Research Article

Antiosteoporotic Effects of Huangqi Sanxian Decoction in Cultured Rat Osteoblasts by Proteomic Characterization of the Target and Mechanism

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1. Introduction

Osteoporosis is the most frequent bone remodeling disease and its incidence increases with advancing age. This disease is characterized by a reduction in bone mass and microarchitectural deterioration of bone tissue, resulting in high risk of fractures [1]. Globally, osteoporosis and associated bone fractures have become a major health hazard afflicting millions of people [2]. Current treatment options for osteoporosis include bisphosphonates, estrogens, selective estrogen receptor modulators, calcitonin, denosumab, and teriparatide. However, many of these drugs generate side effects [3, 4] and their costs are too high to benefit a large population in the developing and developed countries, which may limit their applications.

Traditional Chinese medicine has been a part of healthcare in China for thousands of years and has recently been reevaluated for clinical approach [5]. The traditional Chinese medicine has fewer adverse reactions and is more suitable for long-term administration than synthetic drugs and antibiotics. Popular commercially available prescriptions include Jinkui Shenqi Wan (JKSQW), which exerts a therapeutic effect on the kidney-Yang deficiency and osteoporosis indicated in Chinese Pharmacopoeia (2010), and Huangqi Sanxian decoction (HQSXD), a traditional Chinese formula, which is composed of Radix Astragali, Epimedi Folium, Cistanche Herba, Radix notoginseng, Radix Salviae Miltiorrhizae, Corydalis Rhizoma, Radix Angelicae Sinensis, and Radix Clematis. Our previous study revealed that Huangqi Sanxian decoction treatment significantly increased sex estrogen...
level and bone mineral density (BMD) and repressed bone absorption function in postmenopausal women [6]. This suggested that HQSXD has beneficial effects in the treatment of osteoporosis. However, little is known about the mechanisms and targets underlying the effects of HQSXD on osteoporosis.

The Chinese traditional medicine theory believes that bone activities are controlled by the kidney. Strong "kidney" can nourish bones, but the weak "kidney" might hasten bone deterioration [7]. Kidney deficiency and blood stasis are the main pathological basis of osteoporosis. Huangqi Sanxian decoction is composed of eight Chinese medicinal herbs; of these, Epimedi Folium and Cistanche Herba strengthen kidneys, while Radix Salviae Miltiorrhizae and Radix notoginseng invigorate the circulation of blood. These herbs are an excellent combination for highlighting their superiority in the treatment of osteoporosis [8].

Jinkui Shenqi Wan, an ancient Chinese herbal formula, is indicated in the Chinese Pharmacopoeia (2010) for the treatment of Yang insufficiency of kidney, weakness and soreness of the loins and the knees, cold feeling in the limbs, and frequent urination. Human clinical studies have certified that Jinkui Shenqi Wan exerts a therapeutic effect on the kidney-Yang deficiency [9]. Hence, we used Jinkui Shenqi Wan as a positive control in this study.

In the present study, seropharmacology and functional proteomics technology were used to explore the multiple proteins associated with the antiosteoporotic effect. The results suggest a basis for the clinical use of HQSXD in the treatment of patients with osteoporosis.

2. Materials and Methods

2.1. Animals. Thirty Sprague-Dawley rats (fifteen male and fifteen female), weighing about 250–300 g, were purchased from the Animal Experimental Center of Guangdong Medical College (Dongguan, China). The animals were housed individually in a regulated environment (24 ± 0.5 °C), with a 12-hour light/dark cycle (under light: 08:00–20:00 h). Food and water were given ad libitum throughout the experiment. After three days of acclimation, male and female SD rats were randomly divided into three groups: blank-control group, experimental group treated with Huangqi Sanxian decoction, and positive control group treated with Jinkui Shenqi Wan. The Committee of Experimental Animal Administration of the University approved the study, and the procedures of the experiment were in accordance with generally accepted international rules and regulations.

2.2. Preparation of HQSXD and JKSQW. HQSXD included eight plant extracts, including Radix Astragali (root, Chinese herbal name: Huang-Qi), Epimedi Folium (leaf, Chinese herbal name: Yin-Yang-Huo), Cistanche Herba (succulent stem, Chinese herbal name: Rou-Cong-Rong), Radix notoginseng (root and rhizome, Chinese herbal name: San-Qi), Radix Salviae Miltiorrhizae (root and rhizome, Chinese herbal name: Dan-Shen), Corydalis Rhizoma (rhizome, Chinese herbal name: Yan-Hu-Suo), Radix Angelicae Sinensis (root, Chinese herbal name: Dang-Gui), and Radix Clematidis (root and rhizome, Chinese herbal name: Wei-Ling-Xian) in a ratio of 15: 10 : 5 : 5:10:8:10 [6]. The above eight medicinal extracts were obtained from Dongguan Sinopharm (Dongguan, China) and identified by Professor Zhou (Department of Pharmacy, Guangdong Medical College, Dongguan, China). The eight medicinal plant materials in the mixture (270 g) were powdered and cooiled with 1000 mL water for 2 hours. The extraction was repeated twice. The filtrates were concentrated to 200 mL under reduced pressure and kept at 4 °C.

JKSQW consisted of Radix Rehmanniae Preparata (root, Chinese herbal name: Di-Huang), Dioscoreae Rhizome (root, Chinese herbal name: Shan-Yao), Fructus Corni Officinalis (fructus, Chinese herbal name: Shan-Zhu-Yu), Sclerotium Poriae Cocos (dried sclerotia, Chinese herbal name: Fu-Ling), Cortex Moutan Radicis (root bark, Chinese herbal name: Mu-Dan-Pi), Rhizoma Alismatis Orientalis (stem, Chinese herbal name: Ze-Xie), Ramulus Cinnamomum Cassiae (twig, Chinese herbal name: Gu-Wei), Radix Aconiti Lateralis Praeparata (root, Chinese herbal name: Fu-Zi), Radix Achyranthis Bidentatae (root, Chinese herbal name: Niu-Xi), and Plantaginis Semen (seed, Chinese herbal name: Che-Qian-Zi) [9]. As per the instructions, 5 mg Jinkui Shenqi Wan (Beijing Tong Ren Tang Pharmaceutical Technology Development Inc., Dongguan, China) was dissolved in 100 mL distilled water before administration. The experimental dose for HQSXD and JKSQW in the present study was equivalent to the corresponding clinical prescription dose for a human subject weighing 60 kg.

2.3. Drug Administration and Sample Collections. Rats were randomly divided into three groups of 10 animals each (each group included five males and five females). Chinese medicine HQSXD solution (14 g/kg) and medicine JKSQW solution (0.520 g/kg) were administered orally every day for three days, twice a day. The blank-control group was orally administered distilled water, and they were monitored concurrently with the HQSXD-experimental groups. At the end of the experiment, sixty minutes after the last treatment, the animals were exposed to ether anesthesia; blood samples were collected by heart puncture under aseptic conditions and then centrifuged for 15 min, to obtain serum samples. Serum of HQSXD-treated rats and control serum were inactivated at 56 °C in a water bath for 30 min and filtered through a 0.22 μm filter membrane, termed HQSXD-S, JKSQW-control-S, and BLANK-control-S, respectively, and then stored at −80 °C.

2.4. Primary Osteoblasts Culture and Assay for Osteoblast Proliferation. Primary rat osteoblast cells were obtained from 1-day-old neonatal Sprague-Dawley rats as described previously [10]. Primary osteoblasts were cultured by seeding 96-well plates with a density of 1 × 10⁶ per well and incubated for 24 h. After adhesion of cells, Dulbecco’s Modified Eagle Medium (DMEM) was added and incubated for another 24 hours. Next, after discarding the medium, test (HQSXD-S) and control (JKSQW-control-S, BLANK-control-S) samples
were added at a concentration of 10% (v/v), and the cells were incubated at 37°C in a humid atmosphere containing 5% CO₂ for 72 h. Thereafter, 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 4 hours, after which the medium was discarded, and dimethyl sulfoxide (DMSO) (150 μL) was added. Absorbance was measured at 490 nm using a Synergy 2 multifunctional microplate reader (Bio-Tek) to assess osteoblast proliferation.

2.5. ALP Activity and Staining Assay. Osteoblasts were suspended in DMEM to obtain a cell density of 5 × 10⁵/mL; 2 mL aliquots of the cell suspension were added to 6-well plates. After 24 h of incubation, the medium was changed and cells were incubated with test (HQSXD-S) and control (JKSQW-control-S, BLANK-control-S) at a concentration of 10% (v/v) for 3 days. Alkaline phosphatase (ALP) activity was measured using an ALP assay kit (Sigma) as described previously [11]. For ALP staining, after incubation with HQSXD-S, JKSQW-control-S, and BLANK-control-S (concentration of 10% (v/v)) for three days, the cells were fixed in 70% ethanol for 15 min, washed, and then incubated with ALP staining buffer, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Beyotime Institute of Biotechnology, China), at 37°C for 30 min, air dried, and photographed.

2.6. Mineralization Assay. After 21 days of differentiation, the mineralization of osteoblasts was analyzed as described previously [12]. Briefly, the cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol for one hour. The cells were then rinsed in distilled water, stained with 0.5% Alizarin Red S (ARS) at pH 4.2 with rotation for 30 min at 37°C, and subsequently washed with distilled water and dried in air. Stained cultures were photographed. To analyze ARS activity, the ARS in stained cells was destained with 4% (w/v) Bio-Lyte, 0.1% bromophenol blue, and 1% IPG buffer. The strips were covered with mineral oil (GE Healthcare) to prevent samples from evaporation and rehydrated for 10–20 hours. Isoelectric focusing gel electrophoresis was performed by using an electrophoresis apparatus (GE Healthcare). The running conditions of the IEF process were as follows: 100 V, 1 h; 200 V, 1 h; 300 V, 1 h; 500 V, 1 h; and 8,000 V, up 60,000 V. After the IEF, the IPG strips were equilibrated with equilibration buffer-1 (6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris-HCl [pH 8.8], bromophenol blue dye, and 1% [w/v] DTT) for 15 min and then were repeated for an additional 15 minutes in 5 mL of equilibration buffer-2, except that DTT was replaced by 2.5% [w/v] iodoacetamide. The second dimension was done with a 12.5% acrylamide gel at 10 mA/gel for one hour and then at 38 mA/gel until the bromophenol blue dye reached the bottom of the gel. After electrophoresis, the gels were visualized by silver nitrate staining. Gels were scanned using the ImageScanner (GE Healthcare). The images were analyzed with ImageMaster 2D Platinum v7.0 software (GE Healthcare, San Francisco, CA) including spot detection, background subtraction, gel matching, and normalization. Spots were detected and matched automatically to a master gel and then edited manually. Matched spots from triplicate gel sets that showed overlap ratio with an absolute value ≥2 were recognized as differentially expressed. Differentially
2.9. Mass Spectrometry and Database Search. Protein spots were excised from the 2DE gels using a pipette tip. Gel pieces were destained in a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1:1), washed with deionized water, and dehydrated in 100% acetonitrile (ACN). Samples were rehydrated for digestion with trypsin (12.5 mg/mL) at 4°C for 30 min. Excess trypsin solution was replaced with 25 mM ammonium bicarbonate. The samples were incubated overnight at 37°C. Peptides were then extracted twice with 50% ACN/5% TFA followed by 100% ACN for 15 min each. After drying, the peptide extracts were desalted with ZipTip Pipette Tips (Millipore). Mass spectrometry was done using an ultraflex III MALDI-TOF/TOF-MS (Bruker) with a high voltage of 20 kV, and spectra were externally calibrated using the peptide standard Maker. Protein identification was determined by matching the peptide mass fingerprinting (PMF) and MALDI-TOF/TOF-MS results via MASCOT (version 2.2, Matrix Science) against NCBInr database with BioTools software. Database searches were performed using the following parameters: taxonomy, rice; enzyme, trypsin; and one missed cleavage allowed. Carbamidomethylation was selected as a fixed modification, and the oxidation was allowed as a variable. PMF tolerance set to 100 ppm, and MS/MS tolerance set to 0.7 Da. A protein was regarded as identified if the MASCOT protein score was above the 5% significance threshold for the database (score >64).

2.10. Western Blotting Analysis. To verify the results of 2DE of the identified proteins, we randomly chose four proteins for Western blot: FPR2, TCEB1, PHB, and alpha-spectrin. Cytosolic extracts were prepared from cells, and the protein in abundant spots were selected for mass spectrometry (MS) analysis.
Figure 4: Representative silver nitrate stained gels showing two-dimensional electrophoresis protein profiles of (a) blank-control group, (b) HQSXD-treated group, and (c) JKSQW-treated group. Molecular weight (MW, kDa) and isoelectric point (pI) are indicated along the y- and x-axes, respectively.

the supernatant was quantified using the BCA protein assay kit (Beyotime Institute of Biotechnology, China). A sample (50 μg) was electrophoresed on a 10% SDS-polyacrylamide gel and subsequently transferred onto a PVDF membrane (Millipore). After blocking with 5% nonfat dry milk, the membranes were incubated with anti-FPR2 (M-73, sc-66901, Santa Cruz Biotechnology), anti-alpha-spectrin (C-11, sc-46696, Santa Cruz Biotechnology), anti-TCEB1 (ProteinTech Group, Inc., China), and anti-PHB (ProteinTech Group, Inc., China). The bound antibodies were detected using a horseradish peroxidase- (HRP-) conjugated secondary antibody and visualized by an enhanced chemiluminescence detection system, followed by quantification using the Image J2x.

2.11. Statistical Analysis. Data are presented as mean ± SD of triplicate samples. Comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s test, and the difference was considered statistically significant if \( p < 0.05 \).

3. Results

3.1. Effects of HQSXD-Treated Rat Serum on Proliferation of Osteoblasts. The proliferation of primary osteoblasts showed an upward trend compared to that of blank control. However, there was no significant difference detected in the proliferation between HQSXD-treated and JKSQW-treated groups.
Similar changes in proliferation between JKSQW-treated group and HQSXD-treated group indicated that HQSXD had an influence on osteoblasts (Table 1, Figure 1).

### 3.2. HQSXD-Treated Rat Serum Enhances Primary Osteoblast Differentiation

ALP is an important biochemical marker of differentiated osteoblasts, and the effects of drug on ALP activities in osteoblasts were first determined. Results of ALP staining showed that HQSXD-S and JKSQW-control-S stimulated osteoblast differentiation (Figure 2(a)). The cells cultured with HQSXD-S showed a significantly higher ALP activity than that cultured with BLANK-control-S (Figure 2(b)). ARS staining in osteoblasts was assessed after 21 days of incubation to examine whether HQSXD enhanced bone mineralization during osteoblastogenesis. In osteoblasts treated with BLANK-control-S, the calcium deposition in the mineralized matrix was minimal. The proportional areas of Alizarin Red-positive staining in the HQSXD-treated group and JKSQW-treated group were higher than that in the Blank-control group (Figure 3(a)). As shown in Figure 3(b), the level and intensity of ARS staining indicated the extent of mineralization, which increased upon treatment with HQSXD-S.

### 3.3. Protein Expression Profile in HQSXD-Treated and HQSXD-Untreated Osteoblasts

Two-dimensional electrophoresis and gel silver nitrate staining were conducted to further investigate the differential protein expression between HQSXD-S-treated and HQSXD-S-untreated osteoblasts. After optimization of the 2DE gels, with representative 2DE gel images shown in Figure 4, approximately 938 ± 26, 875 ± 34, and 904 ± 22 protein spots were detected in blank protein sample, HQSXD protein sample, and JKSQW protein sample, respectively. During analysis with ImageMaster 2D Platinum, spots with an overlap ratio absolute value ≥2 were recognized as differentially expressed. Thirty-eight protein spots were found to be significantly regulated among three groups, of which 15 spots were downregulated and 23 spots upregulated. Ten of these 38 spots exhibited a more than twofold increase or decrease in abundance as observed in all replicate gels. These 10 regulated proteins were indicated by the circle in Figure 5, and the selected regions that showed significant differences in protein expression profile of osteoblasts among three groups were shown in Figure 6. All of them were excised from the gels for further identification by MALDI-TOF/TOF-MS analysis.

### 3.4. Identification of the Differentially Expressed Proteins

Proteins were identified by MALDI-TOF/TOF-MS. Ten peptide mass fingerprints (PMFs) and 50 peptide fragment fingerprints (PFF) were successfully obtained. A selected PMF of protein spot 6 is displayed in Figure 7(a), and the TOF/TOF analysis is shown in Figures 7(b)–7(f). All PMFs were evaluated with the Mascot software in NCBI nr database to identify the protein spots. The result had high confidence if the protein was ranked as the best hit with a significant score and high sequence coverage. Finally, we identified eight proteins in these spots. Properties of the identification of eight selected protein spots are summarized in Table 2.

### 3.5. Effect of HQSXD on the Expression of Proteins That Regulate Antiestrogenic Activity

To further investigate the influence of HQSXD on the expression of antioestroporotic proteins, we examined the expression of FPR2, alpha-spectrin, PHB, and TCEB1 by Western blotting. The expression of FPR2 was increased by treatment with HQSXD-S in comparison to the blank-control group. However, HQSXD remarkably decreased alpha-spectrin, PHB, and TCEB1 protein levels compared with blank-control group (Figure 8). Results from Western blot manifested the same trend as from proteomic analysis.
Figure 6: The ten protein spots of osteoblasts that were treated with and without HQSXD-S. Selected regions showed significant differences in the protein expression profile of osteoblasts among the three groups. Upregulated spots are indicated by green circles and downregulated ones by red circles.

Table 2: Summary of differentially expressed proteins in osteoblasts treated with HQSXD.

<table>
<thead>
<tr>
<th>Spot number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein score&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Matching peptides (number)</th>
<th>Theoretical &lt;i&gt;P&lt;sub&gt;i&lt;/sub&gt;&lt;/i&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Theoretical Mr (Da)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Target protein</th>
<th>Species</th>
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<tr>
<td>1</td>
<td>70</td>
<td>6</td>
<td>9.27</td>
<td>39299</td>
<td>N-Formyl peptide receptor 2</td>
<td>Rattus norvegicus</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>7</td>
<td>5.65</td>
<td>54851</td>
<td>Alpha-spectrin</td>
<td>Rattus norvegicus</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>6</td>
<td>10.08</td>
<td>43712</td>
<td>Heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1</td>
<td>Rattus norvegicus</td>
</tr>
<tr>
<td>6</td>
<td>173</td>
<td>12</td>
<td>5.57</td>
<td>29859</td>
<td>Prohibitin</td>
<td>Rattus norvegicus</td>
</tr>
<tr>
<td>7</td>
<td>67</td>
<td>4</td>
<td>4.59</td>
<td>12752</td>
<td>Transcription elongation factor B (SIII), polypeptide 1</td>
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<td>70</td>
<td>11</td>
<td>4.98</td>
<td>127444</td>
<td>Chromosome segregation protein</td>
<td>Rattus norvegicus</td>
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<tr>
<td>9</td>
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<td>4</td>
<td>6.91</td>
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<tr>
<td>10</td>
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<td>12</td>
<td>8.86</td>
<td>48199</td>
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<td>Rattus norvegicus</td>
</tr>
</tbody>
</table>

<sup>a</sup>Protein spot number according to Figure 3.

<sup>b</sup>Protein scores were based on combined mass and mass/mass spectra from MALDI-TOF/TOF identification MS.

<sup>c</sup>Theoretical molecular mass (Mr) and isoelectric point (<i>P<sub>i</sub></i>) from the NCBI nr database.
4. Discussion

In this study, we found that the proliferative activities of osteoblasts between HQSXD-S-treated group and JKSQW-control-S-treated group were similar. This investigation demonstrates that HQSXD can significantly facilitate bone formation through increasing the number of osteoblasts, which is beneficial to the treatment of osteoporosis. By measuring ALP activity, HQSXD was first screened for its ability to induce osteogenesis. HQSXD is capable of significantly promoting osteoblast differentiation, as well as increasing osteoblast mineralization.

Results of the identification of the selected protein spots are summarized in Table 2. The molecular weight (Mr) and isoelectric point (Pl) of each protein spot shown in Table 2 are theoretical values. The eight protein spots were identified as (1) N-formyl peptide receptor 2 (FPR2); (2) alpha-spectrin; (3) heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1 (HS3ST3A1); (4) prohibitin (PHB); (5) transcription elongation factor B (SIII), polypeptide 1 (TCEB1); (6) chromosome
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TCEB1
PHB
FPR2
Blank HQSXD JKSQW

𝛽-actin
Alpha-spectrin

Figure 8: HQSXD-S treatment decreased alpha-spectrin, PHB, and TCEB1 expression and increased FPR2 expression. Cell lysates (50 μg) were processed for Western blot analysis. Normalization performed to 𝛽-actin. The bands shown here were from a representative experiment repeated three times.

5. Conclusions

The results confirm that HQSXD has a beneficial effect on osteoblasts and alters the expression level of some proteins in osteoblasts. The protein expressed by osteoblasts treated with HQSXD may be involved in cell proliferation and differentiation and other physiological processes and in the regulation of cell activation. Further study is needed to investigate the effects of major active constituents in HQSXD on protein expression on osteoblast so as to demonstrate the interaction and synergistic mechanism.

Conflict of Interests

All the authors state that they have no conflict of interests to declare.

Authors’ Contribution

Chong-Chong Guo and Li-Hua Zheng contributed equally to this work.
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