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Jian-Pi-Yi-Shen (JPYS) decoction is a traditional Chinese herbal formula. The present study aimed to investigate the effect of JPYS drug-containing serum on the proliferation and activity of mouse osteoblasts. Sprague–Dawley rats were fed JPYS decoction, calcium supplement, or normal saline for 6 weeks, and the serum was collected. Mouse osteoblasts were treated with JPYS drug-containing serum, calcium supplement serum, or blank control serum. Cell proliferation was assayed using thiazolyl blue tetrazolium bromide. Levels of alkaline phosphatase, nitric oxide, and nitric oxide synthase in the culture medium were measured. The JPYS drug-containing serum significantly improved the proliferation of osteoblasts compared to the blank control serum and the calcium supplement serum. It also significantly increased the levels of alkaline phosphatase in the culture medium compared to the blank control serum and the calcium supplement serum. Treatment with JPYS drug-containing serum for 48 h and 72 h significantly increased the nitric oxide (NO) concentration in the culture medium compared to the blank control serum. The NOS activity of the osteoblasts was significantly increased by JPYS drug-containing serum compared to blank control serum and calcium supplement serum. All these results were enhanced that the JPYS decoction promotes the proliferation and activity of mouse osteoblasts. These effects may be the underlying mechanisms of JPYS decoction in treating osteoporosis.

**Keywords:** Alkaline Phosphatase. Nitric Oxide. Osteoblasts. Osteoporosis.**1. Introduction**

Osteoporosis is still a public health burden and a clinical challenge. Traditional Chinese medicines are widely used in East Asia and many other parts of the world for the treatment of osteoporosis. According to the theory of traditional Chinese medicine, deficiency of Shen (kidney) essence is closely associated with osteoporosis (Gao et al. 2013; Shu et al. 2015). Both animal studies and clinical trials have shown that Shen-tonifying herbs can increase bone mass (He et al. 2017; Liu et al. 2017). Jian-Pi-Yi-Shen (JPYS) is a Chinese herbal decoction that has been used to treat osteoporosis for decades. It can activate blood and resolve stasis by tonifying the kidney and fortifying the spleen. However, the molecular mechanisms of JPYS in treating osteoporosis are not clear.

Serum pharmacology uses drug-containing serum collected from animals that are treated with certain drugs. It may have some advantages in investigating the pharmacological effects of traditional

Chinese medicines, which usually consist of multiple herbs (Yan et al. 2015). The present study aimed to investigate the effects of JPYS drug-containing serum on mouse osteoblasts and elucidate the mechanisms of this Chinese herbal decoction in treating osteoporosis.

## **2. Material and Methods**

### **Animals**

Forty 15-month-old Sprague–Dawley rats, weighing 400 g, were provided by the Experimental Animal Center of Shandong Academy of Medical Sciences. The animals were kept in standard rat cages with 3 rats per cage. The room temperature was 24 °C with a 12-hour light/12-hour dark cycle. Thirty rats received an intramuscular injection of dexamethasone 2.5 mg/kg twice a week for 6 weeks to induce osteoporosis. Meanwhile, 10 rats were reserved as the control group and given the same amount of normal saline injection. The protocol of our study was approved by the Ethics Committee of Shandong Academy of Medical Sciences. Anesthetic drugs and other necessary measures were used to reduce animal suffering during experimental procedures.

### **Preparation of the JPYS decoction**

The following ingredients were mixed with 2 L of water for 60 min: Radix Rehmanniae Preparata 24 g, Epimedium 12 g, Radix Codonopsis 15 g, Cyathula Officinalis 18 g, Poria 15, Atractylodes Macrocephala 15, Rhizoma Dioscoreae 18 g, oster shell 30 g, Eucommia Ulmoides 12 g, Fructus Psoraleae 12 g, Davallia 21 g, Os Draconis Preparata 30 g, Cortex Moutan 12 g, and Chinese licorice root 6 g. The ingredients were boiled for 2 hours and filtered to collect the filtrate. This process was repeated twice with fresh water. Six liters of filtrate was left to stand for 24 h. The supernatant was collected and concentrated to a relative density of 1.02 at 60 °C. The final JPYS decoction was filtered and sterilized by heating to 105 °C for 60 min.

### **Preparation of drug-containing serum**

The animals were divided into 3 groups with 10 rats in each group: (1) JPYS group. The rats were treated with 10 mL/kg JPYS decoction daily by gastric gavage for 6 weeks. (2) Blank control group. The rats were fed normal saline (10 mL/g) daily by gastric gavage for 6 weeks. (3) Calcium supplement group. The rats were treated with 10% calcium gluconate 10 mL/kg daily and vitamin D2 3000 IU/kg daily, both by gastric gavage, for 6 weeks. One hour after the last gavage, the animals were anesthetized, and blood was collected from the abdominal aorta under aseptic conditions. Serum was collected by centrifuging the blood at 3000 r/min for 15 mins. The serum was heated in 56 °C water for 30 min and filtered through a 0.22- $\mu$ m filter membrane. DMEM (Gibco, Shanghai, China) was added to 20% serum from different groups to produce JPYS drug-containing serum, calcium supplement serum, and blank control serum.

### **Cell culture and characterization**

The mouse osteoblast cell line was provided by the China Center for Type Culture Collection. The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 4.0 mM/L glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100  $\mu$ g/mL streptomycin and maintained in an incubator with 5% CO<sub>2</sub> humidified at 37 °C. When the degree of cell confluence reached 80%, cell passage was carried out. The medium was changed every other day until the cells were fully differentiated. To characterize the osteoblasts, alkaline phosphatase staining was performed using the modified Gomori's method. Two hundred cells were randomly selected, and the percentage of alkaline phosphatase positivity was calculated.

## Calcium nodule staining

The osteoblasts were inoculated into a 24-well plate with 0.5 mL cell-containing medium per cell at a density of  $1 \times 10^5$ /mL. After culture for 14 days, the medium was replaced with JPYS drug-containing serum, calcium supplement serum, or blank control serum for 4 days. Then, the cells were fixed with 95% alcohol and stained with 0.1% alizarin red S (Sigma–Aldrich, USA). The calcium nodules were observed and imaged using a light microscope imaging system (Olympus, Japan).

## Thiazolyl blue tetrazolium bromide (MTT) assay

To measure cell proliferation, the osteoblasts were digested and adjusted to a density of  $1 \times 10^5$ /mL. The cells were transferred to a 96-well plate with 80  $\mu$ L cell-containing medium per well. Then, 20  $\mu$ L DMEM supplemented with serum from different animal groups was added to each well. The cells were treated with JPYS drug-containing serum, calcium supplement serum, or blank control serum. Each serum sample was tested with 8 replicates. After culture at 37 °C for 24 h, 100  $\mu$ L of 5 mg/mL MTT was added to each well for 30 min at room temperature. The MTT concentration was measured using a microplate reader (MULTISKAN MK3, Thermo Fisher, USA) at 570 nm.

## Measurement of alkaline phosphatase concentration

The osteoblasts were digested and adjusted to a density of  $1 \times 10^5$ /mL. The cells were transferred to a 96-well plate with 100  $\mu$ L cell-containing medium per well. Then, 100  $\mu$ L DMEM supplemented with serum from different animal groups was added to each well. The cells were treated with JPYS drug-containing serum, calcium supplement serum, or blank control serum for 72 h. Each serum was tested with 8 replicates. Then, the culture medium was removed, and 200  $\mu$ L 0.1% TRITONX-100 lysate was added for 12 h. From each well, 30  $\mu$ L lysate was transferred to a tube for the measurement of alkaline phosphatase concentration using an assay kit (Colorimetric, Abcam, China). The standard tubes were added to 30  $\mu$ L of 0.1 mg/mL phenol standard solution. The blank control tubes were supplemented with 30  $\mu$ L water. All tubes were added to 0.5 mL buffer solution and 0.5 mL substrate and vibrated in 37 °C water for 15 min. The concentration of alkaline phosphatase was measured by reading the optical density using a microplate reader at 520 nm.

## Measurement of nitric oxide (NO) concentration

The osteoblasts were adjusted to a density of  $1 \times 10^5$ /mL and inoculated in a 24-well plate. The cells were transferred to a 96-well plate with 100  $\mu$ L cell-containing medium per well. After adherence, the cells were cultured with serum-free medium for 24 h. Then, the cells were treated with JPYS drug-containing serum, calcium supplement serum, or blank control serum for 24 h, 48 h, or 72 h. Each test was repeated with 8 replicates. The supernatant (0.1 mL) was transferred to a tube for measurement of the NO concentration using an NO assay kit (CheKine, Abbkine, China). Standard tubes were added to 0.1 mL 100  $\mu$ M/L standard solution. The blank control tubes were supplemented with 0.1 mL water. All tubes were vibrated in 37 °C water for 60 min. The concentration of NO was measured by reading the optical density using a microplate reader at 520 nm.

## Measurement of nitric oxide synthase (NOS) activity

The osteoblasts were treated with JPYS drug-containing serum, calcium supplement serum, or blank control serum for 24 h. Each test was repeated with 4 replicates. The cells were adjusted to a density of  $1 \times 10^5$ /mL and inoculated in a 24-well plate. After confluence, the medium was replaced with 500  $\mu$ L lysate (50 mmol/L pH 8.0 Tris, 150 mmol/L NaCl, 0.02% sodium azide, 100  $\mu$ g/mL PMSF, 1  $\mu$ g/mL aprotinin, 1% Triton X100) for 30 min at 4 °C. Then, the lysate was transferred to an Eppendorf tube and centrifuged at 4 °C and 12000 rpm for 20 min. Then, 30  $\mu$ L supernatant was used to measure the concentrations of

NOS and iNOS using a NOS activity assay kit (Colorimetric, Abcam, China). The blank control tube was supplemented with 30  $\mu\text{L}$  and 100  $\mu\text{L}$  water. The total NOS assay tube was added to 100  $\mu\text{L}$  water. All tubes were vibrated in 37 °C water for 15 min. NOS activity was measured by reading the optical density using a microplate reader at 520 nm.

### Statistical analysis

All data are presented as the means and standard deviations. Comparisons were made using one-way ANOVA followed by Tukey's post hoc test. All statistical analyses were performed using SPSS 21.0 (IBM, USA). A p value less than 0.05 was considered statistically significant.

## 3. Results

### Results of rat modeling

By using DPX-ALPHA LUNARTM1, the bone mineral density of the left femur of rats was measured in Hi-res and small animal modes through dual energy X-ray absorptiometry (DEXA). Compared with the control group, the bone mineral density of the three dexamethasone modeling groups was decreased ( $P < 0.05$ ), indicating that the modeling was successful.

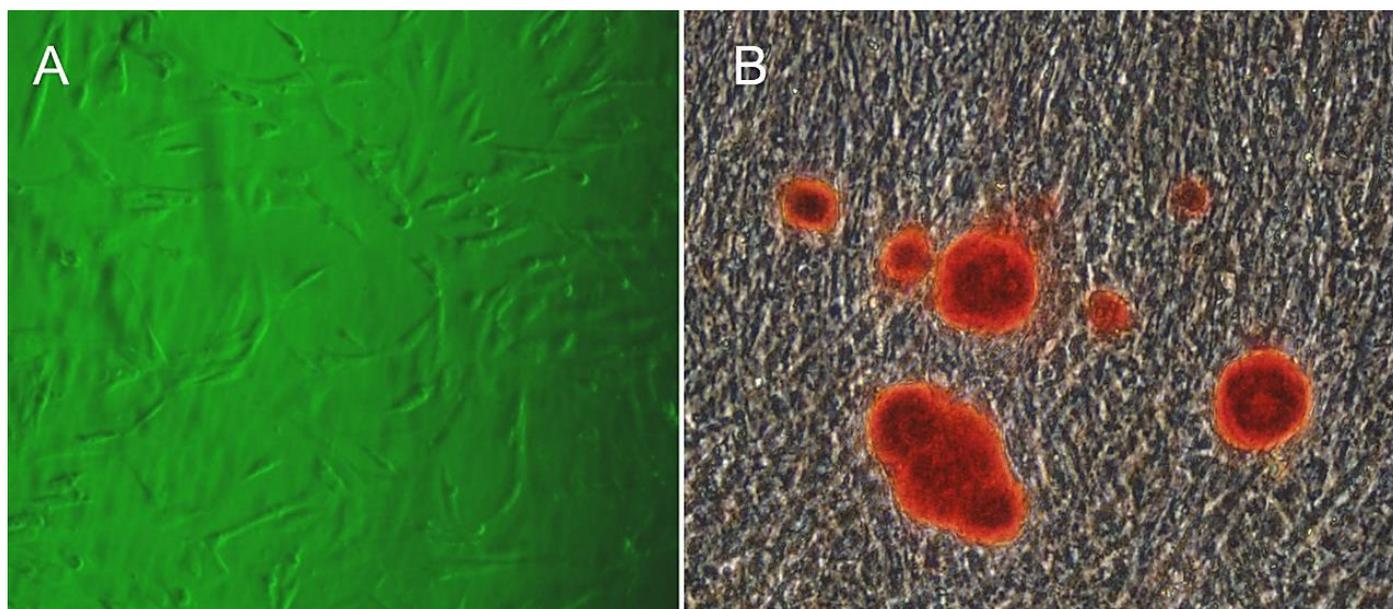
**Table 1.** Bone mineral density measurement of different rat groups.

Group	Number of rats	Bone density ( $\text{g}/\text{cm}^2$ )
Control group	10	$0.28 \pm 0.02$
Pathological model group	10	$0.18 \pm 0.03^*$
JPYS drug-containing group	10	$0.18 \pm 0.02^{* \#}$
Calcium supplement group	10	$0.17 \pm 0.03^{* \#}$

\* $P < 0.05$  as compared to control group; # $P > 0.05$  as compared to pathological model group.

### Cell morphology and characteristics

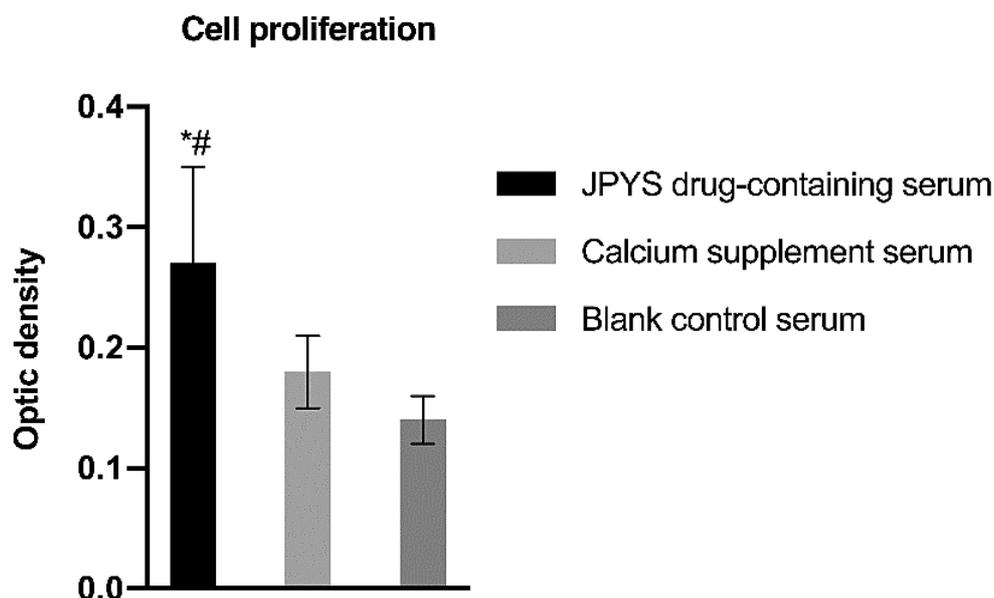
The primary osteoblasts had a morphology of spindle, triangle, or polygon (Figure 1A). Calcium nodules were formed by the cells after the second generation and culture for 3~5 days (Figure 1B). Approximately 78% of the osteoblasts were positive for alkaline phosphatase, showing intracellular gray-black granules after Gomori staining (data now shown).



**Figure 1.** Cell morphology and characteristics. A - Morphology of the primary osteoblasts and B - Calcium nodules produced by the cells.

## Cell proliferation

The MTT assay showed that JPYS drug-containing serum significantly improved the proliferation of osteoblasts compared to the blank control serum ( $0.27 \pm 0.08$  vs.  $0.14 \pm 0.02$ ,  $p < 0.05$ ) (Figure 2). It also significantly improved the proliferation of osteoblasts compared to calcium supplement serum ( $0.27 \pm 0.08$  vs.  $0.18 \pm 0.03$ ,  $p < 0.05$ ). However, cell proliferation showed no significant difference between the calcium supplement serum and the blank control serum ( $0.18 \pm 0.03$  vs.  $0.14 \pm 0.02$ ,  $p > 0.05$ ).

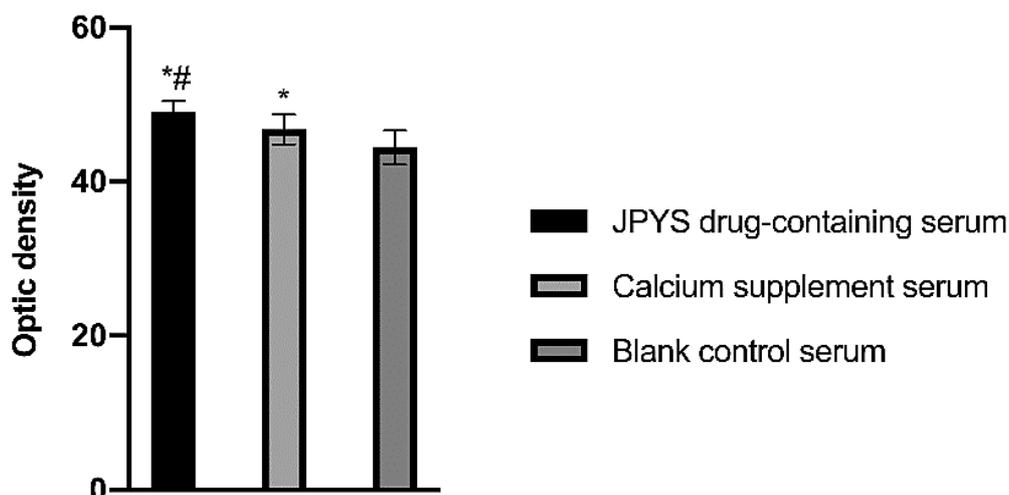


**Figure 2.** Cell proliferation shown by MTT assay. \* vs. the blank control serum,  $p < 0.05$ . # vs. the calcium supplement serum,  $p < 0.05$ .

## Alkaline phosphatase concentrations

The concentration of alkaline phosphatase in the culture medium of the osteoblasts treated with JPYS drug-containing serum was significantly higher than that of the blank control serum ( $49.12 \pm 1.34$  vs.  $44.42 \pm 2.18$ ,  $p < 0.05$ ) and that of the calcium supplement serum ( $49.12 \pm 1.34$  vs.  $46.77 \pm 1.95$ ,  $p < 0.05$ ) (Figure 3). Similarly, the calcium supplement serum significantly increased the alkaline phosphatase concentration in the culture medium compared to the blank control serum ( $46.77 \pm 1.95$  vs.  $44.42 \pm 2.18$ ,  $p < 0.05$ ).

## Concentration of alkaline phosphatase

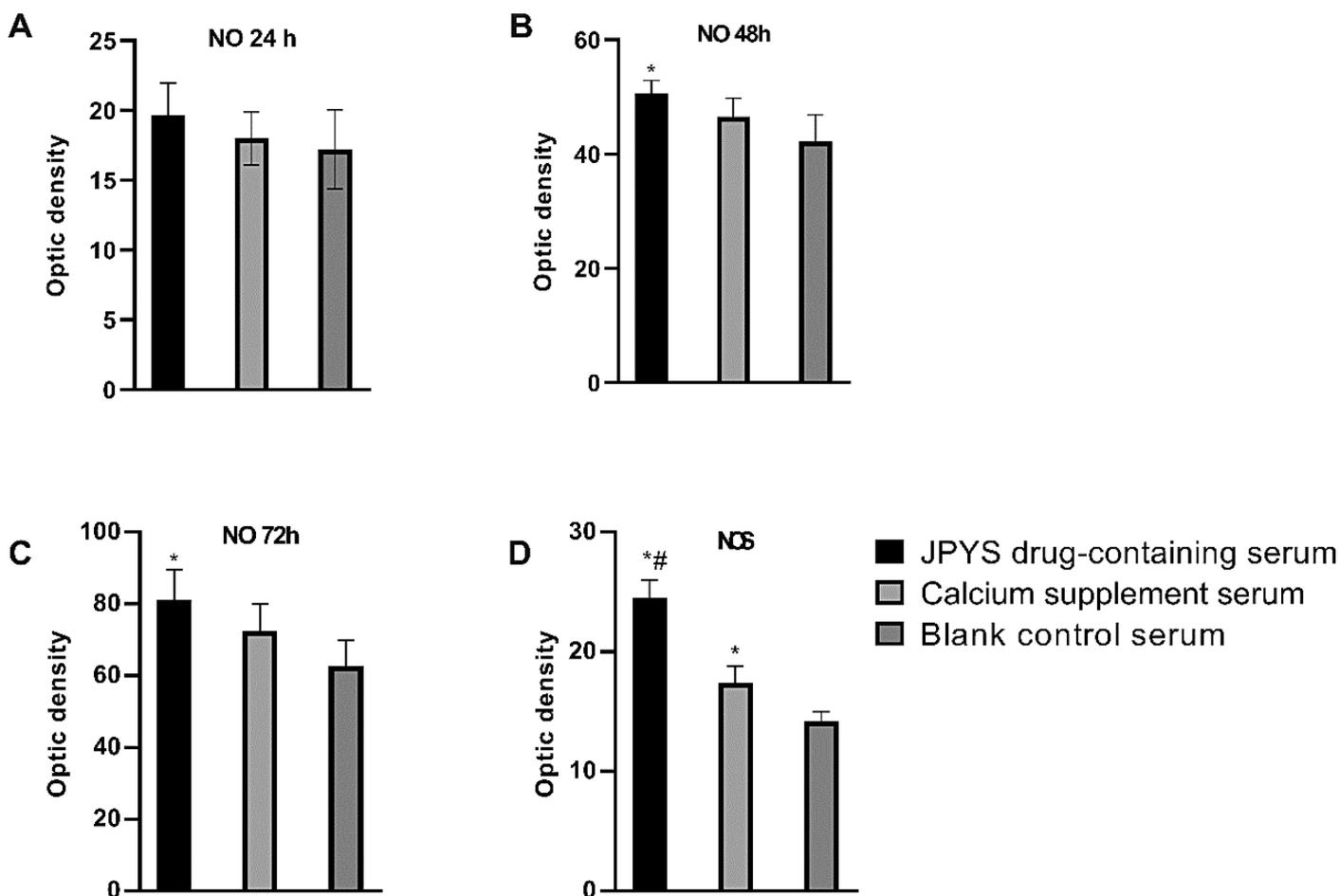


**Figure 3.** Concentration of alkaline phosphatase in the culture medium of the osteoblasts shown by optic density. \* vs. the blank control serum,  $p < 0.05$ . # vs. the calcium supplement serum,  $p < 0.05$ .

## NO concentration and NOS activity

The NO concentration in the culture medium showed no significant difference between the osteoblasts treated with JPYS drug-containing serum, calcium supplement serum, and blank control serum for 24 h ( $19.63 \pm 2.33$ ,  $18.00 \pm 1.90$ , and  $17.22 \pm 2.84$ ,  $p > 0.05$ ) (Figure 4A). Treatment for 48 h and 72 h showed that the JPYS drug-containing serum significantly increased the NO concentration in the culture medium compared to the blank control serum (Figures 4B and 4C). However, treating the osteoblasts with the calcium supplement serum for 48 or 72 h did not significantly increase the NO concentration in the culture medium compared to the blank control serum.

The NOS activity of the osteoblasts was significantly increased by JPYS drug-containing serum compared to blank control serum ( $24.54 \pm 1.47$  vs.  $14.18 \pm 0.83$ ,  $p < 0.05$ ) and calcium supplement serum ( $24.54 \pm 1.47$  vs.  $17.43 \pm 1.39$ ,  $p < 0.05$ ) (Figure 4D). Similarly, the calcium supplement serum significantly increased the NOS activity compared with the blank control serum ( $17.43 \pm 1.39$  vs.  $14.18 \pm 0.83$ ,  $p < 0.05$ ).



**Figure 4.** NO concentration and NOS activity in the culture medium of the osteoblasts shown by optic density. \* vs. the blank control serum,  $p < 0.05$ . # vs. the calcium supplement serum,  $p < 0.05$ .

## 4. Discussion

Osteoporosis is a systemic metabolic skeletal disease caused by many factors, with its core mechanism of imbalance of bone remodeling and bone resorption. JPYS is composed of TCMS, such as drynaria rhizome and longspur epimedium, which have achieved remarkable curative effects in the clinical application of osteoporosis patients, yet the mechanism is still essentially unknown. This paper explored the effect of JPYS on osteoporosis from the perspective of osteoblasts. Our study found that JPYS drug-containing serum significantly increased cell proliferation, the levels of alkaline phosphatase and NO in the culture medium, and the NOS activity of mouse osteoblasts. These findings suggest that JPYS decoction may benefit patients with osteoporosis by promoting the proliferation and mineralization of osteoblasts.

Bone is a highly mineralized connective tissue and is continuously broken down and reformed in a process of turnover known as bone remodeling, which occurs through the interaction and balance between bone-forming cells, called osteoblasts, and bone-resorbing cells, called osteoclasts (Gao et al. 2013). Osteoporosis is a systemic metabolic bone disease that is characterized by microarchitectural deterioration, low bone mass, and increased risk of fractures and is a prevalent disease that results from an increase in bone breakdown relative to bone formation (Armas and Recker 2012; Baccaro et al. 2015; Miller 2016; Lane et al. 2000). Osteoporosis is a major global public health problem that is considered by the World Health Organization to be an important health issue secondary to coronary heart disease. It can occur at any age and in any racial or ethnic group, affecting millions of people all over the world, especially the aging population (Srivastava and Deal 2002).

The ideal strategy for treating osteoporosis is to inhibit bone resorption by osteoclasts and/or increase bone formation by osteoblasts. However, most of the current therapies for treating osteoporosis focus on inhibiting bone resorption, and there are only a few agents available that promote bone formation. To date, most of the effective osteoporosis therapies reduce bone loss but do not restore lost bone mass and strength. It is desirable, therefore, to have satisfactory bone building (anabolic) agents that stimulate new bone formation and correct the imbalance of trabecular microarchitecture characteristic of established osteoporosis, which would create a new alternative for treating osteoporosis (Lorentzon 2019).

In recent years, there has been growing interest in the treatment of osteoporosis with plant-based therapies, including traditional Chinese medicines, for which extensive experience has been accumulated over thousands of years (Liu et al. 2014). Traditional Chinese medicines have been widely used in clinical practice to prevent and treat bone diseases in many countries of the world because they have fewer adverse reactions and are more suitable for long-term use than chemically synthesized medicines. Traditional Chinese medicines contain numerous chemical constituents, which usually exert their therapeutic effects through multiple pathways and targets; these properties correspond with the multifactorial pathogenesis of osteoporosis (Xu et al. 2005). JPYS decoction is a Chinese herbal formula that has been used to treat many diseases, including osteoporosis.

Alkaline phosphatase activity is the most widely recognized biochemical marker for osteoblastic activity. It is the typical protein product of an osteoblast phenotype and of osteoblast differentiation. Composed of homodimeric metalloenzymes that contain zinc at the active site (Siller and Whyte 2018), alkaline phosphatase hydrolyzes a variety of phosphate compounds, such as ATP, releasing the products, specifically inorganic phosphate, into the extracellular matrix (Sasaki 2018). In general, the appearance of alkaline phosphatase activity is represented as an early phenotypic marker for osteoblastogenesis (Deng et al. 2019). Our study found that JPYS drug-containing serum significantly increased the levels of alkaline phosphatase in the culture medium of osteoblasts. This finding suggests that JPYS decoction may improve osteoporosis by increasing the production of alkaline phosphatase by osteoblasts, which further promotes osteoblastogenesis and bone formation.

NO is a short-lived signaling molecule generated from L-arginine by NOS isoenzymes. An accumulating body of evidence has emerged to suggest that NO plays an important role as a paracrine and autocrine mediator of bone metabolism and bone cell activity in response to diverse stimuli (Kalyanaraman et al. 2018; Becerril et al. 2019). The NO-sGC-cGMP-PKG pathway begins with activation of NOS, the enzyme that synthesizes NO in cells. The increased NO levels activate soluble guanylyl cyclase (sGC), thus increasing the level of cyclic guanosine monophosphate (cGMP), which acts to activate protein kinase-G (PKG), a cGMP-dependent protein kinase (Zhao et al. 2015). The serine/threonine protein kinase Akt, also known as protein kinase B, is stimulated by several receptor tyrosine kinases and G protein-coupled receptors through phosphatidylinositol 3-kinase (PI3K) (Costa et al. 2018). In addition, Akt can phosphorylate eNOS, an isoform of NOS (Muhammad et al. 2020). Akt signaling has been reported to be involved in osteoblast proliferation and skeletal development, and Akt is upstream of the NO pathway (Guizoni et al. 2020). Our study found that JPYS drug-containing serum significantly increased the levels of NO and NOS activity in the culture medium of osteoblasts. These findings suggest that the JPYS decoction may improve osteoporosis by promoting bone metabolism and bone cell activity.

## 5. Conclusions

The JPYS drug-containing serum significantly promoted cell proliferation and activity of mouse osteoblasts, which may further promote mineralization and bone formation. These findings may explain the mechanisms of JPYS decoction in treating osteoporosis.

**Authors' Contributions:** CAI, Y.: conception and design, analysis and interpretation of data and drafting the article; LIU, X.: acquisition of data, analysis and interpretation of data; MA, L.: acquisition of data and drafting the article; LV, W.: conception and design and acquisition of data; WANG, W.: analysis and interpretation of data, drafting the article and critical review of important intellectual content; ZHANG, X.: conception and design, critical review of important intellectual content and drafting the article. All authors have read and approved the final version of the manuscript.

**Conflicts of Interest:** The authors declare no conflicts of interest.

**Ethics Approval:** This study was approved by the Ethics Committee of Shandong Academy of Medical Sciences (approval number: 2020-43).

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