

Effect of Laser versus Needle Acupunctures on Bone Regeneration in Rats with Ovariectomy-induced Osteoporosis

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Abstract

Background and Aim: Acupuncture promoting bone mass has long been applied to the treatment of osteoporosis. The aim of this study was to evaluate the protective effects of low-level laser therapy on bone loss to provide new insight into its therapeutic utility for postmenopausal osteoporosis. **Experimental procedure:** 24 female rats were randomly divided into 4 groups: sham operation (sham), ovariectomy (OVX), ovariectomy with needle acupuncture (O-N), and ovariectomy with laser acupuncture (O-L). O-N received needle acupuncture on the acupoints of Shenshu (BL23), Housanli (ST36) and Xuanzhong (GB39), whereas O-L received laser acupuncture on the same points. After the treatment period ended, the rats were euthanized to evaluate bone mass, microstructure, by micro-CT and histological staining. The transcription of bone formation-related genes by RT-PCR. Markers of bone metabolism was determined in serum were quantified by ELISA. **Results and conclusion:** O-L induced significant improvements in bone density and trabecular microarchitecture, showing significant increase in BMD, Tb.Th, Tb.N, BV/TV and Conn.D, as well as significant decline in Tb.Sp and SML. The results mirrored the findings obtained from histological examination. Moreover, O-N versus O-L, Col-1 in O-L significant increase ($P < 0.05$). Both forms of acupuncture treatment significantly stimulated the transcription of ALP, BMP, OCN, Col-1, RUNX-2 and OPG. Laser acupuncture can mitigate bone loss, stimulate osteogenesis, and repair damaged trabecular network in ovariectomized rats. These results provided preliminary evidence that laser acupuncture could be applied as a non-invasive method to osteoporosis treatment.

Key words: osteoporosis; Bone formation; Acupuncture; Low-level laser irradiation

Introduction

Osteoporosis is especially common in postmenopausal women due to reduced estrogen secretion, which causes an imbalance between osteoblasts and osteoclasts¹. Classical bone formation markers such as phosphatase (ALP) and osteocalcin (OCN) can reflect the functional activity of osteoblasts, and play an important role in predicting and managing postmenopausal osteoporosis. Serum ALP and OCN levels have been found to be inversely associated with bone mineral density in postmenopausal women with osteoporosis and can increase with age^{2,3}. This elevation is primarily due to accelerated bone metabolism, so these markers of bone formation do not decrease with age in postmenopausal women⁴, suggesting that postmenopausal osteoporosis may have a high rate of bone turnover⁵.

While medications⁶, Physical therapies⁷ are available and widely employed for osteoporosis treatment, each has limited applications. Osteoporosis drugs

generally produce a range of side effects, and moreover, the patients are often middle-aged or elderly individuals with poor cardiopulmonary functions, for whom long physical therapy sessions are intolerable. Acupuncture, a long-established traditional Chinese medicine therapy, has demonstrated curative effect on osteoporosis with substantially less side effects than medications. Consequently, it has gradually gained popularity among osteoporosis patients and physicians⁸. Many studies have investigated the efficacy of acupuncture for the treatment of primary osteoporosis in clinical practice, and the findings have shown that acupuncture is effective in improving osteoporosis⁸⁻¹¹.

In recent years, photobiomodulation has received increasing attention as a promising approach for the management of tissue injury. Light irradiation has been shown to stimulate osteoblast proliferation, accelerate fracture healing¹², and increase bone strength. Low-level laser therapy (LLLT) stimulated bone nodule formation¹³, accelerated cellular proliferation and differentiation such as osteoblasts, fibroblasts, bone marrow mesenchymal stem cells (BMSC)¹⁴, increased ALP activity and upregulated bone morphogenetic protein (BMP). Furthermore, laser therapy combined with medical therapy produced significantly increased osteoblast number and collagen-1 (Col-1) gene expression and caused a significant decrease in osteoclast number¹⁵. Due to the ameliorative effects of acupuncture and photobiomodulation, medical practitioners have developed laser acupuncture that combines the therapeutic benefits of both treatment modalities. Compared to its needle-based counterpart, laser acupuncture is non-invasive, generally painless, and can be applied to locations difficult to needle without causing swelling or bleeding. Laser acupuncture has achieved satisfactory results in the treatment of musculoskeletal injuries such as adhesive capsulitis¹⁶, lumbago¹⁷, carpal tunnel syndrome¹⁸, temporomandibular disorders¹⁹, osteoporosis²⁰, jumper's knee, Achilles tendinitis, tennis elbow, etc^{21, 22}. However, few studies have explored the difference in efficacy between laser acupuncture with traditional acupuncture. This study aimed to evaluate the therapeutic efficacy of laser acupuncture and needle acupuncture against postmenopausal osteoporosis using a rats ovariectomy model.

1 MATERIALS AND METHODS

2.1 Experimental animals

A total of 24 eight-week-old specific-pathogen-free female Sprague-Dawley rats (weight 284.46 ± 11.97 g, Shanghai Jihui Experimental Animal, China) were housed in separate cages with free access to food and water. SPF-class housing of laboratory followed a cycle of 12-hour day and 12-hour night, with ambient temperature maintained at 25 °C and relative humidity in the range of 40% to 70%.

The rats were randomly divided into four equal-sized groups (n=6): sham (weight 284.78 ± 14.44 g), OVX (weight 285.18 ± 11.19 g), O-N (weight 281.52 ± 11.38 g), and O-L (weight 286.36 ± 13.44 g) by random number table. There was no significant difference in body weight between the groups ($P > 0.05$).

All procedures involving animals in current study were approved by Ethics Committee of Zhoupu Hospital, Pudong New Area, Shanghai (2022-C-017-E01).

2.2 Intervention

All the animals were conditioned to their experimental environment for a week, and then fasted with concurrent water deprivation the night before the operation. For OVX, O-N and O-L, bilateral ovariectomy was performed via longitudinal incision along the midline of the back following anesthesia with sodium pentobarbital at a dose of 30 mg/kg weight. The members of the sham group were subjected to the same operation but without ovary removal (only a similar volume of adipose tissue near the ovaries was resected). After the operation, the rats were returned to the same routine and allowed to rest for 2 weeks.

Table 1 Laser Parameters

Parameters	Value and unit (per point)
Wavelength	808 nm
Output power	150 mW
Spot diameter	0.4 cm
Spot area	0.1256 cm ²
Irradiation time	100 s
Power density	1.2 W/cm ²
Energy	15 J
Energy density	120 J/cm ²

Treatment commenced two weeks after the operation, and varied according to the experiment group. For the O-N group, each rat was fixed in a prone position, and the commonly used acupoints Shenshu (BL23), Housanli (ST36) and Xuanzhong (GB39) were identified based on Experimental Acupuncture and Moxibustion (Fig.1). The skin around each selected acupoint was disinfected and gently pierced with a half-inch needle (0.18 mm × 13 mm, Beijing Zhongyan Taihe Medical Instrument Ltd, China). The lifting-thrusting technique was employed to achieve tonification. Specifically, each needle was

retained in the acupoint for a total of 30 min, with periodic lifting and twisting every 10 min. For the rats in O-L, needle acupuncture was replaced with laser irradiation (LWIRPD, Laserwave Photoelectric Technology, China) based on the parameters in Table 1. Meanwhile, both the sham group and OVX were fixed and handled in the same manner, but did not receive any acupuncture treatment. The entire treatment period spanned 65 days and contained five courses, each consisting of ten consecutive daily sessions, with a 3-day interval between each two courses.

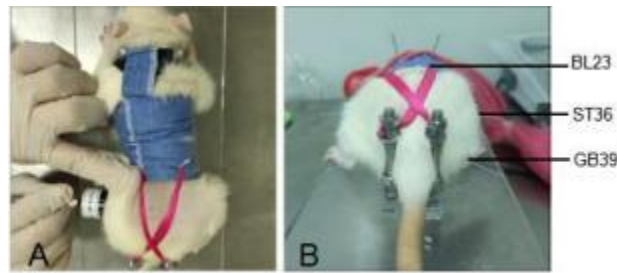


Figure 1A, Laser irradiate acupoint Housanli (ST36).

Figure 1B, acupuncture points, Shenshu (BL23; below the second lumbar vertebrae), Housanli (ST36; located on the posterolateral aspect of the knee joint, 5 mm below the

fibula head) and Xuanzhong (GB39; 10 mm above the tip of the lateral malleolus, at the anterior edge of the fibula)

2.3 *Tissue Sampling*

After the last treatment session (including the three-day interval that followed), the rats were euthanized by intraperitoneal injection of sodium pentobarbital at a dose of 180 mg/kg body weight.

collection. The blood was then centrifuged at 4 °C and 3000 rpm for 10min to obtain the serum. Meanwhile, the bilateral femur and tibia were carefully separated via aseptic surgery, and the surrounding soft tissues were removed. Each bone specimen was gently wrapped in a moist gauze pre-soaked in 0.9% (w/v) NaCl, and subsequently stored at -80 °C until use.

After whole-body disinfection with 75% ethanol, followed immediately by eyeball removal for blood

2.4 *Micro-CT imaging*

The frozen left femur of each animal subject was thawed to room temperature and the distal section (Lower 1/2 of the femur, near the knee joint) was scanned by Micro CT (Bruker Skyscan- 1176, USA), followed by three-dimensional image reconstruction to compare the following bone microstructural parameters between different experimental groups: bone mineral density (BMD), trabecular thickness (Tb.Th), trabecular number (Tb.N), bone volume fraction (BV/TV), trabecular separation (Tb.Sp), connective density (Conn.D), and structure model index (SMI). The micro-CT scan, three-dimensional reconstruction and result analysis were all completed by the same operator, who was blind to the group assignment.

deparaffinized by multiple xylene washes followed by hydration through graded alcohol washes (95%, 85%, 75%, 60%). The tissue sections were stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP), sealed with resin, following by the evaluation of their morphological structures under a light microscope. The number of osteoclasts and adipocytes was counted by ImageJ. TRAP positive cells with more than 3 nuclei were classified as osteoclasts.

2.5 *Histological and morphological assessment*

The frozen right femurs were thawed to room temperature, and decalcified bone tissue sections were prepared as described above. First, it was immersed in 4% paraformaldehyde solution, fixed for 24 hours, then placed in 10% Ethylene Diamine Tetraacetic Acid (EDTA) solution, and decalcified at low temperature. After decalcification, the specimens were dehydrated in 60%, 76%, 85%, 95%, and 100% ethanol solution in turn, then immersed in a mixture of 1:1 absolute alcohol and xylene, and finally immersed in pure xylene to make the tissue transparent. After dehydration, they were immersed in melted paraffin and embedded. The paraffin-embedded blocks were trimmed, frozen, and then serially longitudinally sectioned using a microtome to a thickness of 5µm. The sections were then

2.6 *Immunofluorescent staining*

Briefly, the frozen left tibia was removed from the freezer and decalcified in 10% EDTA solution for 3 weeks. Prepare paraffin sections as described above. The sections were immersed in citrate antigen retrieval solution (50×), boiling water bath for antigen retrieval, and washed with PBS after natural cooling. Add 0.2% TritonX-100 solution to make cell membrane permeabilization and wash with PBS. Add 5% fetal bovine serum (FBS) and incubate for 0.5 hours, then gently shake off FBS, add diluted primary antibody (Rabbit Monoclonal Antibody, Beyotime Biotechnology, China), incubate overnight at 4 °C. After three PBS washes, add fluorescently labeled secondary antibody (Cy3-labeled goat anti-rabbit IgG, Beyotime Biotechnology, China). Incubate for 0.5 hours at 37 °C and then do three PBS washes; Stain the nucleus for 10 minutes in the dark with DAPI (Beyotime Biotechnology, China), then rinse with PBS three times in the dark. The sections are sealed with resin and OCN expression is observed under a fluorescence microscope.

To better understand the molecular effects of ovariectomy and acupuncture, we determined the serum protein levels of four bone turnover markers, including

2.7 *Serum protein levels of bone turnover markers*

ALP, OCN, osteoprotegerin (OPG) and Col-1, for different experiment groups. The serum level of ALP was quantified using the Alkaline Phosphatase Assay Kit (Beyotime Biotechnology, China) following the

manufacturer's instructions. The serum levels of serum OCN, OPG and Col-1 were measured by enzyme-linked immunosorbent assay using appropriate antibodies purchased from Enzyme-Linked Biotechnology, China.

2.8 RT-PCR

The mRNA levels of OCN, ALP, Col-1, OPN, Runx2, OPG and BMP2 were analyzed by RT-PCR. To obtain the total RNA, the fresh right tibia of each rat was frozen in liquid nitrogen and ground to a fine powder. After all liquid nitrogen was evaporated, the bone powder was mixed with Trizol and incubated at room temperature for 10 min to ensure thorough cell lysis. Next, 0.2 vol (relative to Trizol) pre-chilled chloroform was added, and the resultant mixture was violently agitated for 20 min before being allowed to stand at room temperature for 5 min. The suspension was then centrifuged at 12000

rpm, 4 °C for 15 min, and the top layer of supernatant was transferred to a clean microcentrifuge tube, to which an equal volume of isopropanol was added. The resultant mixture was agitated 30 times by gentle pipetting and then incubated for 30 min at room temperature. After centrifugation at 12000 rpm, 4 °C for 10 min, the supernatant was discarded and the white precipitate was washed with 75% ethanol.

After removing the wash by centrifugation, the precipitate was air-dried and dissolved in RNase-free, sterile deionized water to provide the template for RT-PCR, which was subsequently performed using Hifair II 1st Strand cDNA Synthesis Kit (Yeasen Biotechnology, China) and Hieff qPCR SYBR Green Master Mix (Yeasen Biotechnology, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Table 2 List of PCR primers used in this study

Gene	Primer sequence	
OCN	Forward	GCACACCTAGCAGACACCAT
	Reverse	GCTTGGACATGAAGGCTTTGT
ALP	Forward	CAGCGGGTAGGAAGCAGTTTC
	Reverse	GCCACTTGATTCCGTTTCAGC
Col-1	Forward	TCCTTGCTTGGGTTTGCAGT
	Reverse	CATGGTCGTAGTTAGTCCCTCA
OPN	Forward	TAGCACCTTAGTCTTCCGC
	Reverse	CTTGAACACCCACATCCTGC
Runx2	Forward	AGAGTCAGATTACAGATCCCAGG
	Reverse	TGGCTCTTCTTACTGAGAGAGG
OPG	Forward	GAGGAGTCTGGTAGTGGTTCC
	Reverse	GGCGTTTTCGTTGAATATGCG
BMP	Forward	CCGGGACTCCTATGGCTACT
	Reverse	CATCCGTCATGGCACGGTA
GAPDH	Forward	AATGGATTTGGACGCATTGGT
	Reverse	TTTGCACTGGTACGTGTTGAT

2.9 Statistical Analysis

All statistical analyses were conducted with GraphPad Prism 9 (GraphPad Software, USA). The data were expressed as mean ± standard deviation. the normality of all variable's distribution was verified

using Shapiro-Wilk normality test. One-way analysis of variance was employed to evaluate the experimental data that obeyed normal distribution and homogeneity of variance. otherwise Kruskal-Walli's test or Welch test was used. $P < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Micro-CT scanning

No animal death or infection occurred throughout the experiment before the euthanasia. OVX compared to the sham group, Micro-CT scanning of the left femur revealed significant decline in BMD, Tb.Th, Tb.N,

BV/TV and Conn.D, as well as significant increase in Tb.Sp and SMI. Although the 65- day course of needle-based acupuncture treatment appeared to have mitigated the ovariectomy-induced bone remodeling, the changes were found not to be statistically significant. However, the laser acupuncture demonstrated a statistically significant effect on improving the aforementioned bone microstructural parameters in the left figure (fig. 2)

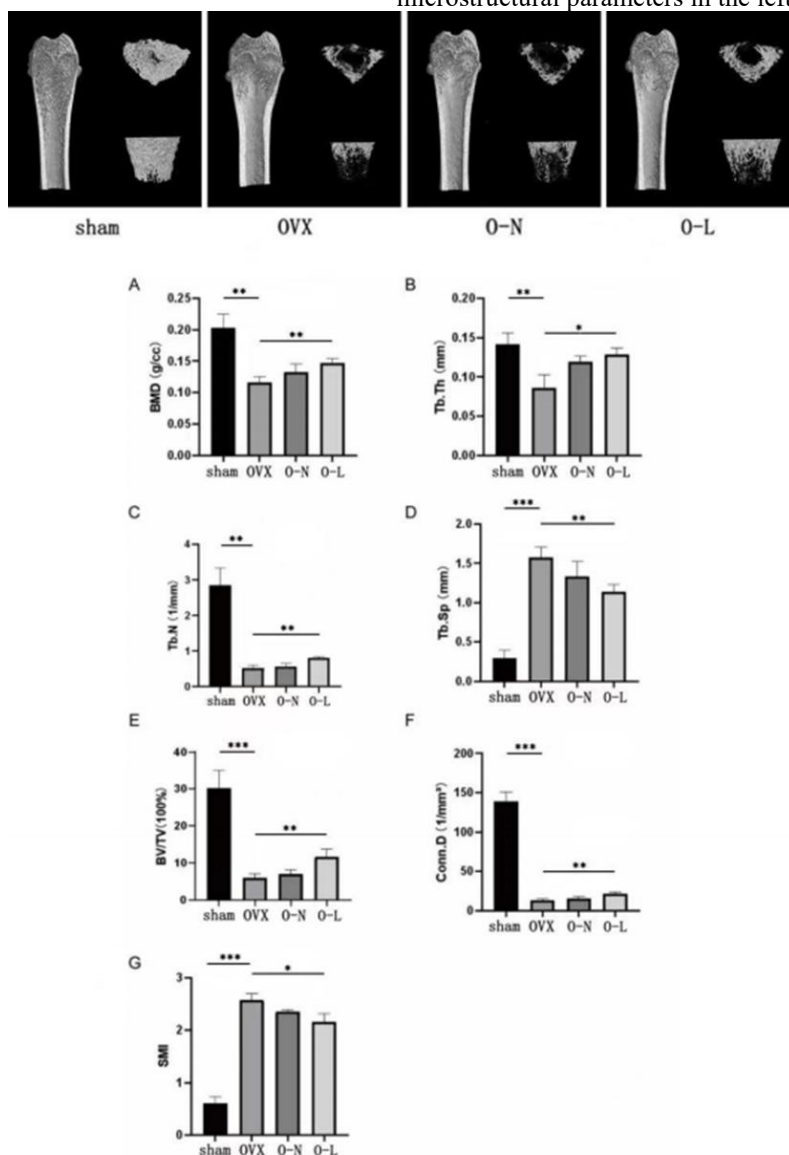


Figure 2 Left: coronal plane; Upper right: transverse plane; Lower right: cancellous bone distal to the sagittal growth plate

Micro-CT images of selected left rat femurs from

3.2 Histological assessment

Histological examination of the right rat femurs indicated that, sham versus OVX, the rats in OVX showed a sparse distribution of bone trabeculae and larger bone marrow cavities, with adipocytes occupying a substantial portion of the microscopic field of view. Osteoclasts and adipocytes in the OVX were significantly increased

different experiment groups and statistical analysis of microarchitectural parameters, including A) BMD, B) Tb.Th, C) Tb.N, D) Tb.Sp, E) BV/TV, F) Conn.D, and G) SMI. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

($P < 0.0001$). OVX versus O-N, osteoclasts and adipocytes in the O-N were significantly decreased ($P < 0.05$, $P < 0.0001$). OVX versus O-L, this reduction is even more significant ($P < 0.001$, $P < 0.0001$). O-N versus O-L, there was no statistically significant difference between the two groups of osteoclasts ($P > 0.005$), but showing a significant decrease of adipocytes in the O-L ($P < 0.001$). (Fig. 3).

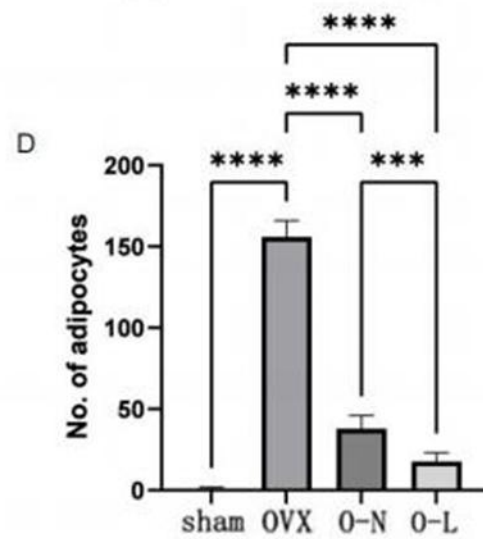
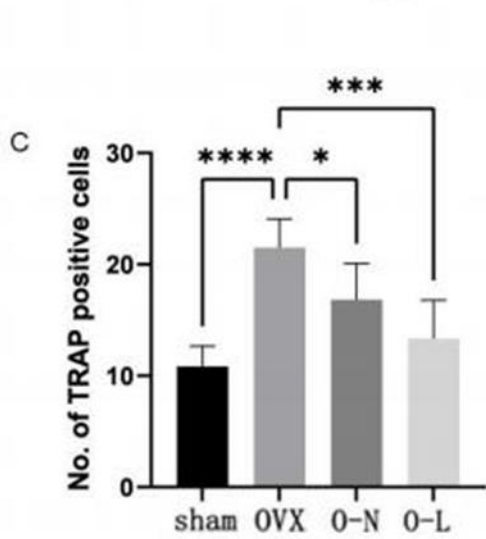
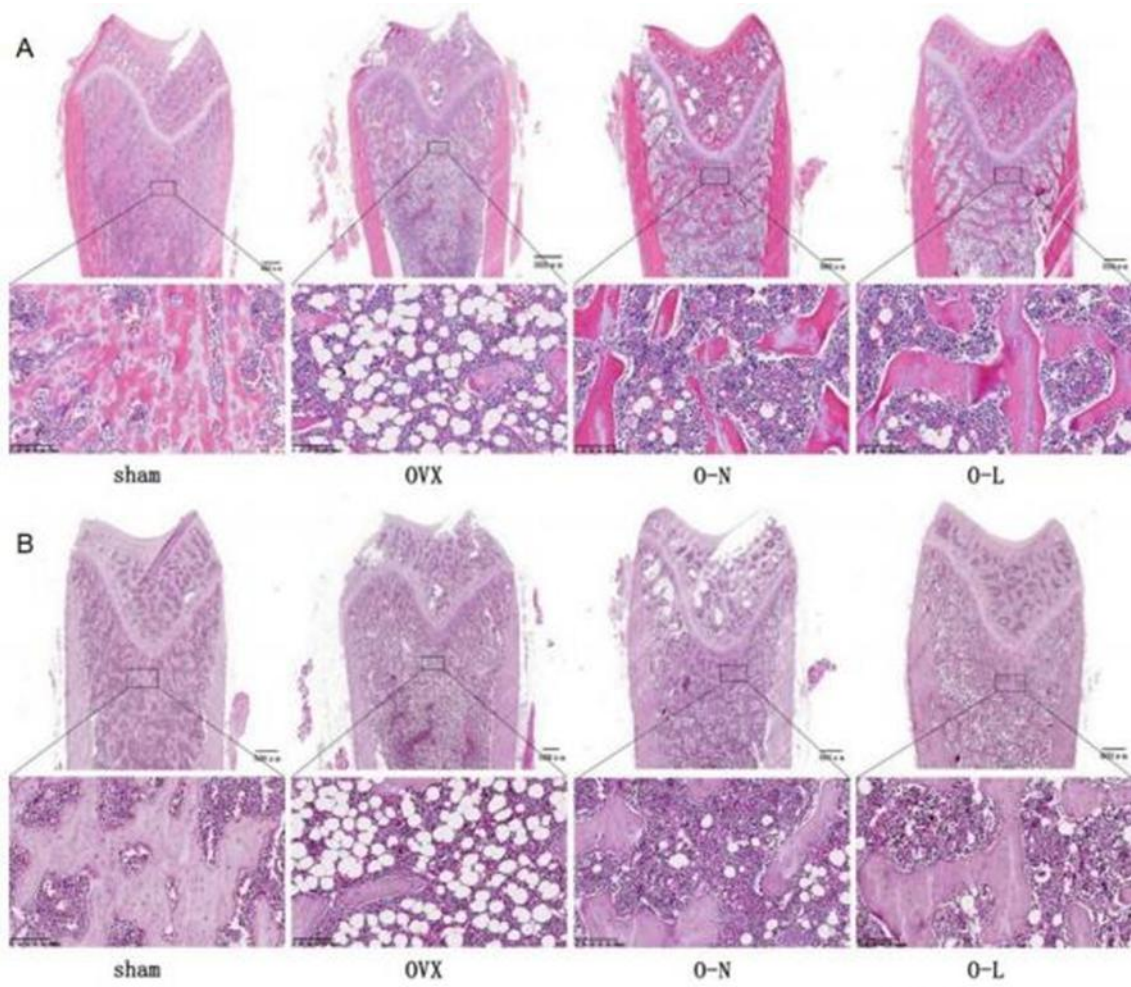


Figure 3 Bone microarchitectural changes in the distal regions of the rats' right femurs stained with H&E(A) and TRAP(B). Means and SD of the number of cells. C osteoclasts (low magnification image), D adipocytes (high magnification image). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3.3 Protein expression of bone turnover markers

Sham versus OVX, the rats in OVX showed up-regulation of ALP and OCN ($P < 0.05$, $P < 0.05$), as well as down-regulation of OPG and Col-1 ($P > 0.05$).

However, only the difference in the level of OCN was statistically significant between the two. The serum levels of ALP and OCN displayed significant elevation in O-N ($P < 0.05$, $P < 0.05$) and O-L ($P < 0.05$, $P < 0.01$) over those in OVX, and even more so when compared to the sham group. Although O-N and O-L also exhibited augmented level of OPG and Col-1 compared to OVX, in both cases the changes were statistically significant only for the laser acupuncture treatment ($P < 0.01$, $P < 0.01$). Notably, O-N versus O-L, we also detected a significantly higher level of Col-1 protein in O-L ($P < 0.05$). (Fig. 4).

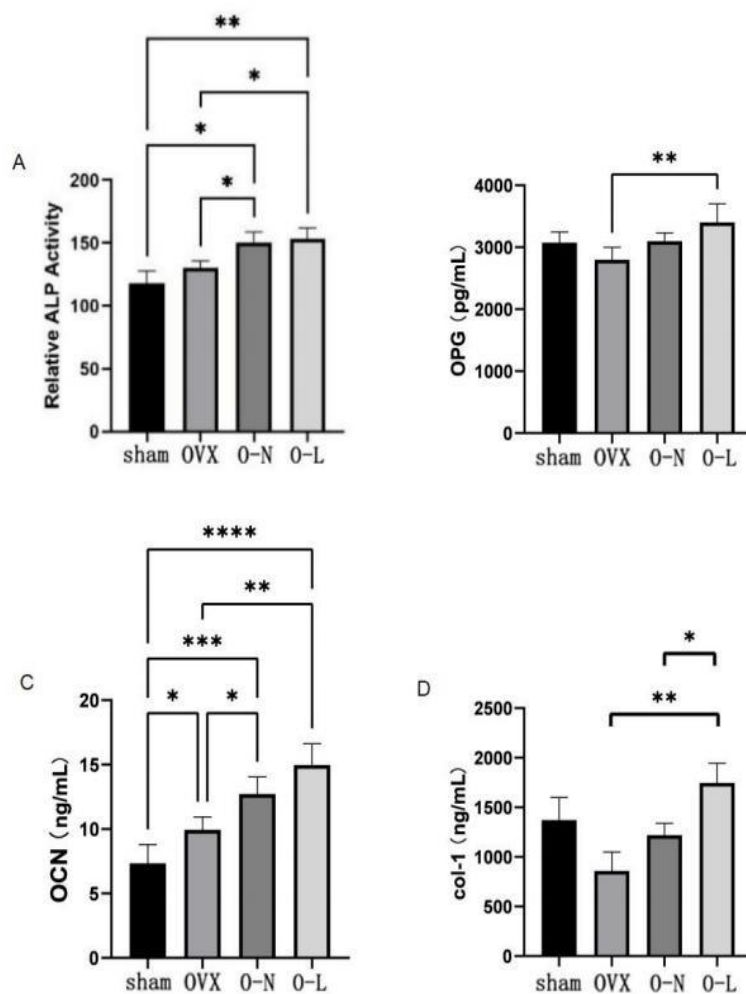


Figure 4 Comparison of serum protein levels of ALP, OPG, OCN and Col-1 between different experiment

groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

3.4 Immunofluorescence staining of OCN

Immunostaining of bone tissue sections prepared from left tibia indicated a slightly increased level of OCN in the trabeculae for the group of OVX compared to the sham group, implying the possible involvement of a compensatory mechanism. The rats in O-N showed a

higher level of OCN in the trabecular bone compared to OVX, though regions with low levels of OCN could still be observed. In contrast, laser acupuncture resulted in strong expression of OCN almost across the entire trabecular network (Fig. 5)

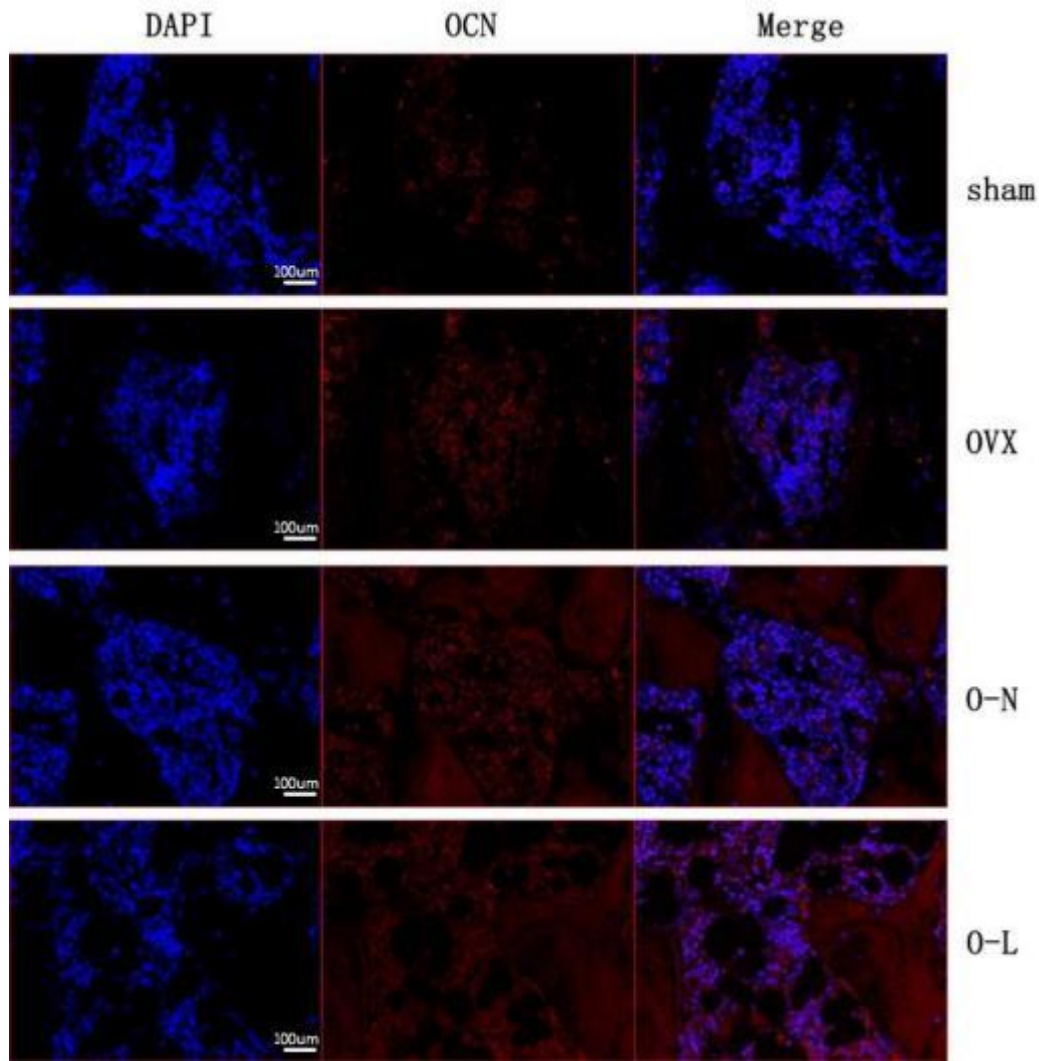


Figure 5 Immunofluorescence staining of OCN, observed at 73× magnification. OCN positive cells the bone marrow cells (blue), OCN positive area (red),

trabecular bone (dark regions around the bone marrow cells) and lipid vacuoles (circular dark regions inside the bone marrow cells) are visible.

3.5 Transcriptional of bone turnover genes

As summarized in Figure 6, the experimental data found the transcription of BMP to be significantly up-regulated, and that of OPG to be significantly down-regulated, in OVX compared to the sham group. Additionally, we observed an apparent increase, albeit without statistical significance, in the mRNA levels of Col-1, OPN and Runx2 in the sham group over OVX, whereas the transcription of ALP and OCN seemed to be similar in both groups. When comparing between OVX and O-N or O-L, all osteogenesis markers except OPN exhibited

significant elevation in transcription activity in the two treatment groups. We also noticed that laser acupuncture induced significantly greater mRNA expression of OCN compared to its needle-based counterpart (Fig. 6). Taken together, these results suggested that needle and laser acupuncture might exert its osteoprotective effects by activating the BMP2/Runx2 signaling pathway to promote the formation and mineralization of osteoblasts, and by diminishing osteoclast activities via the up-regulation of OPG

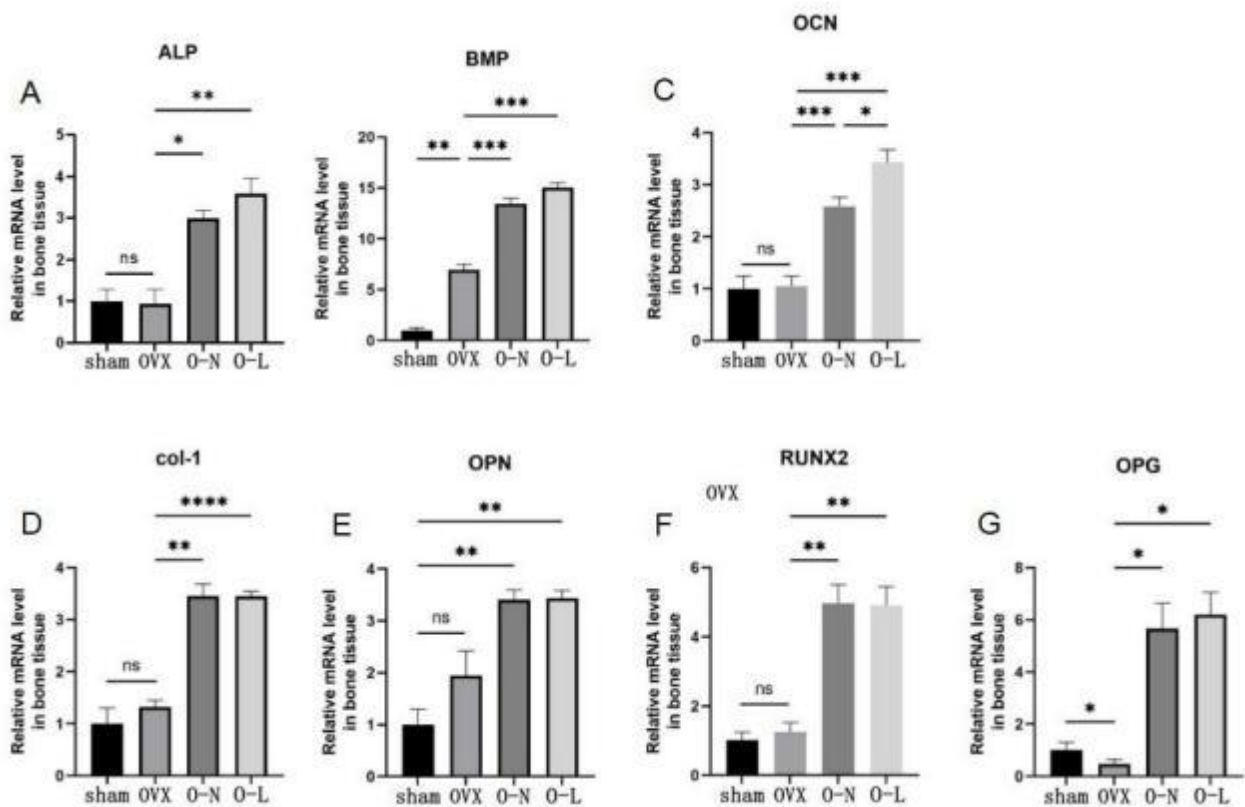


Figure 6 Relative transcription levels of selected

osteogenesis gene markers in the rat tibia *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

4 DISCUSSIONS

Low-level laser therapy utilizes irradiation, typically in the wavelength range of 630 to 1000 nm and at a power output from 10 to 500 mW, to induce beneficial photobiomodulatory effects without causing heat-induced tissue damage 23. Although photobiomodulation therapy has been demonstrated to promote tissue regeneration and alleviate inflammation, swelling and discomfort 22, 24, its clinical application to osteoporosis treatment has been limited by the lack of a well-accepted consensus on the optimal energy density for preventing bone loss and stimulating osteogenesis. Renno AC, et al. compared the effects of laser irradiation on the bone strength of rat femurs at 60 J/cm² and 120 J/cm², and concluded the higher energy density was more effective in reversing ovariectomy-induced osteoporosis 25. Similar findings have also been obtained by Koichiro and coworkers 26. In addition to energy density, cellular response to irradiation treatment depends on the match between its pigment profile and the wavelength of choice. The wavelength of light also correlates to its penetration depth; in general, light of greater wavelengths can reach deeper tissues, and, in the case of osteoporosis, conduce to better bone regeneration. It has been shown that light irradiation in the wavelength range of 700 to 1200 nm was the most effective in penetrating biological tissues 27. Taking all these factors and the results of previous studies into account, we opted to conduct laser acupuncture with semiconductor laser at 808 nm and 120 J/cm², which

achieved gratifying therapeutic results in the ovariectomized rats that we tested. Osteoporosis induces significant bone remodeling characterized by a series of adverse microarchitectural changes such as a loss of bone mass and deterioration of the trabecular network. These pathological alterations are accompanied by decline in Tb.N, Tb.Th, Tb.N and BV/TV, as well as increase in Tb.Sp. SMI is a morphometric parameter valued between 0 and 3 that measures the rods and plates in trabecular bone. In general, SMI increases with a greater rod-to-plate ratio, which is associated with more severe cases of osteoporosis. In our study, sham compared with OVX, the bone density and trabecular bone data in the sham group showed significant improvement, suggesting that estrogen has a protective effect on bone. These changes were confirmed by both micro-CT and histological staining in our rat ovariectomy model, the latter of which also found a sharp increase in the number of adipose cells. Both adipocytes and osteoblasts are derived from bone marrow mesenchymal stem cells 28, and the differentiation shift from osteogenesis to adipogenesis is considered a key aspect of osteoporosis. In this regard, the results of OVX compared to O-N and O-L show that, needle acupuncture seemed to have tilted mesenchymal stem cell differentiation back

toward osteogenesis, as evidenced by the decreased number of adipose cells. In addition, we also observed improvements in trabecular microarchitecture and

morphology, indicating heightened bone formation; however, further analysis using quantitative method did not find these changes to be statistically significant. O-N compared with O-L shows, the therapeutic effects induced by laser acupuncture on bone density and repair of trabecular network were statistically significant and more pronounced on a morphology level. Furthermore, laser acupuncture was also found to be more effective in suppressing adipogenesis. These results support the use of laser acupuncture as a viable and perhaps more desirable alternative to the conventional needle-based practice.

The formation and maturation of osteoblasts, which play an indispensable role in bone metabolism, are tightly regulated by a complex network of osteogenesis markers 29. In this study, the rats in OVX showed no significant drop in the serum protein level of ALP or OCN compared to those in the sham group, and immunostaining of bone tissue sections indicated a slightly increased level of OCN in the trabeculae for the group of OVX compared to the sham group. The finding is consistent with the fact that decline in estrogen level contributes to primary osteoporosis chiefly by accelerating bone resorption, not by inhibiting osteogenesis³⁰. In comparison, diminishing bone formation played a greater role in age-related osteoporosis. As a result, it is not uncommon for middle-aged women with postmenopausal osteoporosis to show relatively normal levels even higher levels of osteoblasts^{31, 32}. On the other hand, OVX compared to O-N and O-L, both needle-based and laser acupuncture led to an up-regulation of serum ALP and OCN proteins, implying enhanced osteoblast activities. Taken together, we tentatively concluded that ovariectomy promoted osteoporosis in our rat model primarily by stimulating bone resorption, and that the acupuncture treatment mitigated this imbalance in bone metabolism by augmenting osteogenesis.

The collagen network is a critical component in bone that supports its stability, toughness and tensile strength. Col-1, secreted by osteoblasts, accounts for 90% of all proteins in the bone matrix, and serves as a linkage between surface integrins and other extracellular matrix proteins 33. It is well-established that the degeneration of the collagen network plays a key role in a wide range of bone diseases^{34, 35}. Although the level of Col-1 in the rat serum samples did not significantly decline or rise following the ovariectomy (compared to sham), it was found to be significantly up-regulated as a result of acupuncture treatment (compared to OVX), with laser acupuncture producing a significantly more pronounced effect in comparison to the conventional needle-based method. And unlike the serum

results, the transcription of Col-1 in OVX showed an apparent increase compared to the sham group, albeit without statistical significance. we speculated that the slight boost in Col-1 transcription might have been offset by the up-regulation of MMP-1 induced by heightened osteoclast activities in the ovariectomized rats, which reduced the amount of collagen released into the blood. This hypothesis is also supported by previous

studies 36. Overall, our results provided clear evidence that acupuncture, particular when combined with low-level laser irradiation, could help repair osteoporotic collagen damage.

The BMP2/Runx2 signaling pathway is a main driver for the production of bone-specific matrix proteins 37, and is instrumental in bone formation. BMP2-dependent up-regulation of Runx2 has been well established, both in vitro and in vivo, to modulate the generation, maturation and differentiation of osteoblasts 38. Once activated, Runx2 directly binds to the promoter regions of several key osteoblast-specific genes, such as OCN and ALP, to trigger the downstream osteogenesis cascade 39. Conversely, knockout of Runx2 in mice has been demonstrated to abolish both intramembranous and endochondral ossification⁴⁰. In our current study, both needle-based and laser acupuncture were shown to dramatically elevate the transcription level of Runx2, lending strong support to their therapeutic benefits for bone formation. The results also echoed previous findings that low-level laser irradiation could exert a positive effect on bone regeneration by promoting osteoblast proliferation, differentiation, and secretion of growth factors 41. On a molecular level, there is increasing evidence that laser therapies could also induce the expression of various osteogenesis markers, including Runx2⁴², BMP2⁴³, Smad144, and OCN⁴⁵. The combination of these studies and our current data demonstrated that the osteoprotective effects of laser acupuncture could be associated with its activation of the BMP2/Runx2 signaling pathway.

The OPG/RANKL/RANK system is considered the dominant and final regulator of osteoclastogenesis⁴⁶. The binding of RANKL to RANK, located on the cell membrane of osteoclast progenitors, triggers the recruitment of tumor necrosis factor receptor-associated factors to activate a number of downstream signaling pathways necessary for osteoclast-dependent bone resorption 47. This process can be negatively modulated by OPG, which serves as a decoy to prevent RANKL-RANK interaction via competitive inhibition. It has been previously shown that decrease in OPG expression exerts a direct effect on inducing osteoporosis in ovariectomized rats 48, which is consistent with our observation of OPG down-regulation in the group of OVX (albeit without statistical significance).

Decline in OPG level, and the concomitant up-regulation of RANKL and osteoclastogenesis, also play a prominent role in age-related osteoporosis⁴⁹. When treated with either form of acupuncture, the rats in our study exhibited a marked increase in both the serum protein and tissue transcription levels of OPG, suggesting that both therapies may suppress the formation and activation of osteoclasts, leading to diminished bone resorption. In conclusion, our current study demonstrated that laser acupuncture is more effective than its needle-based counterpart in enhancing bone density, repairing the damage in trabecular architecture and inhibiting adipogenesis in rats with ovariectomy-induced osteoporosis. On a molecular level, both treatment methods significantly improved the transcription and/or

expression levels of key bone turnover markers, including ALP, BMP, OCN, Col-1, Runx2, OPN, and OPG. Taken together, our current study provided preliminary experimental evidence that supports the use of laser acupuncture as a painless and non-invasive therapy for preventing postmenopausal osteoporosis. However, further therapeutic doses and regimens for laser acupuncture are currently lacking, and the significant heterogeneity of the available studies, particularly in terms of laser parameters and outcome measures, makes it difficult to compare different interventions and expected outcomes. This has led to a lack of consensus on optimal clinical application, which is currently difficult to standardize for large-scale application. In order to overcome these limitations, future studies should first conduct a preliminary evaluation of studies, set different gradients of laser therapeutic doses, screen out the most effective regimens through a large number of randomized controlled trials, and develop uniform operating specifications and standard parameters so that they can be finally translated into daily clinical practice.

5 ACKNOWLEDGMENTS

This study was supported by research funding from the Youth Science and Technology Project of Shanghai Pudong Health Commission (Grant No. PW2022B-02), Demonstration Pilot Project of Central Financial Support for the Inheritance and Innovation of Traditional Chinese Medicine (YC-2023-0601, YC-2023-0201), The National Administration of Traditional Chinese Medicine of the Ministry of Finance Medical Service and Support Capacity Improvement Subsidy Fund (Part of key departments) project "Construction of Key Departments of Collaborative Traditional Chinese and Western Medicine" (Huwei Zhongguan Memorandum No. [2023] 46), the National Natural Science Foundation of China (Grant No. 8217032794)

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