktail T) (Merck, Darmstadt, Germany) using the Kontron Betamatic fluid scintillation counter, Wallac System 1409 (Wallac Oy, Mustionkatu, Turku, Finland). Enzyme activity was calculated as the percentage conversion of [^{3}H] 11-dehydrocorticosterone to [^{3}H] corticosterone from the radioactivity of each fraction.

Bone biochemical markers were determined using enzyme-linked immunosorbent assay (ELISA). The kits used for serum osteocalcin and serum PYD measurements were ELISA Rat-MID™ rat osteocalcin (Nordic Bioscience Diagnostics, Hovedgade Hevlev, Denmark) and EIA Metra serum PYD kit (Quidel Corporation, San Diego, CA, USA), respectively. For structural parameter measurements, which included trabecular bone volume

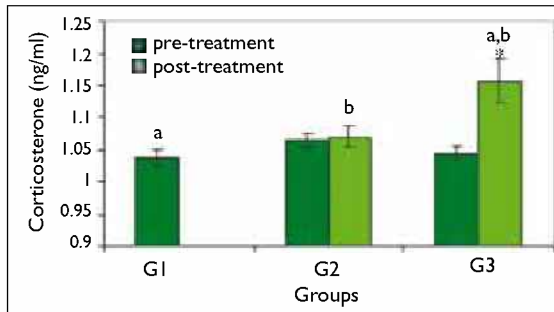


Fig. 1 Graph shows the effect of dexamethasone treatment (120 μg/kg/day) on the plasma corticosterone level of adrenalectomised rats for two months. Data is presented as mean ± SEM. Identical letters (a, b) indicate significant difference between groups at $p < 0.05$. * significant difference before and after treatment for the same group. G1: baseline; G2: sham-operated control; G3: adrenalectomised and given intramuscular dexamethasone 120 μg/kg/day.

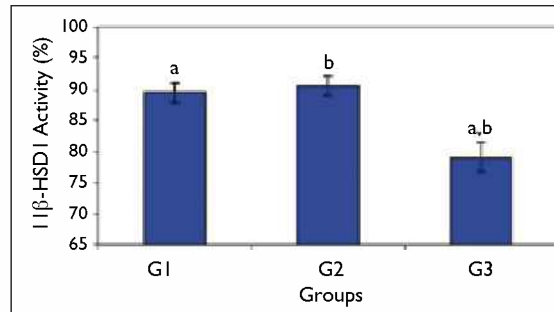
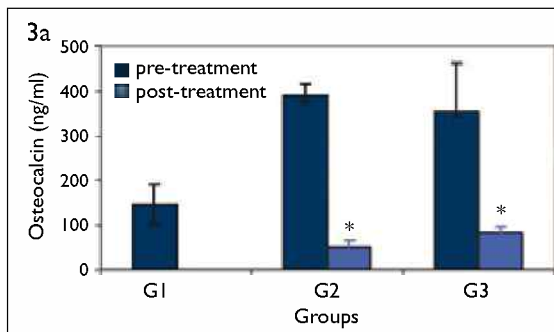


Fig. 2 Graph shows the effect of dexamethasone treatment (120 μg/kg/day) on 11β-HSD1 dehydrogenase activity in the femoral bones of adrenalectomised rats for two months. Data is presented as mean ± SEM. Identical letters (a, b) indicate significant difference between groups at $p < 0.05$. G1:baseline; G2: sham-operated control; G3: adrenalectomised and given intramuscular dexamethasone 120 μg/kg/day.



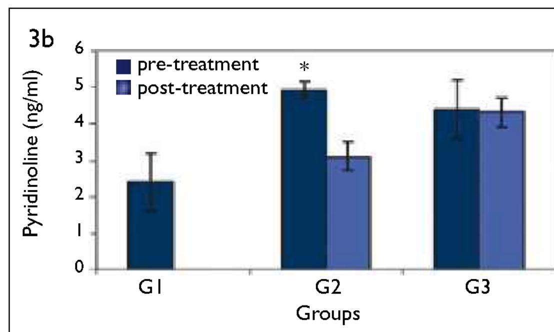


Fig. 3 Graph shows the effect of dexamethasone treatment (120 μg/kg/day) on (a) plasma osteocalcin and (b) plasma pyrodinoline level of adrenalectomised rats for two months. Data is presented as mean ± SEM. * significant difference before and after treatment for the same group. G1: baseline; G2: sham-operated control; G3: adrenalectomised and given intramuscular dexamethasone 120 μg/kg/day.

(bone volume/tissue volume [BV/TV]) and trabecular thickness (Tb.Th), undecalcified bone samples were embedded in the mixture of Osteo-Bed resin solution A (Polysciences Inc, Warrington, PA, USA) with benzoyl peroxide plasticised (catalyst) (Polysciences Inc, Warrington, PA, USA) in the ratio of 100 ml:1.4 g. The samples were sectioned at 9-µm thickness using a Leica RM2155 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and stained using the Von Kossa method. Structural parameters were analysed by a Leica DMRXA2 image analyser (Leica Microsystems GmbH, Wetzlar, Germany) using the VideoTesT-Master software (VideoTesT Ltd, St Petersburg, Russia). Histomorphometric parameter measurements were performed randomly at the metaphyseal region, which was 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 known as the secondary spongiosa area, which is rich in trabecular bone. All parameters were measured according to the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee in 1987.[24]

Data were tested for normality using the Kolmogrov-Smirnov test. Since the groups were found to be normally distributed, the data was analysed by parametric statistics, i.e. the ANOVA test followed by post hoc Tukey test for comparison between treatment groups. Student's *t*-test was used for comparison before and after treatment within the same group. Data was analysed using the Statistical Package for the Social Sciences version 12.0 (SPSS Inc, Chicago, IL, USA). Data was presented as mean ± standard error of the mean (SEM).

RESULTS

After two months, the plasma concentration of corticosterone increased significantly in dexamethasone-treated adrenalectomised rats (G3) compared to the pre-treatment level. The post-treatment plasma corticosterone level was also significantly higher in G3 rats compared to the sham-operated (G2) and baseline (G1) rats. No significant changes were observed between the pre- and post-treatment levels in the sham-operated group (G2) (Fig. 1).

At the end of the treatment period, dexamethasone-treated adrenalectomised rats (G3) had significantly lower

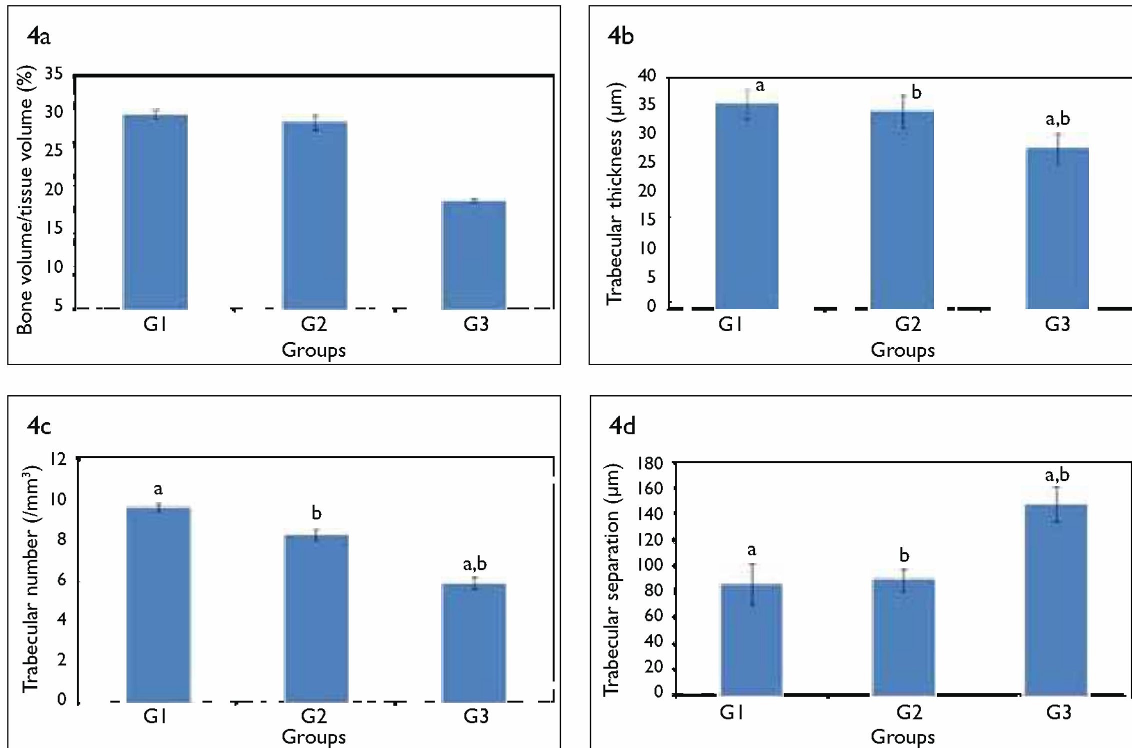


Fig. 4 Graph shows the effect of dexamethasone treatment (120 μg/kg/day) on (a) bone volume/tissue volume; (b) trabecular thickness; (c) trabecular number; and (d) trabecular separation of adrenalectomised rats for two months. Data is presented as mean ± SEM. Identical letters (a, b) indicate significant differences between groups at $p < 0.05$. G1: baseline; G2: sham-operated control; G3: adrenalectomised and given intramuscular dexamethasone 120 μg/kg/day.

11β-HSD1 dehydrogenase activity compared to the sham-operated (G2) and baseline (G1) groups (Fig. 2). Plasma osteocalcin, a marker of bone formation, decreased significantly after two months of treatment in both the sham-operated group (G2) and the adrenalectomised group treated with dexamethasone (G3); however, there was no significant difference in the post-treatment level between the two groups (Fig. 3a). The level of pyridinoline, which indicates the rate of bone resorption, in the sham-operated group (G2) decreased after two months compared to the pre-treatment level, but in the adrenalectomised group treated with dexamethasone (G3), the levels before and after treatment did not show any significant difference (Fig. 3b).

The BV/TV (Figs. 4a & 5), Tb.Th (Figs. 4b & 5) and trabecular number (Tb.N) (Figs. 4c & 5) reduced significantly after two months in dexamethasone-treated adrenalectomised (G3) rats compared to the baseline (G1) and sham-operated (G2) rats. Apart from that, there was also significant increase in trabecular separation (Tb.Sp) (Figs. 4d & 5) in the dexamethasone-treated adrenalectomised rats (G3).

DISCUSSION

The rats were adrenalectomised to remove the endogenous glucocorticoids that were subjected to circadian rhythm, as well as physical and emotional stress. They were then given predetermined doses of dexamethasone to ensure a constant level of glucocorticoids in the body. No replacement of mineralocorticoids was considered necessary, as it has been shown earlier that they have no influence on bone metabolism. However, the rats were given normal saline ad libitum to maintain normal body sodium concentration.[20] The dose and duration of the dexamethasone treatment (120 μg/kg) were based on previous studies, which showed reduced calcium content and femur length in dexamethasone-treated adrenalectomised rats; however, bone histomorphometric analysis was not done in this study.[21]

We observed that long-term dexamethasone-treated adrenalectomised rats had a significantly higher level of plasma corticosterone and lower 11β-HSD1 dehydrogenase activity in the bone as compared to the sham-operated and baseline groups. The increase in plasma corticosterone level is not easily explainable, since most of the previous studies showed that exogenous dexamethasone suppressed both plasma and anterior pituitary adrenocorticotrophic hormone, and subsequently, the production of corticosterone.[25] Despite the increase in plasma corticosterone level, there was a significant reduction of local 11β-HSD1 activity in the bone. However, the corticosterone level was not the

determinant factor of local 11β-HSD1 activity in the bone, as explained by earlier clinical studies, which reported that bone tissue-specific response to glucocorticoids was strongly correlated with circulating inactive hormone (cortisone).[26] Treating the adrenalectomised rats with intramuscular injections of glucocorticoids 120 µg/kg/day for two months also caused a significantly higher level of bone resorption compared to the sham-operated group, and this led to glucocorticoid-induced osteoporotic changes, as demonstrated by the bone histomorphometric analysis.

The high plasma corticosterone level could also be due to defective systemic 11β-HSD1 dehydrogenase activity, causing reduced conversion of inactive glucocorticoid from the active form. This is supported by evidence indicating that defective dehydrogenase activity could lead to an increased conversion of cortisone to cortisol, causing hypercortisolism.[27] 11β-HSD1 was reported to be the local activator that was present at the bone level and acted as the pre-receptorial regulator that produced the biological effect. However, studies have shown that an inverse relationship exists between dexamethasone concentration and 11β-HSD1 reductase activity.[28] Cooper et al reported that dexamethasone causes a dose-dependent increase in 11β-HSD1 dehydrogenase activity.[15] The high level of bone 11β-HSD1 dehydrogenase activity in the baseline and sham-operated group could be explained by the fact that mature osteoblasts and osteocytes do not require endogenous glucocorticoid action for normal skeletal development, peak bone mass and bone cell number. At this stage, glucocorticoids mainly exert a harmful effect.

Development of osteoporosis due to glucocorticoids was indicated by the increase in bone resorption activity. Excess glucocorticoids due to dexamethasone treatment compromises the bone by increasing osteoclastic bone resorption, as shown by the significantly higher plasma pyridinoline levels in the adrenalectomised rats, which were given long-term dexamethasone treatment (G3). The fact that excessive glucocorticoids caused an increase in bone resorption activity was consistent with earlier studies.[29] Moreover, low 11β-HSD1 dehydrogenase activity could have caused higher local generation of active glucocorticoid that contributed to the increase in bone resorption due to inappropriately increased osteoclastic activity. Dexamethasone administration was found to promote human osteoclast formation.[30] It has also been suggested that glucocorticoids stimulate bone resorption directly via the activation of mature osteoclast[8] and by inducing osteoclastogenesis.[16] Glucocorticoids regulate the activity of osteoclasts indirectly through factors derived from osteoblasts. A critical step in osteoclast differentiation is the Receptor Activator of NF-kB (RANK) signalling pathway, whose ligand (RANKL) is a cell surface receptor expressed by osteoblast and kept inhibited by osteoprotegerin (OPG), which is also secreted by osteoblast. Glucocorticoid alters the RANKL/OPG ratio by increasing the expression of RANKL and decreasing the expression of OPG. Glucocorticoids also enhance the expression of colony-stimulating factor-1, which, in the presence of RANKL, induces osteoclastogenesis and rapid early phase of bone resorption.[31]

Despite these facts, the plasma osteocalcin levels decreased significantly after the two-month treatment both in the dexamethasone-treated and sham-operated groups. Previous studies have reported that excess glucocorticoid *in vivo* is associated with a decrease in bone formation, as shown by a rapid decrease in bone formation markers;[32] however, our results did not show any significant difference between the dexamethasone-treated and sham-operated rats. These findings may be related to the age of the animals when they were sacrificed. In our study, serum osteocalcin levels measured after completing six weeks of treatment in the sham-operated group was significantly higher compared to those in the dexamethasone-treated group. Most studies suggest that glucocorticoids exert a profound inhibition of osteoblast functions by decreasing cell proliferation and terminal differentiation, and by promoting apoptosis of mature osteocytes. This contributes to a decreased number of mature osteoblasts, which in turn contributes to decreased bone formation, as reported in previous studies.[9,31]

Bone histomorphometry analysis showed that dexamethasone-treated adrenalectomised rats had significantly lower BV/TV, Tb.Th and Tb.N, while the Tb.Sp was significantly higher compared to that of the baseline and sham-operated groups. These results indicate that lower 11β-HSD1 dehydrogenase activity was associated with an increase in bone loss. As reported in a previous study, defective dehydrogenase activity could lead to increased conversion of cortisone to cortisol, and subsequently, cause an increase in active glucocorticoids in the bone. High levels of active glucocorticoids would causes a reduction in osteoblast number and activity while stimulating osteoclast activity, which would, in turn, contribute to bone loss.[27] This is in accordance with previous studies that reported that the bone formation rate in mice with transgenic expression of 11β-HSD2 (converts active to inactive glucocorticoids) was significantly higher compared to that in glucocorticoid-treated-wild type mice.[8] This result is also supported by the fact that inhibition of 11β-HSD1 activity leads

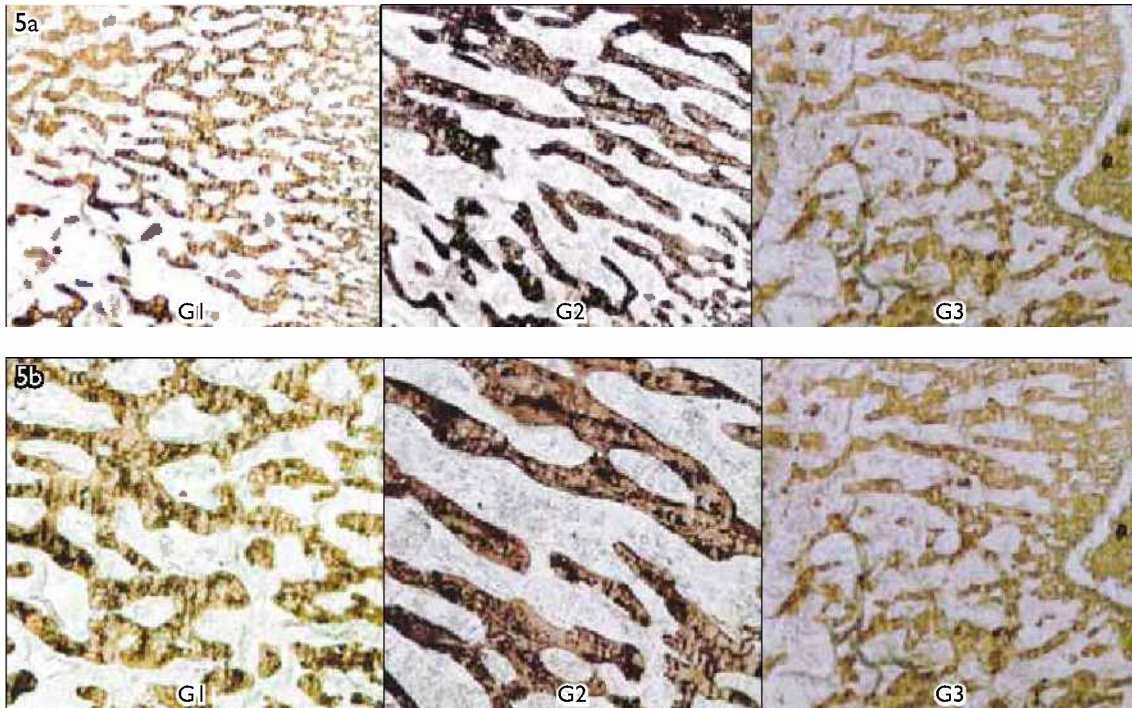


Fig. 5 Photomicrographs show trabecular bones stained with Von Kossa stain at (a) 25 × and (b) 50 × magnification. Dexamethasone-treated rats exhibited more separated and thinner trabecular bone. G1: baseline; G2: sham-operated rats; G3: adrenalectomised and given intramuscular dexamethasone 120 μg/kg/day.

to significant decrease in bone resorption markers. The decreased osteoblast number and function leads to reduced synthesis of bone matrix, and consequently, the Tb.Th and BV/TV, as reported by Dempster et al.[33] This would result in a reduction in the total amount of bone replaced in each remodelling cycle, which is demonstrated by histomorphometric analysis.

In conclusion, our study confirmed that long-term excessive glucocorticoid treatment causes structural bone loss and decreased 11β-HSD1 activity in the bone. Further studies are required in order to ascertain whether specific 11β-HSD1 inhibitors may prevent glucocorticoid-induced osteoporosis.

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