The Effects of Piper Sarmentosum Water Extract on the Expression and Activity of 11β-Hydroxysteroid Dehydrogenase Type 1 in the Bones with Excessive Glucocorticoids

Abstract

Background: Long-term glucocorticoid therapy causes secondary osteoporosis leading to pathological fractures. Glucocorticoid action in bone is dependant upon the activity of 11β-hydroxysteroid dehydrogenase type 1 enzyme (11β-HSD1). Piper sarmentosum is a local herb that possesses the ability to inhibit 11β-HSD1 enzyme activity. We aimed to determine the effects of Piper sarmentosum water extract on 11β-HSD1 expressions and activity in the bones of glucocorticoid-treated adrenalectomized rats.

Methods: Forty male Sprague–Dawley rats (200-250 g) were used. Twenty-four animals were adrenalectomized and received intramuscular injection of dexamethasone (120 μg/kg/day). They were simultaneously administered with either Piper sarmentosum water extract (125 mg/kg/day), GCA (120 mg/kg/day) or distilled water as vehicle by oral gavage for two months. Eight animals were sham-operated and given vehicle daily, i.e. intramuscular olive oil and oral distilled water.

Results: Following two months treatment, dexamethasone-treated adrenalectomized rats had significantly lower 11β-HSD1 dehydrogenase activity and higher 11β-HSD1 expression in the femoral bones compared to the sham-operated and baseline group. The rats supplemented with Piper sarmentosum water extract had significantly higher 11β-HSD1 dehydrogenase activity and lower 11β-HSD1 expression in the bones.

Conclusion: The results showed that Piper sarmentosum water extract had the ability to prevent glucocorticoid excess in the bones of glucocorticoid-treated adrenalectomized rats through the local modulation of 11β-HSD1 expression and activity, and may be used as prophylaxis for osteoporosis in patients on long-term glucocorticoid treatment.

Keywords ● Piper sarmentosum ● 11β-hydroxysteroid dehydrogenase type 1 ● dexamethasone ● glucocorticoids ● osteoporosis

Introduction

Long-term glucocorticoid therapy induces osteoporosis which is clearly seen in glucocorticoid-induced osteoporosis.
Corticosteroid concentration and receptor expression, duration of exposure, and stage of cellular differentiation are the factors influencing glucocorticoids action in vitro. Bone forming cells needs sufficient glucocorticoid availability for normal bone development, but mature osteoblast and osteocytes do not require endogenous glucocorticoids. Circulating active steroids have negative correlations with bone mineral density (BMD) of the lumbar spine. At molecular level, glucocorticoid signaling is mediated via the glucocorticoid receptor (GRα), which is expressed in tissues responsive to glucocorticoid action including osteoblasts. Patients with endogenous glucocorticoid excess develop osteoporosis mainly due to rapid suppression of bone formation. Glucocorticoids inhibit osteoblast functions by promoting apoptosis of osteoblasts and mature osteocytes together with the inhibition of cell proliferation and differentiation. Increase in osteoclastic activity which causes the increase in bone resorption also contributes to the development of osteoporosis due to excess of glucocorticoids.

Bone tissue response to glucocorticoids does not correlate with serum levels of active glucocorticoid, but it is strongly correlated with the serum levels of the inactive glucocorticoid, cortisone. This indicates that there may be a local factor that modulates the sensitivity of glucocorticoids in the tissue. 11β-hydroxysteroid dehydrogenase has been shown to be regulating glucocorticoid action in the tissue at the pre-receptor level. This enzyme is found in almost all glucocorticoids target tissues including the skeletal tissues. There are two isoenzymes of 11β-hydroxysteroid dehydrogenase, 11β-HSD1 and 11β-HSD2 with 11β-HSD1 being the predominant isoenzyme expressed in human osteoblasts and osteoclasts. It interconverts inactive cortisone to active glucocorticoids, cortisol. The activity and synthesis of 11β-HSD1 depends on the glucocorticoids concentration in the bone. Both dehydrogenase and reductase activities are present in adult bones, but the 11β-HSD1 activity in adult human osteoblast is primarily reductase, which converts cortisone into biologically active cortisol. In contrast, dehydrogenase activity converts active glucocorticoids to inactive metabolite. Bone tissue cells can self-regulate the local concentration of active glucocorticoids by modulating the expression and activity of 11β-HSD1. At the physiological levels of endogenous glucocorticoids, 11β-HSD1 activity is predominantly reductase in order to generate active glucocorticoids. However, with excessive levels of glucocorticoids, particularly with some synthetic steroids, dehydrogenase activity is diverse ranging from increase to attenuation of active glucocorticoids local availability.

Liquorice and its derivatives, carbenoxolone and glycyrrhetinic acid (GCA), inhibit 11β-HSD activity. Treatment with carbenoxolone led to a significant fall in bone resorption markers, but did not show any effect on bone formation markers. Glycyrrhetinic acid was also shown to totally inhibit 11β-HSD 1 activity in an ‘in vitro’ study. Piper sarmentosum, locally known as “daun kadok” is a glabrous creeping terrestrial herb usually used to flavor local cuisine. The methanolic extract of Piper sarmentosum also possesses a natural superoxide scavenger, naringenin. A recent study reported that it inhibited the 11β-HSD1 activity in the liver and adipose tissue of ovariectomized female rats. In addition, Piper sarmentosum was also found to reduce the bone resorption marker pyridinoline, in glucocorticoid treated adrenalectomised rats.

Both osteoblasts and osteoclasts exhibit the expression of 11β-HSD1, which is responsible for the local generation of glucocorticoids in bone. The aim of this study was to determine the effects of Piper sarmentosum extract on the bones of glucocorticoid treated adrenalectomized rats through the modulation of local 11β-HSD1 activity and expression in bone tissues.

**Materials and Methods**

**Preparation of Piper sarmentosum Water Extract**

Fresh leaves of Piper sarmentosum were collected from the Ethnobotanic Garden, Forest Research Institute Malaysia (FRIM). They were identified and confirmed by a plant taxonomist from the Medicinal Plant Division, FRIM, and were given a voucher specimen number (FRI 45870). The extraction procedures were performed at the FRIM laboratory. Fresh Piper sarmentosum leaves were cleaned with tap water and dried at room temperature before being chopped into small pieces. The leaves were then boiled in distilled water (90%, v/v) at 80°C for three hours. The water extract was then concentrated and dried into powder by freeze-drying. The powdered extract was stored at 4°C until further use.

**Animals and Treatment**

All procedures were carried out in accordance with the guidelines of the Universiti Kebangsaan

Forty three-month-old male Sprague-Dawley rats weighing 220-250 grams were obtained from the UKM Animal Breeding Centre. Animals, which were sick and underweight, were excluded from the study. The rats were divided into groups of eight rats and given following treatments: G1; the control group, which did not receive any treatment, G2; sham operated control group, which was given intramuscular (IM) olive oil as vehicle (0.05 ml/100 g), G3; adrenalectomized (adrx) group, which were given IM dexamethasone (120 µg/kg/day); G4: adrx group, which was given IM dexamethasone (120 µg/kg/day) and glycirrhizic acid (GCA, 120 mg/kg/day) by oral gavage, and G5: adrx group, which was given intramuscular dexamethasone (120 µg/kg/day) and water extract of *Piper sarmentosum* leaves (125 mg/kg/day) by oral gavage. Adrenalectomy was done two days after receiving the animals. The animals were first anaesthetized with Ketapex and Xylazil (Troy Laboratories, 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, Malaysia) was applied to the wound daily to prevent infection and aid wound healing. The rats were also given Intramuscular injection of Baytril 5% (Bayer Health Care, Thailand) for 5 days. The sham-operated rats underwent a similar procedure except that the adrenal glands were not removed.

The treatment was started two weeks after adrenalectomy. Dexamethasone (Sigma, USA) was dissolved in olive oil (Bertolli, Italy) and administered IM (120 µg/kg/day) six days-a-week for two months. The dose and duration of treatment was determined by a pilot study. The *Piper sarmentosum* leaves extract was provided by the Forest Research Institute of Malaysia (FRIM). *Piper sarmentosum* and GCA (Sigma, USA) were dissolved in normal saline and administered for two months. The sham-operated rats were administered equivalent volumes of vehicle (olive oil) intramuscularly and vehicle (normal saline) by oral gavage. The dexamethasone-treated adrenalectomized rats (G3) were also administered with vehicle (normal saline, 0.1 ml/100 g) by oral gavage. The administrations of *Piper sarmentosum* leaves water extract, GCA and dexamethasone were started simultaneously two weeks after the adrenalectomy. The treatment was given for two months.

The animals were kept in clean cages under natural sunlight during daytime and darkness at night. They had free access to rat pellets (Gold Coin, Malaysia). The sham-operated animals had free access to tap water, while the adrenalectomized animals had free access to normal saline instead of tap water to replace the salt loss due to post-adrenalectomy mineralocorticoid deficiency. The activity and expression of 11β-HSD1 in femoral bone were measured at the end of two months of treatment.

**Sample Collection**

The right femoral bones were cleared of surrounding tissues, wrapped in a piece of gauze and aluminium foil, and frozen at -70°C until analyzed. The left femoral bones were cut at the mid shaft 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 lateral part of the bone was then subjected to decalcification process in a mixture of ethylenediaminetetraacetate (EDTA) and 10% formalin.

**Assay for 11β-HSD1 Dehydrogenase Activity**

The activity of 11β-HSD1 dehydrogenase was measured using the modified technique of Cooper et al. The right femoral bones were dissected, cleared from soft tissues and washed extensively in phosphate-buffered saline to reduce the fat content. They were ground into small pieces before being suspended in Krebs-Ringer bicarbonate buffer and homogenized overnight at 4°C. The bone homogenate was centrifuged at 12,100 g for 20 min at 4°C, and the supernatant was decanted. The total protein content was estimated calorimetrically (Bio-Rad, Hercules, CA, USA). Two hundred micromolar NADP and 12 nM [1,2,6,7-3H] corticosterone (specific activity:84 Ci/mmol; Amersham, Buckinghamshire, England) were added to the tissue homogenates containing 0.5 mg protein. Krebs-Ringer bicarbonate buffer containing 0.2% glucose and 0.2% bovine serum albumin (BSA) was then 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. After incubation in a water bath at 37°C for two hours, the reaction was terminated by the addition of ethyl acetate and the steroids were then extracted. The organic layer was separated by centrifugation at 3000 rpm and 4°C for 10 min. The top layer was then transferred into new test tubes and evaporated to dryness at 55°C in a vacuum concentrator. Steroid residues were dissolved in an ethanol containing nonradioactive carrier of 11-dehydrocorticosterone and corticosterone.
They were then separated by thin layer chromatography, TLC (Whatman, UK) in 92:8 ratio of chloroform and 95% ethanol. The fractions corresponding to the steroids were located by UV lamp absorption at 240 nm, scraped, transferred into scintillation vials and counted in scintillation fluid (Cocktail T) in a Kontron Betamatic fluid scintillation counter (Merck, Germany). Enzyme activity was calculated as the percentage conversion of the active [3H] corticosterone to inactive [3H] 11-dehydrocorticosterone from the radioactivity of each fraction. Enzyme activity was measured by the method used by previous studies with some modification. The statistical software used was the Statistical Package for Social Sciences (SPSS) version 12. The data was tested for normality using the Kolmogrov-Smirnov test. Since the groups were found to be normally distributed, they were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey pos-hoc test for pairwise comparisons. A P value of < 0.05 was taken as significant. Data are presented as mean±standard error of the means (SEM).

Measurement of 11β-HSD1 Expression

The formalin-fixed paraffin-embedded bone sections were deparaffinized and rehydrated. For antigen retrieval, the sections were incubated in 0.01M citrate buffer at 90°C for 5 minutes. The sections then were incubated in 0.3% hydrogen peroxide for 10 minutes to block the endogenous peroxide activity and subsequently incubated in 1:50 normal goat serum (Vector Laboratories; Burlingame, CA) for 20 minutes to block nonspecific antibody binding. Sections were incubated for 60 minutes with primary rabbit 11β-Hydroxysteroid Dehydrogenase (Type1) Polyclonal Antibody, and detected by goat anti-rabbit peroxidase (Vector Laboratories, Burlingame California) using DAB as a chromogen according to the manufacturer’s instructions and counterstained with haematoxylin. Controls were done by using positive tissues (liver and adipose tissue) and omissions of the primary antibody. Photomicrographs taken were scored by two blind observers at 25 and 50 times magnification. Five slides from each group were analyzed for intensity and percentage of immunopositive stained area. The scores for the cells stained were as follows: 0; no staining in the majority of cells, 1; staining in 25-50% of cells, 3; staining in 50-75% of cells, 4; staining in more than 75% of cells. The grades were summed up, and the averages were taken as the readings. This procedure followed previous studies with some modifications. The statistical software used for data analysis was the Statistical Package for Social Sciences (SPSS) version 12. The 11β-HSD1 evaluations by the two independent viewers were tested by Pearson Correlation prior to the normality test. The data were tested for normality using Kolmogrov-Smirnov test. Since the groups were found to be normally distributed, the data were analyzed using ANOVA test followed by the Tukey post-hoc test for pairwise comparison. A P value of < 0.05 was taken as statistically significant. Data are presented as mean±SEM.

Results

The adrenalectomized rat treated with intramuscular injection of dexamethasone (G3) had significantly lower 11β-HSD1 dehydrogenase activity in femoral bone compared to the sham-operated (G2) and baseline (G1) group. Supplementing the dexamethasone-treated adrenalectomized rats with GCA (G4) and Piper sarmentosum water extract (G5) extract significantly increased the 11β-HSD1 dehydrogenase activity compared to the control group. There were no significant difference in the 11β-HSD1 dehydrogenase activity of rats treated with GCA and Piper sarmentosum extract, and control or sham-operated group (figure 1).

![Figure 1: The activity of 11β-HSD1 dehydrogenase activity in femoral bones of G1: control group, G2: sham-operated group, G3: adrenalectomized rats given intramuscular dexamethasone (120 µg/kg/day), G4: adrenalectomized rats given intramuscular dexamethasone (120 µg/kg/day) and glycyrrhizic acid (120 mg/kg/day) and G5: adrenalectomized rat given intramuscular dexamethasone (120 µg/kg/day) and Piper sarmentosum water extract (125 mg/kg/day). Data are presented as mean±SEM. Same alphabets indicate significant (P<0.05) difference between groups.](image-url)
*Piper sarmentosum* extract (G5) caused a significant reduction in 11β-HSD1 dehydrogenase expression compared to the G3 group. Despite that, the 11β-HSD1 dehydrogenase expression in the control group (G1) and the dexamethasone-treated group supplemented with GCA (G4) was significantly higher than those in the sham-operated (G2) or the *Piper sarmentosum* extract supplemented (G5) group (figures 2, 3).

**Discussion**

The rats were adrenalectomized to remove the main source of endogenous glucocorticoids which are influenced by the circadian rhythm, as well as by the physical and emotional stress. The endogenous glucocorticoids were replaced by predetermined doses of dexamethasone to ensure a constant level of glucocorticoid in the body. The animals were also
given normal saline ad libitum to maintain normal sodium homeostasis. The dose and duration of dexamethasone treatment for the induction of osteoporosis were determined by a pilot study. The doses of the GCA and *Piper sarmentosum* water extract were also determined based on previous studies. 

Dexamethasone is a synthetic glucocorticoid, which is 20-30 and five times more potent than hydrocortisone and prednisolone, respectively. It is able to bind to glucocorticoid receptors (GRα). Long-term dexamethasone treatment causes significant reduction in mineral density, calcium content, and length of the femur of adrenalectomized rats. That study showed that long-term dexamethasone treatment caused a significant reduction in local 11β-HSD 1 dehydrogenase activity, but increased the expression of 11β-HSD1 in the bone. The study also showed that long-term glucocorticoid treatment led to a defect in dehydrogenase activity in the bone. Defective dehydrogenase activity might have been associated with an increase in the reductase activity, which led to an increase in the conversion of inactive cortisone to active cortisol. This would lead to an increase in the local availability of active glucocorticoids in the bone, which subsequently would increase the risk of developing glucocorticoid-induced osteoporosis. The expression of 11β-HSD1 enzyme in the bones of dexamethasone-treated rats was greater than that in the sham-operated group. This is consistent with the findings of a previous study, which reported that cortisol and dexamethasone increased the expression of 11β-HSD1 mRNA in a primary osteoblast culture. Increase in the 11β-HSD1 enzyme expression could be due to an increase in synthesis of the enzyme. Possibly, a larger proportion of 11β-HSD1 enzyme expressed in the bone demonstrated a higher reductase activity, and this caused an increase in the local availability of active glucocorticoids in the bone in agreement with a previous study.

Supplementing the dexamethasone-treated adrenalectomized rats with *Piper sarmentosum* water extract and GCA resulted in a significant increase in dehydrogenase activity. Supplementing dexamethasone-treated rats with *Piper sarmentosum* extract may have inhibited the reductase activity of the enzyme, and switched its action to dehydrogenase activity. It was reported in a previous ‘in vitro’ study that GCA totally inhibited the dehydrogenase activity of dexamethasone-treated osteoblast cells. The increase in dehydrogenase activity by *Piper sarmentosum* extract leads to a reduction in the local availability of active glucocorticoid in the bone, thereby protecting the bone from the detrimental effects of glucocorticoids excess. Previous studies also showed that treatment with *Piper sarmentosum* extract and GCA had reduced bone resorption markers. Apart from that, the expression of 11β-HSD1 enzyme in the bones of dexamethasone-treated rats supplemented with *Piper sarmentosum* extract significantly decrease. This could be explained by the fact that 11β-HSD1 synthesis is glucocorticoid-dependant. There is a possibility that *Piper sarmentosum* extract might have inhibited the synthesis of 11β-HSD1 in the bones. Such an effects, however, was not seen in the groups supplemented with GCA. This could be due to repetitive stress, during which the inhibition of the 11β-HSD 1 synthesis is overcome, and return to basal level. Despite that, GCA is a known 11β-HSD1 inhibitor, was able to completely block 11β-HSD1 reductase activity, and caused to switch the enzyme activity from reductase to dehydrogenase. It would be interesting to look for the active component(s) of the *Piper sarmentosum* extract that has the inhibitory effect on the activity and expression of 11β-HSD 1 enzyme.

Conclusion

From the results obtained, it can be concluded that *Piper sarmentosum* water extract is a potential 11β-HSD1 inhibitor that was able to switch the 11β-HSD1 action from reductase to dehydrogenase activity. This subsequently reduced the local availability of active glucocorticoids in the femur bone. As a potential inhibitor of 11β-HSD1 synthesis in the bone, *Piper sarmentosum* extract has the potential to act as a protective agent against glucocorticoid-induced osteoporosis.

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Conflict of Interest: None declared

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