Salvianolic Acid B Prevents Bone Loss in Prednisone-Treated Rats through Stimulation of Osteogenesis and Bone Marrow Angiogenesis

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Abstract

Glucocorticoid (GC) induced osteoporosis (GIO) is caused by the long-term use of GC for treatment of autoimmune and inflammatory diseases. The GC related disruption of bone marrow microcirculation and increased adipogenesis contribute to GIO development. However, neither currently available anti-osteoporosis agent is completely addressed to microcirculation and bone marrow adipogenesis. Salvianolic acid B (Sal B) is a polyphenolic compound from a Chinese herbal medicine, Salvia miltiorrhiza Bunge. The aim of this study was to determine the effects of Sal B on osteoblast bone formation, angiogenesis and adipogenesis-associated GIO by performing marrow adipogenesis and microcirculation dilation and bone histomorphometry analyses. (1) In vivo study: Bone loss in GC treated rats was confirmed by significantly decreased BMD, bone strength, cancellous bone mass and architecture, osteoblast distribution, bone formation, marrow microvessel density and diameter along with down-regulation of marrow BMPs expression and increased adipogenesis. Daily treatment with Sal B (40 mg/kg/d) for 12 weeks in GC male rats prevented GC-induced cancellous bone loss and increased adipogenesis while increasing cancellous bone formation rate with improved local microcirculation by capillary dilation. Treatment with Sal B at a higher dose (80 mg/kg/d) not only prevented GC-induced osteopenia, but also increased cancellous bone mass and thickness, associated with increase of marrow BMPs expression, inhibited adipogenesis and further increased microvessel diameters. (2) In vitro study: In concentration from 10^{-6} mol/L to 10^{-7} mol/L, Sal B stimulated bone marrow stromal cell (MSC) differentiation to osteoblast and increased osteoblast activities, decreased GC associated adipogenic differentiation by down-regulation of PPARγ mRNA expression, increased Runx2 mRNA expression without osteoblast induction, and, furthermore, Sal B decreased Dickkopf-1 and increased β-catenin mRNA expression with or without adipocyte inducement in MSC. We conclude that Sal B prevented bone loss in GC-treated rats through stimulation of osteogenesis, bone marrow angiogenesis and inhibition of adipogenesis.

Introduction

Glucocorticoid (GC) therapy is commonly used for inflammatory and autoimmune diseases. The long-term administration of GC can lead to glucocorticoid-induced osteoporosis (GIO), which significantly increases the patients’ morbidity and mortality. Due to limited treatment options, the side effects of GC often have to be tolerated during treatment [1]. Currently, the clinical management of GIO relies on medications similar to those used for treatment of post-menopausal osteoporosis, such as calcium, vitamin D, bisphosphonates, raloxifene, PTH, hormone replacement and calcitonin. These drugs do not address the multi-factor driven GIO. In particular, they do not target the detrimental effect of GC on bone marrow fat metabolism and circulatory system [2–5]. Thus more studies on these GC induced effects may lead to development of a novel therapeutic approach to prevent and treat GIO. The pathogenesis of GIO involves multiple factors, of which some suggest the decrease in number and functions of osteoblasts is the main contributing factor [2]. However, recently increased apoptosis of osteoblasts, osteocytes and endothelial cells, suppression of osteoblasts and osteoclasts, and endothelial cell precursor production as well as prolongation of the life span of osteoclasts have all been shown to contribute to the skeletal side effects of GC [4–6]. Recent studies suggested that the regulation of marrow stromal cell (MSC) differentiation into bone or fat cells [3] and the inhibition of bone marrow microvasculature play a very important role in GIO development [4,5]. GC can inhibit osteoblast production of bone morphogenetic protein 2 (BMP-2), which causes decreased MSC differentiation into bone cells [7]. GC also directly induce differentiation of marrow stromal cells into adipocytes and inhibit osteogenic differentiation [8]. Kitajima et al. showed that mature fat cells exposed to high dose of GC were larger than control cells derived from bone marrow [9]. The latter would lead to narrowing and obstruction of capillaries in bone marrow microvasculature from increased adipose tissue that
results in increased intraosseous pressure and decreased blood flows [5,10]. Excessive GC treatment was also found to inhibit the growth of vascular endothelial cells that further contributes to microcirculation disturbance [11]. Marx et al. have previously demonstrated that the peroxisome proliferator-activated receptor γ (PPARγ) can induce apoptosis in vascular endothelial cells via caspase-3 activation, thus inhibiting vascular endothelial cell proliferation and angiogenesis [12]. GC can activate PPARγ in MSCs through different pathways to promote adipogenesis, which reduces osteoblast differentiation, and eventually leads to fat tissue accumulation in bone marrow [6]. Taken together, these studies suggest that the GIO bone loss is comprised of multiple mechanisms involving the increase in bone marrow adipogenesis and decrease in marrow angiogenesis leading to a decrease in bone marrow microvasculature and consequent decrease in osteogenesis [4,5].

_Salvia miltiorrhiza Bunge_ is a traditional Chinese medicine, called dan Shen, widely used in clinical practice for the prevention and treatment of cardio-cerebral vascular diseases. Pharmacological testing showed that dan Shen has anticoagulant, vasodilatory, increased blood flow, anti-inflammatory, free radical scavenging, mitochondrial protective and other activities [13]. Salvianolic acid B (Sal B), the aqueous bioactive component from _Salvia miltiorrhiza Bunge_, is a polyphenolic compound found in abundance in this plant. The structure of Sal B is shown in Figure 1 which consists of three molecules of Tanshinol (D (+) β-3,4-dihydroxyphenyl lactic acid, danshensu) and a molecule of caffeic acid. Sal B can be converted _in vivo_ to Tanshinol, another water-soluble bioactive ingredient of _Salvia miltiorrhiza Bunge_, with similar function to Sal B.

Multiple pharmacological studies have found that Sal B can attenuate the effect of myocardial ischemia-reperfusion injury [15]. Interestingly, Sal B can also increase angiogenesis and reduce myocardial ischemia via vascular endothelial growth factor (VEGF) activation [16]. It also relieves brain injury by reducing neuronal damages in cerebral ischemia [17]. Sal B can improve cellular hypoxia-ischemia by expanding micro-arteries, improving microcirculation and increasing the blood flow velocity. Its beneficial effects on blood vessel dilation and protection are believed to be mediated by blocking calcium channels and angiotensin-converting enzyme [18,19]. Moreover, _Salvia miltiorrhiza Bunge_ and its aqueous extract can increase the activity of superoxide dismutase, scavengen

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**Figure 1. Structure of Salvianolic acid B and Tanshinol.** Salvianolic acid B consists of three molecules of Tanshinol (D (+) β-3,4-dihydroxyphenyl lactic acid, danshensu) and a molecule of caffeic acid. Sal B can be converted _in vivo_ to Tanshinol, another water-soluble bioactive ingredient of _Salvia miltiorrhiza Bunge_, with similar function to Sal B.

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**Table 1. Experimental Design.**

| Groups | Code | Description for Treatment and Dosage |
|--------|------|-------------------------------------|
| (I) Basal control | BAS | Scarified at 0 day of the study |
| (II) Age control | CON | Vehicle treatment of distilled water at 5 ml/kg/d |
| (III) Intact-low dose Sal B | B40 | Sal B treatment at 40 mg/kg/d |
| (IV) Prednisone model | GC | Prednisone acetate (Pred) at 3.5 mg/kg/d |
| (V) GC-low dose of Sal B | GC+B40 | Pred at 3.5 mg/kg/d and Sal B at 40 mg/kg/d |
| (VI) GC-high dose of Sal B | GC+B80 | Pred at 3.5 mg/kg/d and Sal B at 80 mg/kg/d |

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reactive oxygen species (ROS) and therefore reduce the damage of ROS to the vascular endothelium. Thus, Sal B functions as a vasodilator, maintains red blood cell deformability and increases the function of the hematopoietic system [20]. Recently, our in vitro studies have demonstrated that Sal A can inhibit glucocorticoid–induced bone marrow stromal cells adipogenesis, promote osteoblast differentiation, bone matrix formation and bone mineralization [21]. Therefore, we hypothesize that the clinical use of Sal B will hold promise for a more effective and safe treatment for GIO.

The aim of the current study is to validate our hypothesis in a GIO rat model and additional study on in vitro.

Materials and Methods

Animals

Ethical Treatment of Animals: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Guangdong Laboratory Animal Monitoring Institute under by National Laboratory Animal Monitoring Institute of China. The experiments have been conducted according to protocols approved for Specific Pathogen Free animal care of Animal Center of Guangdong Medical College, and approved by the Academic Committee on the Ethics of Animal Experiments of the Guangdong Medical College, Zhanjiang, P.R. China, Permit Number: SYXK (GUANGDONG) 2008-0007. All surgery was performed under sodium pentobarbital anesthesia.

Table 2. Primer sequences used in RT-PCR.

| Gene   | Primer sequence                  | Products Length (bp) |
|--------|----------------------------------|----------------------|
| Runx2  | 5'-ACTGAAAGCGCTTGGACACG-3' (sense) | 122                  |
|        | 5'-TCACCCAGCCACCCAGGAG-3' (antisense) |                       |
| PPARγ  | 5'-GGGCTCTGCTGGATGCT-3' (sense) | 240                  |
|        | 5'-CGAAATGCCACCTTGGAAAAAT-3' (antisense) |                       |
| Dickkopf-1 | 5'-GCTCGGTCATCAAGACCACTG-3' (sense) | 224                  |
|        | 5'-GGAGGCTGGAAGGACTTAA-3' (antisense) |                       |
| β-catenin | 5'-TGAGCAGCGACTAAGCAG-3' (sense) | 198                  |
|        | 5'-TCACCACTGGAGAAG-3' (antisense) |                       |
| GAPDH  | 5'-ACCCACGTCATACACCAC-3' (sense) | 451                  |
|        | 5'-CCACACCCTTGGCCTGA-3' (antisense) |                       |

Table 3. Primer sequences used in RT-PCR.

| Gene   | Primer sequence                  | Products Length (bp) |
|--------|----------------------------------|----------------------|
| BMP-2  | 5'-AAATTATAAAGCGCTCCAC-3' (sense) | 326                  |
|        | 5'-TCACCACCTTTTCTGGTG-3' (antisense) |                       |
| BMP-7  | 5'-AGAGCCAAAGACACACAGG-3' (sense) | 323                  |
|        | 5'-GCTGTCGCAGAAAGAG-3' (antisense) |                       |
| GAPDH  | 5'-CGGAGGAGGACGGTGGG-3' (sense) | 195                  |
|        | 5'-CAGATGTGCATGTTAGAAG-3' (antisense) |                       |

The Sprague-Dawley male rats were acclimated to local vivarium conditions (temperature 24–26°C, humidity 67%) and allowed free access to water and diets containing 1.33% calcium, 0.95% phosphorus, and vitamin D3 60 IU %. All rats received subcutaneous injection of tetracycline (20 mg/kg, Sigma Chemical Co. St. Louis, MO) on days 13 and 14, and calcine (10 mg/kg, Sigma Chemical Co. St. Louis, MO) on 3 and 4 days before sacrifice.

Experimental Protocols

Forty-six 6-month-old male Sprague-Dawley rats weighing 390±25 grams, were randomly divided into 6 groups with 8 rats per group, except 6 for the basal control group (Table 1). The groups were: 1) a basal (BAS), an age control (distilled water, CON), 3) 40 mg Sal B/kg/d (B40), 4) 3.5 mg prednisone acetate/kg/d (GC), 5) GC + 40 mg Sal B/kg/d (GC + B40) and 6) GC + 80 mg Sal B/kg/d (GC + B80). Prednisone acetate was obtained from Guangdong Xianju Pharmacy Co. China and the Sal B prepared as described in below. All treatments were by daily oral gavage for 12 weeks.

Salvianolic Acid B (Sal B) Preparation

The original herbal medicine *Radix Salviae miltiorrhiza* was selected according to the standard protocol of the pharmacopoeia of the People’s Republic of China [22]. The aqueous bioactive component from *Radix Salviae miltiorrhiza* was extracted as previously reported [23]. Salvianolic acid B (Sal B) and Tanshinol found in the aqueous extract of *Radix Salviae miltiorrhiza* were...
### Table 4. Effects of Sal B, GC and GC+Sal B on the soft tissue weights (g/1000 g body weight).

| Groups | Thymus | Liver | Adrenal gland (/100 g) | Testicles | Soleus |
|--------|--------|-------|------------------------|-----------|--------|
| CON    | 0.53±0.06 | 26.45±1.28 | 0.90±0.10 | 4.17±0.29 | 0.46±0.04 |
| B40    | 0.52±0.06 | 27.13±1.52 | 0.87±0.19 | 4.19±0.27 | 0.47±0.03 |
| GC     | 0.40±0.07** | 28.98±1.30** | 0.72±0.19* | 4.12±0.36 | 0.42±0.02* |
| GC+B40 | 0.48±0.08 | 28.33±2.86 | 0.89±0.12** | 4.10±0.07 | 0.48±0.06** |
| GC+B80 | 0.43±0.09* | 26.97±1.96** | 0.90±0.17 | 4.11±0.26 | 0.47±0.04*** |

Note: Value are mean ± SD,
*P<0.05,
**P<0.01 vs CON;
#P<0.05,
##P<0.01 vs GC,
++P<0.05+
++P<0.01 vs GC+B40,
Not available for BSA.
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### Table 5. Effects of Sal B, GC, and GC+ Sal B on femur bone biomechanics.

| Groups | Maximum force (N) | Fracture Force (N) | Elastic Force (N) | Maximum deflection (mm) | Stiffness coefficient (N/mm) |
|--------|-------------------|--------------------|------------------|------------------------|-----------------------------|
| BAS    | 162.93±13.36**    | 134.58±22.16**    | 94.99±13.74**    | 0.97±0.19              | 531.09±49.67               |
| CON    | 207.91±13.01      | 178.77±30.39      | 126.97±24.39     | 0.90±0.10              | 617.97±95.61               |
| B40    | 208.52±21.50**    | 171.37±38.07      | 121.35±38.26     | 0.90±0.17              | 678.24±51.53####           |
| GC     | 184.76±17.72x     | 177.97±17.86####  | 127.21±19.45#### | 0.70±0.11*####         | 601.12±78.48               |
| GC+B40 | 213.40±12.82####  | 191.98±21.22####  | 131.75±10.85#### | 0.94±0.14####          | 774.13±75.89***####       |
| GC+B80 | 218.68±15.32####  | 184.52±30.08####  | 136.11±26.49#### | 0.93±0.13####          | 738.87±57.53***####       |

Note: Value are mean ± SD,
*P<0.05,
**P<0.01 vs CON;
#P<0.05,
##P<0.01 vs GC,
++P<0.05,
++P<0.01 vs GC+B40,
Not available for BAS.
doi:10.1371/journal.pone.0034647.t005

### Table 6. Effects of Sal B, GC, and GC+ Sal B on proximal tibial metaphysis bone structure histomorphometry, osteoblast and osteoclast contents.

| Parameters | BAS | CON | B40 | GC | GC+B40 | GC+B80 |
|------------|-----|-----|-----|----|--------|--------|
| BV/TV (%)  | 16.34±3.52 | 13.18±3.87 | 13.44±3.09 | 9.42±3.34#### | 14.04±2.56## | 17.58±3.20## | 17.19±3.44## |
| Tb.Th (µm) | 59.52±4.53 | 59.77±8.86 | 59.80±6.92 | 49.09±9.11## | 59.36±6.19## | 69.82±8.85## | 60.82±8.12## |
| Tb.N (mm−1)| 2.72±0.43 | 2.18±0.43 | 2.25±0.43 | 1.87±0.38## | 2.37±0.31## | 2.51±0.25## | 2.51±0.25## |
| Tb.Sp (µm) | 316.2±68.6 | 416.3±103.1 | 399.0±93.3 | 505.3±123.5## | 369.51±51.3## | 332.4±47.7## | 332.4±47.7## |
| OcS/BS (%) | 0.74±0.33 | 0.65±0.28 | 0.47±0.16## | 0.86±0.36 | 0.79±0.31 | 0.55±0.30 | 0.55±0.30 |
| ObS/BS (%) | 1.21±0.33 | 1.21±0.42 | 1.50±0.68 | 0.31±0.13## | 0.80±0.31## | 1.01±0.36## | 1.01±0.36## |

Note: Value are mean ± SD,
*P<0.05,
**P<0.01 vs CON;
#P<0.05,
##P<0.01 vs GC,
++P<0.05,
++P<0.01 vs GC+B40,
**P<0.01,
**P<0.01 vs BAS.
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characterized by HPLC using a standard reference from the Chinese Biological Appraisal Institute, Beijing, China (>99.0%) [24]. HPLC chromatograms of the control compounds and aqueous extraction samples are shown in Figure 2. The content of Sal B was 25 mg per gram of Radix Salviae miltiorrhiza.

### Body weight and serum markers assay

Rats were weighed every week. At the end of the experiments, rats were sacrificed by cardiac puncture under anesthesia. Soft tissues were removed and weighed. Blood and serum samples were collected for measurements of serum calcium (Ca), the serum bone biomarkers alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase-5b (TRACP-5b) according to manufacturers’ instructions (Immunodiagnostics Systems Inc, USA).

### Immunohistochemical analysis of bone tissues

The distal femoral bone marrow cavity was exposed to prepare decalcified bone slides. Samples were decalcified at room temperature in 15% EDTA for 5 weeks. After decalcification, the sample was placed in 70% alcohol and paraffin embedded. Four micrometer paraffin slides were prepared on glass slides coated with egg white-glycerol, or polylysine, then dried for one hour at 60°C and stored at 4°C for future use.

Immunohistochemical analysis of bone marrow microcirculation factor VIII-related antigen (Von Willbrand Factor, vWF) and peroxisome proliferator-activated receptor γ (PPARγ) were performed following the manufacturers’ instructions. Rabbit anti-human vWF polyclonal antibody was purchased from CHEMICON International, Inc, USA, and PPARγ mouse monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc, USA.

Briefly, the endogenous peroxidase activity of slides were blocked by 10% goat serum. The slides were then incubated with primary antibody was purchased from Santa Cruz Biotechnology Inc, USA.

### Table 7. Effects of Sal B, GC, and GC+ Sal B on proximal tibial metaphysis cancellous bone dynamic parameters.

| Parameters | BAS | CON | B40 | GC | GC4B40 | GC4B80 |
|------------|-----|-----|-----|----|--------|--------|
| LGR (µ/d)  | 8.31±0.45** | 5.13±0.43 | 5.36±0.52** | 4.03±0.51** | 5.10±0.25§ | 5.32±0.42§ |
| MS (%)     | 23.11±2.92** | 17.31±2.62 | 20.78±2.68** | 17.20±1.53** | 19.76±1.55§ | 18.40±0.99§ |
| MAR (µ/d)  | 1.23±0.08 | 1.18±0.13 | 1.17±0.09 | 1.08±0.11** | 1.21±0.07§ | 1.21±0.10§ |
| BFR/BS (µm/d*100) | 28.40±4.32* | 20.49±3.93 | 24.40±4.08** | 18.68±3.41** | 23.95±2.86§ | 22.21±2.06§ |
| BFR/BV (µ%/year) | 291.5±43.7** | 214.5±57.3 | 251.0±49.0 | 238.6±68.7** | 247.1±32.3 | 196.8±22.2** |
| BFR/TV (µ%/year) | 47.02±10.1** | 26.53±4.13 | 32.67±4.94** | 21.19±4.97** | 34.23±3.59§ | 34.39±4.46§ |
| Ec-MS (%) | 84.95±10.7** | 62.74±6.89 | 64.63±11.16** | 30.94±5.17** | 50.95±9.55§ | 59.08±9.88§ |
| Ec-MAR(µ/m) | 3.55±0.75** | 1.82±0.16 | 2.00±0.22** | 1.16±0.13** | 1.59±0.25§ | 1.48±0.03§ |
| Ec-BFR/BS (µm/d*100) | 298.7±61.3** | 114.3±15.7 | 130.6±32.3** | 36.1±8.4** | 79.5±11.3§ | 89.1±32.9§ |

Note: Value are mean ± SD,
*P<0.05,
**P<0.01 vs CON;
§P<0.05,
# P<0.01 vs GC,
†P<0.05,
;PP<0.01 vs GC+B40,
;P<0.05,
;P<0.01 vs BAS.

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### Table 8. Effects of Sal B, GC, and GC+ Sal B on distal femoral metaphyseal bone marrow.

| Parameters | BAS | CON | B40 | GC | GC4B40 | GC4B80 |
|------------|-----|-----|-----|----|--------|--------|
| F.Ar/TV (%) | 10.67±3.61 | 17.67±6.9 | 11.13±2.84 | 39.59±7.15** | 19.67±5.71§ | 21.23±4.53§ |
| PPARγ (A) | 0.18±0.05* | 0.21±0.03 | 0.18±0.04 | 0.31±0.05** | 0.23±0.03§ | 0.20±0.03§ |
| DMV (µm) | 9.74±2.92* | 15.03±5.21 | 17.18±4.02** | 6.11±1.64** | 20.68±10.25§ | 21.23±6.86§ |
| MVD (vWF #/view) | 24.7±1.64 | 26.1±1.61 | 27.0±1.56 | 20.1±3.68* | 25.4±1.63§ | 27.9±2.57§ |

Note: Value are mean ± SD,
*P<0.05,
**P<0.01 vs CON;
§P<0.05,
# P<0.01 vs GC,
†P<0.05,
;PP<0.01 vs GC+B40,
;P<0.05,
;P<0.01 vs BAS.

A: absorbance; vWF: positive staining of Von Willbrand Factor in the endothelial cells.

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DAB was applied on the slide for color development. The reaction was stopped when a uniform brown color became visible on the slide by rinsing in running water. Counterstaining was done with hematoxylin for 5–10 seconds. A control experiment was performed by replacing primary antibody with PBS. This method was a modification at the manufacturers’ instructions. After a wash in PBS, sections were incubated with hydrogen treatment of the sections in citrate buffer (0.01 mol/L, pH 6.0). Retrieval was performed by high temperature and pressure. Immunohistochemical analysis of bone marrow BMP-2 and BMP-7 expressions were performed following the manufacturers’ instructions: Antigen retrieval was performed by high temperature and pressure treatment of the sections in citrate buffer (0.01 mol/L, pH 6.0). After a wash in PBS, sections were incubated with hydrogen

Table 9. Percentage change of all parameters from basal (BAS), vehicle control (CON) and prednisone (GC).

| %Change | Aging | B40 | GC | B40+GC | B80+GC |
|---------|-------|-----|----|--------|--------|
|         | v.s. BAS | v.s. CON | v.s. BAS | v.s. CON | v.s. BAS | v.s. CON | v.s. BAS | v.s. CON | v.s. BAS | v.s. CON |
| BW      |        |       |     |        |        |     |       |     |        |        |        |
| Thymus  | /     |       |     | /     |       |     | /     |     |       |     |
| Liver   | /     |       |     | /     |       |     | /     |     |       |     |
| Adrenal Gland | /   |       |     | /     |       |     | /     |     |       |     |
| Testicles | /   |       |     | /     |       |     | /     |     |       |     |
| Soleus  | /     |       |     | /     |       |     | /     |     |       |     |
| Calcium | /     |       |     | /     |       |     | /     |     |       |     |
| TRACP-5b | /   |       |     | /     |       |     | /     |     |       |     |
| ALP     | /     |       |     | /     |       |     | /     |     |       |     |
| PF-BMD  | /     |       |     | /     |       |     | /     |     |       |     |
| Whole Femur BMD | −8 | −15 | −11 | 22 | 10 |        |     |     |     |
| Max force | 28 | 13 | −11 | 31 | 16 | 34 | 18 |        |     |
| Fracture Force | 33 | (27) | 32 | 43 | 37 |        |     |     |     |
| Elastic Force | 34 | (28) | 34 | 39 | 43 |        |     |     |     |
| Max Deflection | −28 | −22 | 34 | 33 |        |     |     |     |
| Stiffness | 28 | 46 | 25 | 29 | 39 | 20 | 23 |        |     |
| BV/TV   | (−19) | −42 | −29 | 49 | 33 | 87 | 25 |        |     |
| Tb.Wi   | −18 | −18 | 21 | 17 | 17 | 42 | 18 |        |     |
| Tb.N    | (−20) | −31 | 27 | (15) | 34 |        |     |     |     |
| Tb.Sp   | (32) | 60 | −27 | (−20) | −34 |        |     |     |     |
| Ocs/BS  | (−14) | −38 | (−28) | (32) | (22) | (−8) | (−15) |        |     |
| Ob5/BS  | (24) | −74 | −74 | (−34) | 158 | (−17) | 226 |        |     |
| LGR     | −38 | −35 | −52 | −21 | −39 | 27 | −36 | 32 |        |     |
| MS/BS   | −25 | −10 | 20 | −26 | −14 | 14 | 15 | −20 |        |     |
| MAR     | −12 | −8 | 12 | 12 |        |     |     |     |     |
| BFR/BS  | −28 | −14 | 19 | −34 | (−9) | −16 | 28 | −22 |        |     |
| BFR/BV  | −26 | −18 | −32 | −17 | −20 |        |     |     |     |
| BFR/TV  | −44 | −30 | 24 | −55 | −20 | −27 | 29 | 62 | −27 | 30 | 62 |     |
| Ec-Ms/BS | −26 | −24 | −64 | −51 | −40 | 65 | −30 | 91 |        |     |
| Ec-MAR  | −49 | −44 | −67 | −36 | −55 | 37 | −58 | (−19) | 28 |        |     |
| Ec-BFR/BS | −62 | −56 | (14) | −88 | −68 | −73 | −30 | 120 | −70 | (−22) | 147 |
| PPARy   | (17) | (−14) | 72 | 48 | 28 | −26 | −35 |        |     |
| Fat/TV  | (66) | (4) | (−37) | 271 | 124 | 84 | −50 | 99 | −46 |        |     |
| DMV     | 54 | 76 | (14) | −37 | −59 | 112 | (38) | 238 | 118 | 41 | 247 |
| MVD (vWF#view) | −23 | 26 | 13 | 39 |        |     |     |     |     |

Note: This table shows significant %change from BAS, CON and GC, respectively. [ ]: useful non-significant %changes. #: not available.

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#Salvianolic Acid B Stimulates Osteogenesis

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The evaluation of Von Willbrand Factor expression and marrow microvessel density (MVD) were determined by counting the number of endothelial cells showing positive staining Von Willbrand Factor, and the MVD was determined by the total number of vWF stained cells divided by the measured area in previous studies [25,26]. The diameter of marrow microvessels (DMV) was determined as the average diameters of microvessels in the same area used to measure MVD, using a digitizing image analysis system (Osteometrics, Inc. Decatur, GA, USA). To evaluate PPARγ positive expression in distal femur, the slides were scanned by LeicaQ550CW image analyzer and the average of absorbance from 30 positive expression cells on each slide was presented as mean ± SD (absorbance/30 cells). Immunohistochemical analysis of bone marrow BMP-2 and BMP-7 expressions were performed following the manufacturers’ instructions: Antigen retrieval was performed by high temperature and pressure treatment of the sections in citrate buffer (0.01 mol/L, pH 6.0). After a wash in PBS, sections were incubated with hydrogen
peroxide blocking agent to quench endogenous tissue peroxidases and then washed in PBS. The slides were incubated with Ultra V Block for 30 min at room temperature. The primary antibodies were incubated according to the manufacturer’s protocol (Maixin-Bio, China). Slides with serial sections were incubated with the primary antibodies (Santa Cluz Corporation) in a humidified chamber at 4°C overnight, followed by being incubated with streptavidin-perosidase avidin for 10 min at room temperature. Diaminobenzadine (DAB) staining was done by incubating the sections with coloring reagent. The sections were counterstained with Harris’ haematoxylin, dehydrated through increasing concentrations of alcohol and mounted with coverslips. One section on each slide was used as a control to assess nonspecific binding. For this section, dilution buffer without antibody was used.

Bone mineral density (BMD) determination

The right femurs of the rats were wrapped with saline saturated gauze to maintain moisture and stored at −20°C. After thawing at room temperature, the femurs were moisturized by soaking in saline solution, the residual muscle removed and the length of femur measured with a ruler. The femur bone mineral density between the midpoint and the distal end of the femur was scanned with a SD-1000 single-photon bone mineral density instrument (Nuclear Industry Beijing Institute of Geology) to measure the bone mineral content (BMC, g/cm) and bone width (BW, cm). The bone mineral density (BMD) measurements were performed at the midpoint of the femur and 2 cm proximal and the BMD was calculated as BMC/BW.

Bone mechanical properties determination

After measuring BMD, the femur was used to determine the bone mechanical properties through three-point bending using Bose ElectroForce Testing system (ELF3510, Bose, USA). Bone samples were tested with a 1 mm indenter, at speed of 0.01 mm/s with a 15 mm span (L). Force (F) and deflection (D) that automatically recorded. The output parameters include elastic force (the force required to cause bone specimens to deform, N), maximum force (the maximum force the bone can resist, N), fracture force (the force required to cause bone fracture, N) and the maximum deflection (maximum degree of the bone displacement, mm). The stiffness coefficient (load-displacement curve slope, N/mm) was also calculated based on the above parameters.

Bone Histomorphometry

The right proximal tibial growth plate and metaphyses were processed for cartilaginous longitudinal growth rate and cancellous bone histomorphometric analyses. The samples were opened to expose the bone marrow cavity using an Isomet Low Speed Saw (Buehler, Lake Bluff, Illinois, USA) and fixed in 10% phosphate buffered formalin for 24 hours. They were then dehydrated in graded ethanol, defatted in xylene, and embedded undecalcified in methyl methacrylate [27]. Frontal sections were cut at thicknesses of 4- and 10-μm. The 4-μm sections were stained by Goldner’s Trichrome for static histomorphometric measurements. The 10-μm unstained sections were used for dynamic histomorphometric analyses [28]. A digitizing image analysis system (Osteometrics, Inc. Decatur, GA, USA) was used for quantitative bone histomorphometric measurements. Briefly, the regional of interest were the proximal tibial growth plate and the proximal tibial metaphysis (PTM) located between 1 and 4 mm distal to the growth plate-epiphyseal junction. Static measurements included total tissue volume (TV), trabecular bone volume (BV), marrow fatty area (F.Ar), trabecular bone surface (BS), osteoclast surface (OcS) and osteoblast surface (ObS). Dynamic measurements include interlabel width in the growth plate (G-Int.Wi) of PTM, trabecular single-labeled surface (sL.S), double labeled surface (dL.S) and endocortical interlabel width (Ec-Int.Wi), and endocortical single-labeled surface (Ec-S.L.S), double labeled surface (Ec-dL.S) and endocortical interlabel width (Ec-Int.Wi). These parameters were used to calculate longitudinal growth rate (LGR), percentages of trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), marrow fatty area (F.Ar/TV), osteoclast surface (OcS/BS), osteoblast surface (ObS/BS), longitudinal growth rate (LGR), mineralizing surface (MS/BS), mineral
apposition rate (MAR), bone formation rate (BFR) per unit of bone surface (BFR/BS), bone volume (BFR/BV), and tissue volume (BFR/TV), endocortical mineralizing surface (Ec-MS/BS), endocortical mineral apposition rate (Ec-MAR), endocortical bone formation rate (Ec-BFR) per unit of bone surface (Ec-BFR/BS) as previously described [29,30].

Culture of rat osteoblast and marrow stromal cell (MSC)

Osteoblastic cell was isolated from newborn rat calvaria (rOB). After removing the periosteum, the bones were cut into small flaps of 1 mm³ and subjected to digestion with 0.25% trypsin (Life Technologies Gibco-BRL) for 20 min at 37°C. After centrifugation, supernatants were discarded to remove the fibroblast population. Then the flaps were digestion with 0.2% collagenase type I and 0.1% hyaluronidase (Life Technologies Gibco-BRL) for six 20-min intervals at 37°C. The samples were then washed thoroughly with Dulbecco’s modified eagle’s medium (DMEM, Gibco). The precipitates and bone flaps were transferred to 25 cm² culture flasks and cultured in Dulbecco’s modified eagle’s medium, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified...
incubator with 5% carbon dioxide (CO2). The DMEM culture medium was changed every 3 days. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Bio-Rad, USA) assay was use for the observation of cell growth/death.

Primary rat bone marrow stromal cells (rMSC) were collected from marrow of femur in 4-week-old Wistar rats (obtained from the Laboratory Animal Center of Guangdong Medical College) [21]. The rMSC then were prepared by gradient centrifugation at 900 g for 30 min on a Percoll-Paque gradient (Amersham Pharmacia, USA) at a specific gravity of 1.073 g/mL. The low-density mononuclear cells were washed twice in Hanks' balanced salt solution and cultured in DMEM supplemented with 10% FBS and 1% antibiotic (10 000 U/mL penicillin G sodium, 10000 μg/mL streptomycin sulfate) at 37°C in a humid atmosphere containing 5% CO2. The medium was changed every 3 to 4 d to remove the non-adherent hematopoietic cells. The adherent cell population was expanded and passaged every 12 to 14 d. All cells used for the experiments have been through three passages. To identify the abilities of rMSC with regard to osteogenesis and adipogenesis, the following studies were performed.

Induction of osteogenic differentiation of rMSC (OB-in): When the rMSC from passage number 2 became 80% confluent in the culture plates, the culture medium was changed to an osteoblast inducing medium containing 50 μg/mL L-ascorbic acid, 10−2 mol/L β-glycerophosphate, and 10−8 mol/L dexamethasone. When the cells became layered and confluent up to 100%, visible symmetric colonies were observed after osteoblast induction.

Induction of adipogenic differentiation of rMSC (Ad-in): The rMSC of each group were replated at 2×104 cells/cm2 in a 25 cm2 culture dish and maintained until 80% confluence. Then, the culture medium was switched to adipogenic medium consisting of control medium supplemented with 10 μg/ml insulin (Sigma, St. Louis, MO), 10−5 mol/L dexamethasone (Sigma, St. Louis, MO), 2×10−4 mol/L indomethacin (Sigma, St. Louis, MO) and 5×10−5 mol/L isobutylmethyl xanthine (IBMX, Sigma, St. Louis, MO) for an additional 5 days followed by total RNA isolation.

Identification for OB-in and Ad-in of MSC had according to the methods referred to Cui. et al. [21].

Determination of alkaline phosphatase (ALP) and osteocalcin secretion

ALP activity assay: Cells were seeded in 96-well plates and the confluent cells were cultured for the indicated period with or without Sal B treatment. Cells were washed with PBS and 150 μL of substrat buffer (6.7 mmol/L disodium p-nitrophosphophate hexahydrate, 25 mmol/L diethanolamine and 1 mmol/L MgCl2) was added. After the mixtures were incubated at 37°C for 3 min, we measured the absorbance at 405 nm.

Determination of osteocalcin secretion: Cells were seeded in 6-well plates at a seeding density of 2×104 cells/cm2 and were further incubated for 14 days in osteogenic medium. At the end of the treatment period, the cells were harvested and subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis.
cultured with or without Sal B treatment for 14 d. At the end of the culture, the conditioned media were collected for assessment. The concentration of free osteocalcin was measured by radioimmunoassay (RIA) according to the manufacturer's instructions (Tian Jin Nine Tripods Medical& Bioengineering Co, LTD). The intra-assay variance of the measurements of osteocalcin RIA was 1.26%.

Reverse transcription polymerase chain reaction (RT-PCR) assay

Test of cultured cells: Total RNA was extracted from cultured cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) and subjected to RT-PCR analysis using the 9600Gen Amp PCR system (PerkinElmer Applied Biosystems, USA) with PCR reagents (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Primers used are listed in Table 2. Amplified products were then loaded on a 2% agarose gel and subjected to electrophoresis. Digital pictures were taken and analyzed with gel image system. Each gene expression was normalized to GAPDH.

Test of rat femur bone tissue: The upper femur halves obtained were snap-frozen in liquid nitrogen and stored at \(-270^\circ\)C. Total RNA was prepared using TRIZOL reagent and stored at \(-20^\circ\)C in ribonuclease (RNase)-free water until use. To determine mRNA levels, a one-step reverse transcription-polymerase chain reaction (RT-PCR) procedure was performed using the 9600Gen Amp PCR system (PerkinElmer Applied Biosystems, USA) according to the manufacturer’s protocol. Primers used are listed in Table 3. Amplified products were then loaded on a 2% agarose gel and subjected to electrophoresis. Digital pictures were taken and analyzed using Bandscan analysis software. Each gene expression was normalized to GAPDH.

Statistic Analysis

Data were presented as mean± SD. The statistical differences among groups were evaluated using variance (ANOVA) with Fisher’s PLSD test. Probabilities (p) less than 0.05 were considered significant.

Results

Effect of Aging

Aging has a significant impact on the bone architecture and microvasculature density. Compared with the 6 month old rat, the aging associated changes seen at 9 months included significantly increased femoral cortical bone strength parameters (i.e. the maximum, fracture and elastic forces increased by 28, 33 and 34%), decreased proximal tibial (PT) longitudinal growth rate (LGR) (down by 38%), decreased proximal tibial metaphyses (PTM) cancellous bone formation (i.e. MS/BS, BFR/BS, BFR/BV, BFR/TV down by 25, 28, 26 and 44%) and endocortical bone formation indices (i.e. Ec-MS/BS, Ec-MAR and Ec-BFR/BS by 26, 49 and 62%), these changes were associated with significantly increased distal femoral marrow microvessels diameter (DMV), up by 54%. No available data of soft tissue in 6 month old rat. (Tables 4–9, Figures 3–9).

There were non-significant reductions in PTM cancellous bone mass (BV/TV down by 19%, Tb.N down by 20%, Oc.S/BS down by 14%), and increased Tb.Sp (up by 32%). In addition, PPARγ...
Effect of GC in Intact Male Rats

Sal B alone (B40), prednisone treated (GC), GC plus Sal B40 mg/kg/d staining of Von Willbrand Factor (vWF) in microvessel endothelial cells increased expression by 14%, reduced marrow fatty area (F.Ar/TV) by 37%, and increased DMV by 238%, when compared to GC control. The rats treated with Sal B40 showed reduced PTM cancellous bone Ob.S/BS by 34%, while the GC alone was reduced by 74%. The results suggest that both osteoblasts and microvessel diameters were increased by Sal B40. (Tables 4–9, Figures 3–5)

Effect of Sal B80 in GC treated Male Rats

Sal B80 treatment of GC-treated rats for 12 weeks not only prevented GC-induced changes but also showed additionally stimulated osteogenesis that increase cancellous bone mass and increased marrow angiogenesis by enlargement of microvessels diameter.

Compared to vehicle (aging) control, the 80 mg of Sal B/kg/d (Sal B80) - treated GC rats showed no significant differences in terms of body and soft tissues weights, serum biomarkers, BMD, biomechanical properties (except for increase in stiffness by 25%), PT LGR, PTM cancellous bone mass, architectures, MAR, BFR/BS, BFR/BV, Ob.S/BS and Ob.S/BS, endocortical formation indices (i.e. Ec-MS/BS, Ec-MAR and Ec-PTM cancellous bone formation indices (i.e. MAR and BFR/TV) by 8 and 20%, Significant reductions were seen in the endocortical bone formation indices (i.e. Ec-MS/BS, Ec-MAR and Ec-BFR by 51, 36 and 68%), and microvessels diameter (DMV, down by 59%) and density (MVD) down by 23%, while fatty marrow content was increased by 124% and PPARγ expression by 48% (Table 4–9, Figure 6–9). Immunohistochemical analysis of bone marrow BMP-2 and BMP-7 expression significant decreased with decrease of total BMP-2 and BMP-7 mRNA expression (Figure 10).

Effect of Sal B40 in GC treated Male Rats

Sal B40 treatment of GC-treated rats prevented most of the GC-induced changes. Compared to vehicle (aging) controls, the 40 mg Sal B/kg/d (Sal B40) - treated GC rats showed no significant differences in terms of body and soft tissues weights, serum biomarkers, BMD, biomechanical properties (except for increase in stiffness by 25%), PT LGR, PTM cancellous bone mass, architectures, MAR, BFR/BS, BFR/BV, Ob.S/BS and Ob.S/BS, endocortical formation indices (i.e. Ec-MS/BS, Ec-MAR, except for a decrease in Ec-BFR/BS by 30%), DMV and MVD, F.Ar/TV and distal femoral marrow PPARγ expression, and both mRNA and positive expression of BMP-2 and BMP-7. (Tables 4–9, Figures 3–5, 9–10)

Furthermore there were no differences in skeletal cell and tissues values, except for significantly increased PTM cancellous MS/BS (up by 14%) and BFR/TV (up by 29%), and decreased Ec-BFR/BS (down by 30%). (Tables 4–9, Figures 3–5)

The Sal B40 treated GC rats tended to exhibit a larger marrow DMV (up by 38%) versus aging control, that resulted in significantly increased DMV by 230%, when compared to GC control. The rats treated with Sal B40 showed reduced PTM cancellous bone Ob.S/BS by 34%, while the GC alone was reduced by 74%. The results suggest that both osteoblasts and microvessel diameters were stimulated by Sal B40. (Tables 4–9; Figures 3–5)
Effect of Sal B in osteoblast viability and MSC differentiation in vitro

When cultured different concentration of Sal B with rOB, Sal B at concentration from $10^{-8}$ mol/L to $10^{-6}$ mol/L stimulated rOB cell growth and proliferation, increased ALP activity and osteocalcin secretion, this effect appeared in a time and dose-dependent manner, when the concentration reached $10^{-5}$ mol/L the cell death occurred with function decrease. (Figure 11).

Sal B at $5 \times 10^{-7}$ mol/L stimulated ALP secretion in MSC which was similar to the action of osteoblast induction media (OB-in), Sal B from $10^{-6}$ mol/L to $10^{-5}$ mol/L further increased OB-in treated-MSC secretion of ALP and osteocalcin, which reveal the ability of MSC differentiation into osteoblast by Sal B. (Figure 12)

Effect of Sal B on Dickkopf-1(DKK-1)/β-catenin mRNA expression in MSC

DKK-1 is an inhibitor of Wnt signaling and GC is a strong stimulator of DKK-1. When MSCs were exposed to adipocyte induction medium (Ad-in, containing high concentration of GC), PPARγ mRNA expression increased accompanied with increases of DKK-1 mRNA expression while β-catenin mRNA expression decreased when compared to control. Treatment with $10^{-5}$ mol/L Sal B and $5 \times 10^{-7}$ mol/L Sal A decreased PPARγ and DKK-1 mRNA expression and increased β-catenin mRNA expression with or without adipocyte inducement medium. Sal B $10^{-7}$ mol/L and Sal A $5 \times 10^{-7}$ mol/L also increased Runx2 mRNA expression without osteoblast inducement medium (Figure 13).

Discussion

The current study found that GC treatment decreased bone formation by inhibiting osteoblast activity and marrow production of BMP-2 and BMP-7, increased marrow adipocytes with elevated PPARγ expression that promoted bone marrow stromal cell differentiation into adipocytes. Since osteoblasts and adipocytes share common bone stromal progenitors [31], GC treatment stimulate the differentiation of marrow stromal cells to adipocytes, thereby, reducing the pool of local progenitor cells to differentiate into osteoblasts. The reduction in osteoblast number in turn contributes to decreased osteogenesis. Furthermore, we specifically labeled marrow vascular endothelial cells with Von Willbrand Factor and observed similar GC disruption of marrow microvessels as previously observed in rabbit femoral head [32]. The disruption of marrow microvasculature would reduce the source of circulating progenitor cells supporting osteogenesis [33–39]. The reduction in local and circulating progenitors would then lead to reduce osteogenesis. Our findings suggest that bone formation, bone growth, and bone remodeling are affected by GC treatment via GC-induced changes in gene expression in local progenitor cells.
Figure 11. Effects of Sal B on the proliferation and differentiation in new born rat calvarium osteoblast. Cells were inoculated in 96-well plates and cultured, then transferred to a medium containing various concentrations of vehicle and Sal B. MTT was tested at 24, 48, 72 and 96 h of incubation (A), ALP activity (represent by OD value) was determined later until 7 days of incubation (B), and the content of osteocalcin (µg/L) in culture medium was determined in day 21 after incubated with different treatment (C). Data shown are mean ± SD. n = 6. **P<0.01, *P<0.01 versus control (vehicle treatment).
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Figure 12. Effects of Sal B on rat marrow stromal cell (rMSC) differentiation into osteoblast. Cells were collected from the femur marrow of one month old SD rats. Cells inoculated in 25 mm² culture flask and cultured. The cells used in the study were the 3 passage, then transferred to the medium containing osteoblast induction medium (OB-in), Sal B and OB-in plus various concentrations of Sal B respectively. A: ALP activity (represent by OD value) was determined at 3, 5 and 7 days. B: the content of osteocalcin (µg/L) in culture medium was determined in day 21 after incubated with different treatment. Data shown are mean ± SD. n = 6. **P<0.01, *P<0.05 versus control (vehicle treatment).
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Salvianolic Acid B Stimulates Osteogenesis

A

Runx2
GADPH

OB-in  -  +  -  -
SAA    -  -  +  -
SalB   -  -  -  +

Runx2 mRNA Expression (Gray scale ratio)

B

PPARγ
GADPH

AD-in (GC)  -  +  +  -
SAA    -  -  +  -
SalB   -  -  -  +

PPARγ mRNA Expression (Gray scale ratio)

C

β-catenin
GADPH

AD-in (GC)  -  +  -  -
OB-in    -  -  +  -
SAA    -  -  -  +
SalB   -  -  -  +

β-catenin mRNA Expression (Gray scale ratio)

D

β-catenin
GADPH

OB-in    -  +  -  -
AD-in (GC)  -  +  +  +
SAA    -  -  +  -
SalB   -  -  -  +

β-catenin mRNA Expression (Gray scale ratio)

E

DKK-1
GADPH

AD-in (GC)  -  +  +  +  -
OB-in    -  -  +  -
SAA    -  -  +  -
SalB   -  -  -  +

DKK1 mRNA Expression (Gray scale ratio)
marrow fat metabolism and microcirculation are closely related to each other and confirm that the latter two factors also contribute significantly to the development of GC-induced bone loss and the decline in bone strength [4,5].

Our data found 80 mg Sal B/kg/d for 12 weeks depressed adipogenesis and stimulated angiogenesis and osteogenesis in GC-treated rats. The lower 40 mg Sal B/kg/d dose in GC-treated rats only depressed adipogenesis and stimulated cancellous bone formation rate. The 40 mg dose also appeared to increase angiogenesis by enlarging marrow microvessels diameter but the observations were not statistically significant. The prevention of GC-stimulation of adipogenesis maintains marrow stromal and red marrow cell levels at control levels. Since osteogenesis and adipogenesis share common bone stromal progenitors [31], this means the pool of marrow progenitors available to differentiate into osteoblasts will be maintained and not be preferentially stimulated to form adipocytes by GC treatment. Additionally the maintenance of red bone marrow means that the bone marrow will retain more microvessels, unlike fatty bone marrow, which is not as well vascularized [40,41]. The Sal B treatment further increased marrow angiogenesis that increases blood flow to improve nutrition and source of circulating progenitors [33-39]. In support of osteogenesis, stimulated angiogenesis reduced intraosseous pressure that increases blood flow and in turn stimulates osteoblastic activity (i.e. mineral apposition rate) [42]. Further red bone marrow sites with better vasculature exhibit a factor of 10 higher bone remodeling and formation rates than fatty marrow bone sites [43–46]. Also the improved blood flow increases osteocyte lacunocanaluli and blood vessel fluid volume that increase bone strength and reduce the risk of fracture [4,47].

To clarify the mechanism by which Sal B promotes osteoblast differentiation and alleviates GC-induced impairment of bone formation, we examined the effects of Sal B on primary rat calvarial osteoblasts and rat bone marrow stromal cell by up-regulating Wnt/β-catenin signaling. Our data showed that Sal B stimulates osteoblast cell growth and MSC differentiation into osteoblast maturation (secretion of ALP and osteocalcin) with a decline in bone strength [4,5].

Figure 13. Effects of Sal B on the gene expression of Dkk-1/β-catenin pathway in rat marrow stromal cell (rMSC) differentiation. rMSC were cultured with osteoblast induction medium (OB-in), adipocyte induction medium (Ad-in, i.e. high concentration of GC), with or without Sal B (10^{-7} mol/L) and Sal A (5×10^{-7} mol/L, a derivant of Sal B, Figure 1) for 7 days. Expression levels of Runx2 (A), PPARγ (B), β-catenin (C and D) and DKK-1 (E) were measured by RT-PCR. Sal B increased Runx2 and β-catenin mRNA expression and decreased DKK-1 mRNA expression which was similar to the action of OB-in. Ad-in marked increased PPARγ and DKK-1 mRNA expression. When treated the Ad-in rMSC with Sal B, the PPARγ and DKK-1 mRNA expression decreased obviously. Sal A had similar effects to Sal B.

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In summary, Sal B treatment of GC-treated male rats not only prevented GC-induced osteopenia but also increased cancellous bone mass by the combination of improved marrow microenvironment (i.e. increased local and circulatory progenitors, blood flow and nutrition) and Sal B stimulation of bone formation rate not only prevented GC-induced osteopenia but also increased cancellous bone mass.

The current study used doses of 40 and 80 mg/kg gavage in rats, which is similar to the dose used in humans. Salvianolic Acid B (parental form) was approved by the State Food and Drug Administration (SFDA) of China in 2007 for clinical use in the prevention and treatment of cardiovascular diseases. The recommended dose of Salvianolic Acid B for humans is 4 mg/kg/d i.v. Our study examined the drug impact on body weight (Figure 5), organ weight (table 4), gross necropsy and histopathology (negative data not shown) both in intact rats (SalB40 group) and glucocorticoid-treated rats. No significant adverse effects on these parameters were observed. Recently Li et al. [55] reported on the toxicity of Sal A, a derivative of Sal B, in male and female dogs after a 3-month continuous intravenous infusion at doses of 17, 50, and 150 mg/kg/day. No significant cumulative toxicity was observed either during or 90 days following treatment. Importantly, the doses of Sal A used in dogs were very high as compared to clinical practice and to rats; the 17, 50, and 150 mg/kg/day in dogs are equivalent to 61.2, 180 and 540 mg/kg in rats.

Supporting Information

Figure S1 Working scheme: the mechanism of action of Salvianolic acid B on glucocorticoid induced bone loss. Decrease of osteogenesis and angiogenesis, increase of adipogenesis are considered contributions to glucocorticoid induced bone loss. Salvianolic acid B could withstand the impairment induced by glucocorticoid. (BMPs: bone morphogenetic proteins; MSCs:...
marrow stromal cells; PPARγ: peroxisome proliferator-activated receptor γ; VEGF: vascular endothelial growth factor; Black: effect of glucocorticoid; Red: effect of Salvianolic acid B; ↓: decrease.

(DOC)

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