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Piper sarmentosum Prevents Glucocorticoid-Induced osteoporotic Bone Resorption by Increasing 11β-Hydroxysteroid Dehydrogenase Type 1 Activity

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Abstract

Aims. Osteoporosis is a proven complication of long-term glucocorticoid therapy. Concern on glucocorticoid induced osteoporosis has increased dramatically in recent years with the widespread use of synthetic glucocorticoids. Glucocorticoid action in bone depends upon the activity of 11β-hydroxysteroid dehydrogenase type 1 enzyme (11β-HSD1). This enzyme plays an important role in regulating corticosteroids by locally interconverting cortisone into active cortisol. This has been demonstrated in primary cultures of human, mouse or rat osteoblasts. Therefore, inhibition of this enzyme may reduce bone resorption markers. *Piper sarmentosum (Ps)* is a potent inhibitor of 11β-HSD1 in liver and adipose tissue. In this study we determined the effect of *Ps* on 11β-HSD1 activity in bones of glucocorticoid-induced osteoporotic rats.

Materials and Methods. Three-month old male *Sprague-Dawley* rats were adrenalectomised to remove the main source of circulating glucocorticoids. The animals were administered with dexamethasone 120 µg/kg body weight/day. Treatment with *Ps* 125 mg/kg body weight and glycirrhizic acid (GCA) 120 mg/kg body weight were given simultaneously.

Results. The results showed that *Ps* extract reduced plasma corticosterone concentration (1.05±0.02µg/ml) and induced 11β-HSD1 dehydrogenase activity in bone (87.69±1.41%). Consequently, it also reduced the bone resorption marker, pyridinoline, in dexamethasone-treated adrenalectomised rats (2.07±0.62/L). Despite that, our data showed an inverse relationship between the plasma corticosterone level and the dehydrogenase activity of 11β-HSD1 in the bone.

Conclusions. This suggests that 11β-HSD1 acts as the local regulator of glucocorticoid and its activity in bone was not correlated to systemic corticosterone level. ***Clin Ter 2011; 162(4):313-318***

Key words: *dexamethasone, glucocorticoids, osteoporosis, piper sarmentosum, 11β-hydroxysteroid dehydrogenase type 1*

Introduction

Long-term glucocorticoid therapy induces osteoporosis. Glucocorticoids are required for the differentiation of osteoblast cells and formation of mineralized extracellular matrix (2-4). However, if present in excess, glucocorticoids have adverse effects on the skeleton by reducing proliferation and inducing apoptosis. This is clearly seen in glucocorticoid-induced osteoporosis. Concern on glucocorticoid-induced osteoporosis has increased with the widespread use of exogenous glucocorticoids.

The effects of glucocorticoids in vitro depend upon corticosteroid concentration and receptor expression, timing of exposure and stage of cellular differentiation (5). At the molecular level, glucococorticoid signaling is mediated via the glucocorticoid receptor (GRα) which is expressed in almost all cell types including osteoblasts and osteoclasts.

Patients with endogenous glucocorticoid excess, develop osteoporosis (6) mainly by suppression of bone formation which was shown by rapid decrease in bone formation markers (7,8) and also by increase in bone resorption through the increase in osteoclastic activity (9-11). Glucocorticoids also cause impairment of bone growth and development (12). Despite this, the susceptibility to the bone specific effects of endogenous and exogenous glucocorticoids in individuals correlates only weakly with systemic glucocorticoid levels (11, 13) or variation in the affinity or concentration of the glucocorticoid receptor (GR) (14).

Clinical studies showed that bone tissue specific response to glucocorticoids was strongly correlated to serum levels of the inactive glucocorticoid hormone, not the active form (15). Glucocorticoid action has been shown to be regulated at the pre-receptor level by the isoenzyme, 11β-hydroxysteroid dehydrogenase, which is found in almost all glucocorticoid target tissues. There are two isoenzymes of 11β-hydroxysteroid dehydrogenase, 11β-HSD1 and 11β-HSD2 but 11β-HSD1 is the predominant isoenzyme expressed in human osteoblasts and osteoclasts (5). It interconverts inactive cortisone to active cortisol in humans and inactive 11-dehydrocorticosterone to active corticosterone in rodents.

The activity and synthesis of 11β-HSD1 is glucocorticoid dependent. Expression of this enzyme has a functional consequence, as it reduces expression of alkaline phosphatase in response to corticosterone therapy. 11β-HSD1 in adult bone demonstrated both dehydrogenase and reductase activities

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(4). Several additional studies have replicated the expression of 11β-HSD1 in adult human osteoblast in which the activity is primarily reductase, converting cortisone into biologically active cortisol. (16, 17). Reductase activity in osteoblasts in vivo provides a potent mechanism for local generation of active glucocorticoids and this activity is predominant in physiological conditions. This activity would have important effects on proliferation and differentiation but could lead to potentially deleterious effects on bone integrity. In contrast, dehydrogenase activity, i.e conversion of active to inactive glucocorticoids may play an important role in attenuating local availability of active glucocorticoids. Its importance increases at supraphysiological doses of glucocorticoids. The kinetic characteristics of 11β-HSD1 suggest that, with physiological levels of endogenous glucocorticoids, reductase activity would be prominent. However, with the supraphysiological doses of glucocorticoids, particularly some synthetic steroids, the importance of dehydrogenase activity may increase (5).

Both 11β-HSD isoenzymes are inhibited by liquorice and its derivatives, carbenoxolone and glycyrrhetinic acid (GCA) (18). GCA was found to inhibit reductase activity of 11β-HSD1 in differentiating human osteoblast cell line (SV-HFO). Carbenoxolone, a liquorices derivative had no impact on bone formation markers but did result in suppression of bone resorption markers pyridinoline and deoxypyridinoline, suggesting a positive role of 11β-HSD1 in the modulation of osteoclastic activity (5). *Piper sarmentosum (Ps),* locally known as "daun kadok" is a glabrious creeping terrestrial herb, usually used to flavor local cuisine. The leaves and roots of this plant were used in the Malay and Indonesian Archipelago for the treatment of toothache, fungoid dermatitis, coughing asthma and pleurisy. In Thailand it was also used as an expectorant (16). The methanolic extracts of *Ps* leaves also possessed a natural superoxide scavenger, narigenin. Recent study reported the fact that *Ps* extract inhibits the 11β-HSD1 activity in the liver and adipose tissue of ovariectomised rats (1).

The enzyme11β-HSD1 is present in osteoblasts and osteoclasts and is responsible for the local generation of glucocorticoids in bone. The effects of *Ps* on glucocorticoid-induced osteoporosis had not been explored till date. Hence, the present study aimed to look into the effects of *Ps* in the regulation of 11β-HSD1 activity in the bone. The results of this study may have an important impact in combating glucocorticoid induced osteoporosis.

Materials and Methods

Animals and treatment

All procedures were carried out in accordance with the institutional guidelines for animal research surgical procedures of the Universiti Kebangsaan Malaysia UKM Research and Animal Ethics Committee (UKMAEC) (No: ANT/2007/ FARIHAH/14-NOV/201-NOV-2007-SEPT-2010).

Forty, 3 month-old male Sprague-Dawley rats weighing 220-250 grams were obtained from the National University of Malaysia Animal Breeding Centre. The animals were divided into groups of 10 rats and given the following treatment: G1: sham operated control; G2: adrenalectomised (adrx) and given intramuscular dexamethasone (DEX), G3: adrx and given intramuscular DEX and glycirrhizic acid (GCA) and G4: adrx and given intramuscular DEX and Ps. 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 left in-situ.

The treatment period was started 2 weeks after adrenalectomy. Dexamethasone (Sigma®, USA) was dissolved in olive oil (Bertolli®, Italy) and administered intramuscularly (120 μg/kg/day) for 6 days a week (19). The *Ps* extract was provided by the Forest Research Institute of Malaysia (FRIM). *Piper sarmentosum* and GCA (Sigma, USA) were diluted in normal saline and administered by oral gavage at the dose of 1.25 mg kg^{-1} (1) and 1.20 mg kg^{-1} (20), respectively for two months. The sham-operated rats were administered equivalent volumes of vehicle olive oil intramuscularly and vehicle normal saline by oral gavage. The DEX (G2) rats were also administered vehicle normal saline by oral gavage. The administration of *Ps*, GCA and dexamethasone were started simultaneously ten days after the adrenalectomy. The treatment was given for two months.

The animals were placed in clean cages under natural sunlight and darkness at night. They were given rat pellets (Gold Coin, Malaysia) *ad libitum.* The sham operated animals were given tap water, while the adrenalectomized animals were given normal saline to drink *ad libitum.* This is to replace the salt loss due to mineralocorticoid deficiency post-adrenalectomy.

The following parameters were measured at the end of two months of treatment:-11β-HSD1 enzyme activity in bone, osteocalcin, pyridinoline (Pyd) and corticosterone levels in plasma.

Collection of samples

Blood samples were taken before and after completion of the treatment period. The blood samples were centrifuged at 3000 rpm at 4°C for 15 minutes and the plasma was kept in aliquots at -70°C until analysis. At the completion of 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 analyzed.

Plasma corticosterone level measurements

Plasma corticosterone levels were analyzed using the High Performance Liquid Chromatography (HPLC) (Waters®, USA) machine utilizing the mobile phase which consists of 60:40 of methanol and deionized water. 100 μl of dichloromethyl containing 0.025mg/L cortexolone was added to 100 μl of plasma. The mixture was shaken vigorously for 10 minutes and centrifuged for 5 minutes at 1000 g. The

upper aqueous phase was discarded and 0.05M NaOH was added and centrifuged for 5 minutes at 1000g. The aqueous phase was again discarded, and this process was repeated. Following the washing steps, the dichloroethane layer was transferred into another tube and evaporated to dryness in a vacuum concentrator. Then, it was resuspended in 1 ml HPLC mobile phase and placed in the vials. Steroids were detected at 254 nm using Empower software.

Assay for bone 11β-HSD1 enzyme activity

The right femoral bones were dissected, cleared from soft tissues and washed extensively in phosphate-buffered saline to reduce fat content. The frozen bones were ground into small pieces using mortar and pestle, then suspended in Krebs-Ringer bicarbonate buffer. The suspended bone was homogenized and kept overnight at 4ºC. The homogenized bone was centrifuged at 12,100 g for 20 min at 4 °C and the supernatant was decanted. Total protein content was estimated calorimetrically (Bio-Rad®, Hercules, CA, USA) on aliquots of each homogenate. Two hundred micromolar NADP and 12nM (1,2,6,7-^{3}H) corticosterone® (specific activity:84 Ci/mmol; Amersham, Buckinghamshire, England) were added to the tissue homogenates containing 0.5 mg protein. The cofactor NADP drives the enzyme's oxidative activity. Krebs-Ringer bicarbonate buffer containing 0.2% glucose and 0.2% BSA 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. After incubation in a water bath at 37ºC for 2 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 4ºC for 10 min. 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 nonradioactive carrier 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 (^{3}H) corticosterone to inactive (^{3}H) 11-dehydrocorticosterone from the radioactivity of each fraction.

Measurement of plasma bone biochemical markers

Bone biochemical markers were determined using ELISA. The kits used for plasma osteocalcin and Pyd measurements were ELISA Rat-MID™ rat osteocalcin (Nordic Bioscience Diagnostics A/S®, Denmark) and EIA Metra serum PYD kit (Quidel Corp®, San Diego, USA) respectively.

Statistical Analysis

Data were tested for normality using the Kolmogrov-Smirnov test. Since the groups were found to be normally distributed, the data was analyzed by parametric statistics, i.e. the ANOVA test followed by pos-hoc Tukey's test for comparison between treatment groups. The student *t* test was used for comparison before and after treatment between groups. Statistical software used was the Statistical Package for Social Science (SPSS) version 12. Data were expressed as the mean ± standard error of the mean (SEM).

Results

Effects of Ps and GCA on plasma corticosterone level in dexamethasone-treated adrenalectomised rats

Plasma concentration of corticosterone increased significantly after 2 months in dexamethasone-treated adrenalectomised rats (G3) (1.16±0.03 µg/ml) compared to sham operated (G2) (1.07.0±0.02 µg/ml). Rats supplemented with GCA (G4) (1.06.0±0.01 µg/ml) and *Ps* (G5) (1.05.0±0.01 µg/ml) prevented the increase of corticosterone level in the plasma of dexamethasone-treated adrenalectomised seen in G2 (Fig. 1).

Effects of Piper sarmentosum on 11β-HSD1 activity in dexamethasone-induced osteoporotic bone

The adrenalectomised rat treated with intramuscular injection of dexamethasone (G3) has significantly lower 11β-HSD1 dehydrogenase activity (79.16±2.28%) in the bone compared to the sham-operated (G2) (90.69±1.52%). Supplementing the dexamethasone treated adrenalectomised rats with GCA (G4) and *Piper sarmentosum* (G5) extract has maintained the 11β-HSD1 dehydrogenase activity at sham operated control levels (G1) (87.67±0.85%) (87.69±1.41%) (Fig. 2).

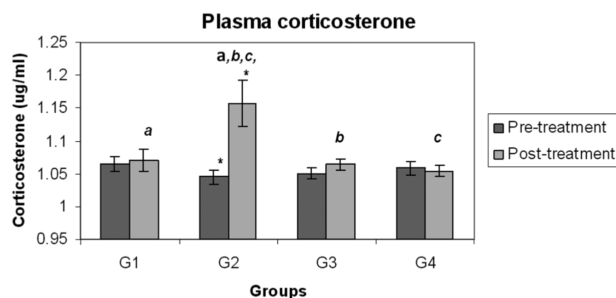

Fig. 1. The effect of supplementation of GCA (120 mg/kg/day and PS (125 mg/kg/day) on plasma corticosterone level of dexamethasone treated adrenalectomised rats (120 µg/kg/day) for two months. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at p< 0.05. * indicate significant difference before and after treatment for the same group.
G1= sham operated control; G2= adrenalectomised (adrx) and given intramuscular dexamethasone 120 µg/kg/day (DEX); G3= adrx and given intramuscular DEX 120 µg/kg/day and GCA 120 mg/kg/day and G4= adrx and given intramuscular DEX 120 µg/kg/day and Ps 125 mg/kg/day.

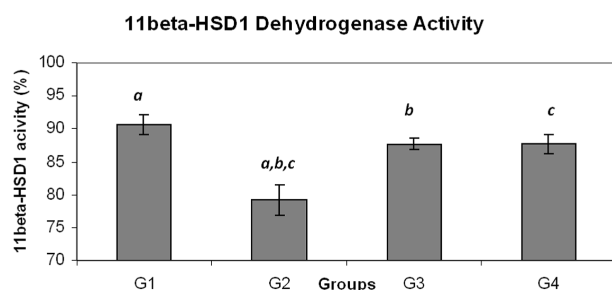

Fig. 2. The effect of supplementation of GCA (120 mg/kg/day) and *Ps* (125 mg/kg/day) on 11β-HSD1 dehydrogenase activity in femoral bones of dexamethasone treated adrenalectomised rats (120 µg/kg/day) for two months. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at $p < 0.05$.
G1= sham operated control; G2= adrenalectomised (adrx) and given intramuscular dexamethasone 120 µg/kg/day (DEX); G3= adrx and given intramuscular DEX 120 µg/kg/day and GCA 120 mg/kg/day and G4= adrx and given intramuscular DEX 120 µg/kg/day and *Ps* 125 mg/kg/day.

Plasma osteocalcin level

The plasma osteocalcin levels decreased significantly after two months of dexamethasone treatment in adrenalectomised rats compared to the pretreatment level in all the treatment groups including the sham-operated rats (Fig. 3). However, no differences between the groups was observed before and after the treatment.

Plasma pyridinoline

The pyridinoline levels decreased after two months compared to the pre-treatment levels in all the groups except for G2, i.e., the adrenalectomised group given dexamethasone where the levels did not show any obvious changes (4.32±0.37 nm/L) (Fig. 4). The post-treatment pyridinoline levels of the dexamethasone treated group supplemented with GCA (G4) (2.07±0.62 nm/L) and *Ps* (G5) (2.07±0.33 nm/L) extract were significantly lower compared to G2.

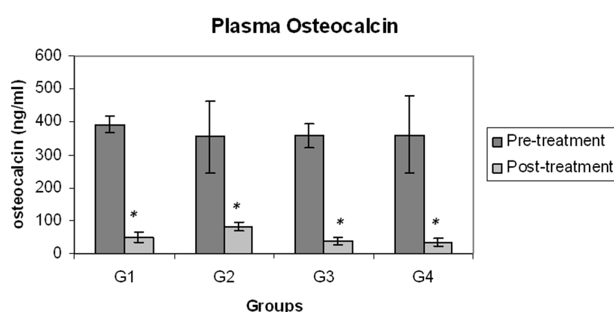

Fig. 3. The effect of supplementation of GCA (120 mg/kg/day) and *Ps* (125 mg/kg/day) on bone formation marker osteocalcin of Dexamethasone treated adrenalectomised rats (120 µg/kg/day) for two months. Data presented as mean ± SEM. * indicate significant difference before and after treatment for the same group at $p<0.05$.
G1= sham operated control; G2= adrenalectomised (adrx) and given intramuscular dexamethasone 120 µg/kg/day (DEX); G3= adrx and given intramuscular DEX 120 µg/kg/day and GCA 120 mg/kg/day and G4= adrx and given intramuscular DEX 120 µg/kg/day and *Ps* 125 mg/kg/day.

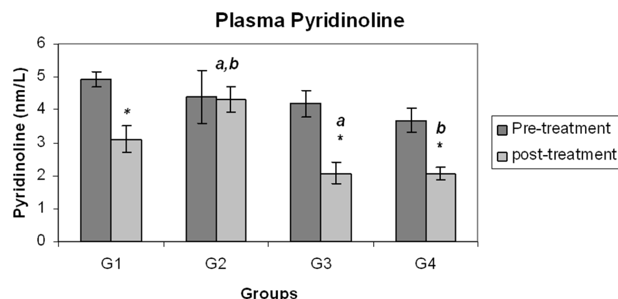

Fig. 4. The effect of supplementation of GCA (120 mg/kg/day) and *Ps* (125 mg/kg/day) on of bone resorption marker, pyridinoline of dexamethasone treated adrenalectomised rats (120 µg/kg/day) for two months. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at $p< 0.05$. * indicate significant difference before and after treatment for the same group at $p<0.05$.
G1= sham operated control; G2= adrenalectomised (adrx) and given intramuscular dexamethasone 120 µg/kg/day (DEX); G3= adrx and given intramuscular DEX 120 µg/kg/day and GCA 120 mg/kg/day and G4= adrx and given intramuscular DEX 120 µg/kg/day and *Ps* 125 mg/kg/day.

Discussion

The rats were adrenalectomised to remove the endogenous glucocorticoids that were subjected to circadian rhythm, as well as physical and emotional stress. The endogenous glucocorticoids were replaced by predetermined doses of dexamethasone to ensure a constant level of glucocorticoid in the body. No replacement of mineralcorticoids was considered necessary as it was shown earlier to have no influence on bone metabolism (19) However, the animals were given normal saline *ad libitium* to maintain normal sodium homeostasis. The dose and duration of dexamethasone treatment (120 µg/kg) were determined by previous studies (12, 19). The dose of the GCA and *Ps* were also determined by previous studies (20, 21).

In the present study, it was shown that long term dexamethasone treatment in adrenalectomised rats increased plasma corticosterone, but lowered 11β-HSD1 activity in bone. This was associated with increased nett bone resorption, as seen in increased serum pyridinoline levels, while plasma osteocalcin levels were unchanged. These findings appear consistent, since glucocorticoid treatment causes a time and dose dependent increase in 11β-HSD1 reductase activity (22). Reduced activity of 11β-HSD1 dehydrogenase may imply increased activity of 11β-HSD1 reductase in bone, leading to increased local glucocorticoid production (5). Increased glucocorticoid concentration in bone will lead to increased bone resorption activity, and this was seen in the increased pyridinoline levels in the plasma, whilst the osteocalcin activity remained unchanged. However we postulate that the 11β-HSD1 dehydrogenase may be suppressed systemically, and not just only in the bone, leading to systemic increase in 11β-HSD1 reductase activity, which may explain the increase in plasma corticosterone levels (22). These changes support the observation of increased bone resorption leading to osteoporosis in condition of glucocorticoid excess (5).

11β-HSD1 enzyme activity is the key modulator of intracellular glucocorticoid concentration. Dexamethasone administration was found to promote human osteoclast formation (23). It has also been suggested that glucocorticoids stimulate bone resorption directly via activation of mature osteoclasts (24) and by inducing osteoclastogenesis (25). 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) signaling pathway, whose ligand (RANKL) is a cell surface receptor expressed by osteoblast and kept inhibited by osteoprotegerin (OPG) also secreted by osteoblast. Glucocorticoid alters the RANKL/OPG ratio by acting on both factors resulting in osteoclastogenesis and rapid early phase of bone resorption (26).

Treatment of the adrenalectomised, dexamethasone-treated rats with GCA did not cause any changes in the plasma corticosterone levels. This is consistent with the unchanged activity of 11β-HSD1 dehydrogenase activity in bone. These observations imply that GCA was able to prevent the suppression 11β-HSD1 dehydrogenase activity locally and maybe even systemically, leading to normal circulating plasma corticosterone levels. Plasma PYD was also maintained at the same level as seen in the sham-operated group. Thus, administration of GCA was able to prevent the increased of bone resorption seen in rats given longterm excessive glucocorticoids, and the mechanism is by preventing in the inhibition of 11β-HSD1 dehydrogenase in the bone, and perhaps also systemically. This observation is consistent with other researchers (5).

Treatment of adrenalectomised, dexamethasone-treated rats with *Ps* gave very similar results to those seen in the GCA treated group. Therefore, we may conclude that *Ps* is effective in preventing glucocorticoid-induced osteoporosis in rats most probably by preventing the inhibition of 11β-HSD1 dehydrogenase in the bone, and perhaps also systemically. Thus there is a real potential for *Ps* to be used to prevent and treat osteoporosis in patients o longterm glucocorticoid therapy, subject to further animal and human studies.

The high level of bone 11β-HSD1 dehydrogenase activity in the 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 but at this stage, glucocorticoids mainly exert a harmful effect (27).

Despite these facts, the plasma osteocalcin levels decreased significantly after two months treatment in all groups, including the sham compared to the pre-treatment levels and there were no significant differences between the groups. This suggests that dexamethasone, GCA and *Ps* did not have significant effects on osteoblasts.

In conclusion, from the result, we can conclude that *Ps* was effective in preventing bone resorption in dexamethasone treated rats by increasing systemic and osteoclastic 11β-HSD1 dehydrogenase leading to inhibition of bone resorption. This suggest that *Ps* can be used as an agent to protect the bone against the action of glucocorticoid and this may benefit the patients who needs to be on long-term glucocorticoid treatment. However, the mechanisms involved need further exploration.

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