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Original Article

An investigation over the impact of conventional Chinese bone setting to cartilage and the VEGF expression in cartilage cells in rats with knee osteoarthritis disease

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Abstract: The conventional Chinese bone setting, named Xiao Yu San Plaster has been used for treating knee osteoarthritis for hundreds of years and achieved dramatic effects. However, very few work has been done to investigate the impact these bones settings experimentally or pre-clinically. In this work, we investigated the impact of this bone setting on cartilage tissue structure. In particular, we also studied the influence of this bone setting on VEGF metabolism in the cartilage cells. To illustrate the treatment efficacy, an FDA approved medicine, Voltaren Gel, was employed as a control group. Morphological observation and immunohistochemical staining demonstrated that the bone setting improved the disease to a degree comparable to Voltaren Gel. This study illustrated the potency of the Chinese bone setting in treating knee osteoarthritis disease, and partially revealed the mechanism behind the treatment.

Keywords: Chinese bone setting, osteoarthritis, cartilage, vascular endothelial growth factor

Introduction

Osteoarthritis (OA) is a disease featured by the chronic degenerative joint disorder. The disease has a huge negative impact the healthcare system in almost every country all over the world. Major symptoms in OA include pain, decreased joint range of motion, muscle weakness, and joint swelling [1, 2]. Although this disease happens to a huge population of people, the mechanisms of the disease are not clear. In most occasions, OA is associated with joint structure changes such as a loss of articular cartilage thickness or increased thickness associated with the radiological changes. However, despite the negative impact of the disease to individuals, there is no cure for OA. Most current strategies for treating OA are mainly symptoms-reducing drugs, or surgery that preserve or replace the joint tissue [3, 4]. In addition, recent studies also suggested that OA may be related with the muscle weakness due to the atrophy [1, 2, 5, 6]. Clinical practice also suggested the age-related muscle decrease accompanies with the high occurrence rate of OA [3, 4, 7, 8].

Vascular endothelial growth factor (VEGF) is a signal protein that is a characteristic of vasculogenesis and angiogenesis [9]. VEGF is widely expressed in some of the major cells residing in human osteoarthritic joints, including synoviocytes, macrophages. Current studies demonstrated that VEGF affects chondrocytic proliferation, apoptosis, and metabolism. In particular, the expression of VEGF in the superficial zone of the cartilage disc indicated the association between VEGF and OA [7, 10-13]. Clinically, synovial fluid extracted from OA patients has 20 times higher of VEGF than that from healthy subjects, further indicating the VEGF was related to OA progression [3, 14-16].

Chinese bone setting was a therapy that has been in clinic to improve OA in patients for a long history [17-19]. In particular, clinical study found that the pad improved OA joints and reduced the decrease of cartilage in OA joint. In

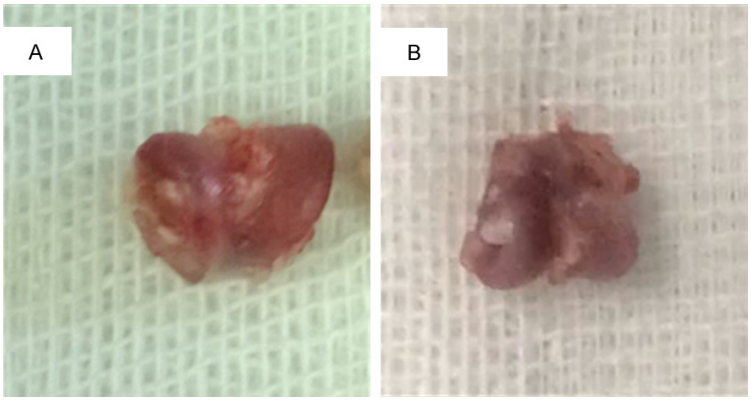

Figure 1. The observation of cartilage of disease and normal group after 6 weeks. (A) Cartilage from rats in normal (B) and disease model group.

this work, we compared the impact of the bone setting with a conventionally used OA drug (i.e. Diclofenac).

Materials and methods

Materials

Chinese bone setting was provided by the Pharmacy Department at Xinhua hospital. The setting was purchased commercially (Yunan Bai Yao Inco., China). Diclofenac diethylamine (DFTA) gel was provided by Novartis. First and secondary antibody against VEGF was from abcam (UK). DEPC water was from Genview (US); Triton X-100, and DAB kit was from Blue Sky Biotech Institute. Pentobarbital was from Shanghai Xitang Biotech. Kits for hematoxylin and eosin stain (H&E staining) were from Blue Sky Biotech Institute.

Animals

Sprague-Dawley (SD) rats (8 weeks old, 50% male and 50% female) were obtained the animal room at Xinhua Hospital affiliated to Shanghai Jiaotong University. All Animal experiments were approved by the Animal Care Committee at our institute, and followed the local and governmental laws on animal care and protection.

Animal model

The animals were divided into four groups randomly: Naïve, Disease group, Setting group, DS group, each group contains 8 rats. All rats were raised at the same conditions. The OA animal model was established according to Hulth method [8]. Briefly, 3% pentobarbital sodium (30 mg/ml, dose: 2 ml/kg) was injected to rats via intraperitoneal injection. After anesthesia, the rats were put on the bench and cut medial ligament for around 2 cm in depth to remove the meniscus completely. After cleaning the blood, the cut was closed with surgical suture. The rats were then injected with 800 K units of penicillin through muscle injection for three days to prevent infection. After 6 weeks, random rats were picked to test the whether the model was successfully established or not.

Animal treatments

The animals were divided to four groups randomly: 1) Naïve, 2) Disease group (rats were induced with the model disease for 6 weeks, followed with no treatment), 3) Setting group, the rats were induced with the disease model. Rats legs were then attached with the bone setting. The sticker was changed with a new one every other day. This treatment lasted for 4 weeks. 4) Voltaren Gel group (i.e. DS group). The rats were induced with the disease for 6 weeks, followed by treating with DS for 4 weeks. After all the study, rats were sacrificed with CO_2, followed with conical dislocation to ensure complete sacrifice. The meniscus and the surrounding tissues were collected in culture hood. 0.5 cm×0.5 cm×0.5 cm arthrodial cartilage were collected to SE staining and other tests. Briefly, the tissue was fixed in formadehyge for 24 hour, followed by washing with DI water for 2 hour. The tissue was moved to 10% EDTA solution for around 6 weeks until needles can penetrate the tissue easily. The tissue was then embedded with wax and cut into thin slide (5 μm) for further experiment.

Immunohistochemical staining

Standard HE and VEGF staining was employed to stain the cartilage tissue and VEGF expression. The experiments were performed by standard protocols [20, 21].

Clinical score of pathological study of HE staining

Markin's [12] standard was employed to assess the cartilage structure, cells staining degree

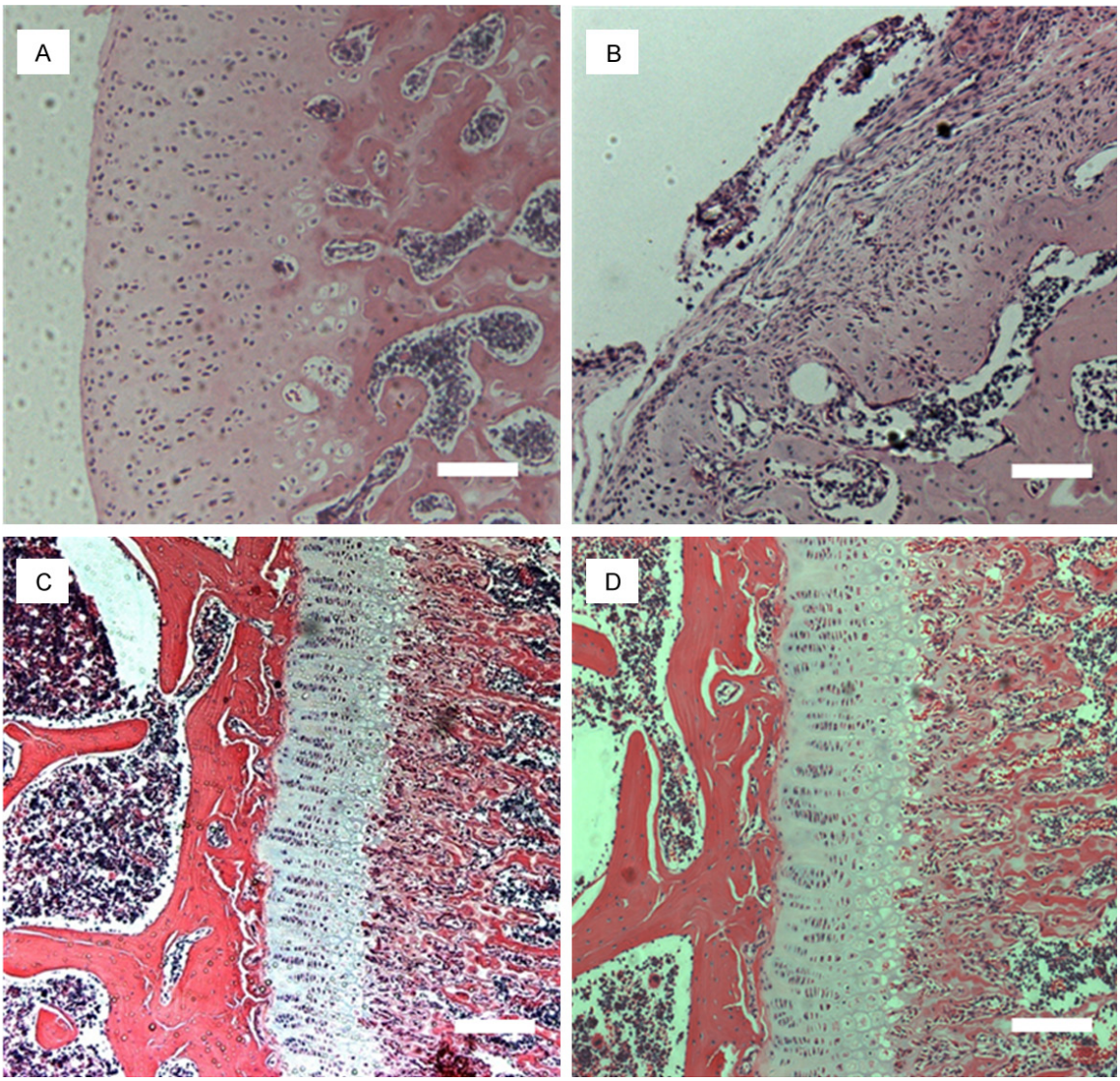

Figure 2. Observation of metaphyseal morphology of (A and C) normal and (B and D) diseased groups, respectively. Rats in the diseased group received no treatment. Scale bars are 500 μm.

Table 1. Score of cartilage disease degree in rats with different treatment ($\bar{x} \pm s$)

Groups/Score	0	1	2	3	4
Naive	7	1	0	0	0
Disease model	0	1	1	3	3
Sticker treatment	0	5	2	1	0
DS treatment	0	2	1	3	2

$P < 0.05$ for disease model group vs. Naïve group. $P < 0.05$ fro sticker group vs. diseased model group. $P < 0.05$ for DS group vs. diseased model group. The disease model group had no treatment.

and morphology of the tissue. Those cells that had brown particles was defined as VEGF positive cells, according to the methods reported in literature [13]. 5 view fields (200×) were selected from each slice. The number of VEGF positive cells as well as the total number of cells in the view field was counted, where the ratio (i.e. VEGF positive cells/total number of cells) was used to show the expression level of VEGF.

Statistical analysis

SPSS19.0 was used for statistical analysis. The data was expressed as $\bar{x} \pm s$, as analyzed via one-way ANOVA. $P < 0.05$ was employed as statistical importance.

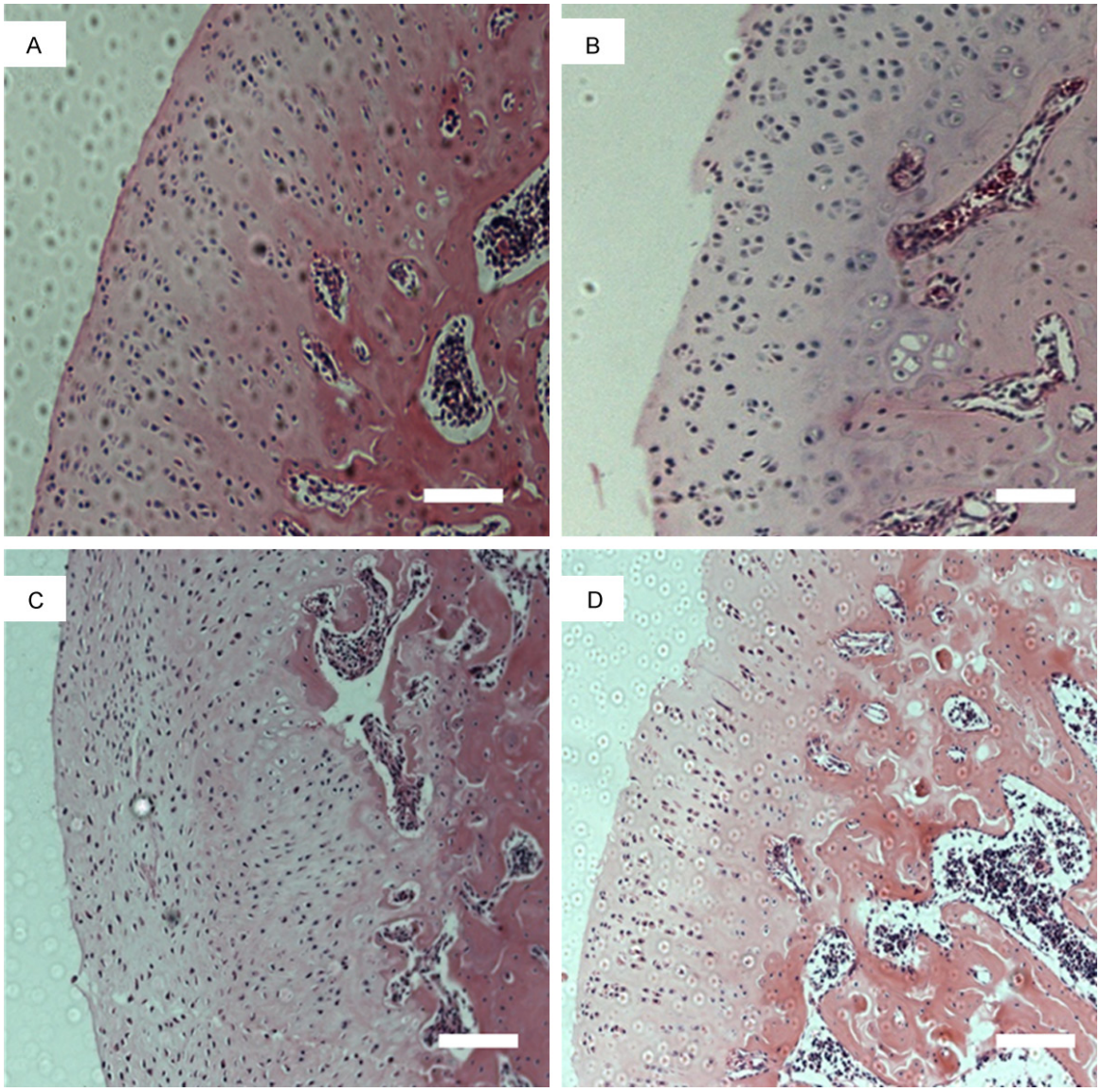

Figure 3. The observation of cartilage morphology of different groups after different treatments. (A) Naïve group, the tissue had a smooth surface, organized cell structure (B) disease model group, the tissue had a rough surface. There was aggregates of cartilage cells. (C) The Bone setting group, the cells had improved structure compared to the disease group. The tissues had an organized cell structure. (D) In DS group, roughness and damages were observable on the tissue surface. Scale bars are 500 μm.

Table 2. Mankin's clinical score ($\bar{x} \pm s$) of rats with OA

Group	Rat numbers	Mankin score
Naive	8	0.25±0.46
Disease model	8	10.75±1.98*
Sticker	8	4.13±1.55*
DS group	8	8.63±0.52*

*P < 0.05 for disease model group vs. Naïve group. *P < 0.05 for sticker group vs. disease model group. P < 0.05 for DS group vs. disease model group. The disease model group had no treatment.

Results

The disease model was successfully established. After treating the rats for 6 weeks, the rats joints was collected for pathological observation. The cartilage was also collected as control to assess whether the disease model was successfully established or not (**Figure 1A**). The photograph of cartilage from untreated rats. The cartilage had a smooth surface and complete structure. As a comparison, the cartilage

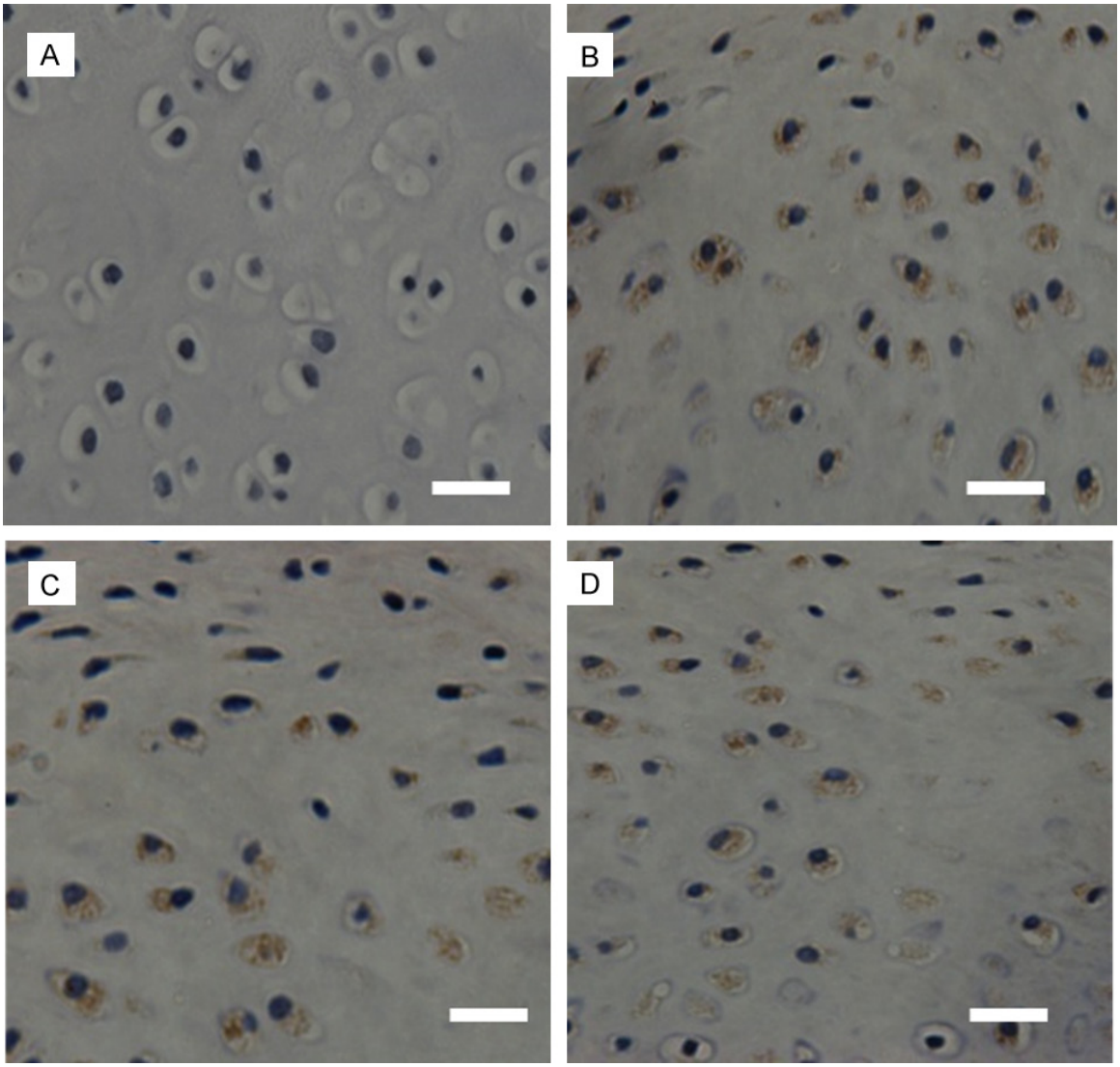

Figure 4. The expression of VEGF of cartilage in the knee OA rats in different groups. A. The image showed very few VEGF positive cells in the control group. B. A significant population of VEGF positive cells were observable in rats induced with OA. C. The population of VEGF positive cells reduced in the samples from rats in the sticker group compared to rats induced with OA. D. DS treatment also reduced the population of VEGF positive cells in the cartilage compared to rats induced with OA. However, compared to the sticker group there was still a higher amount of VEGF positive cells in this group compared to the sticker group. Scale bars are 100 μm.

Table 3. The expression level of VEGF positive cells in the OA cartilage cells ($\bar{x} \pm s$) %

Group	Rats numbers	VEGF (%)
Naive	8	0.74±0.06
Disease model	8	1.78±0.14*
Sticker group	8	0.96±1.55*
DS group	8	1.16±0.52*

Note: *P < 0.05 for disease model group vs. Naïve group. *P < 0.05 fro sticker group vs. disease model group. *P < 0.05 for DS group vs. disease model group.

from the disease rats had a rough, eroded surface, where some parts of cartilage was missing as well (**Figure 1B**).

Immunohistochemical staining demonstrated the establishment of the disease model. HE staining was used to assess the establishment of model as well. For the naïve group, the cartilage had a smooth structure. The cells had a well-organized structure (**Figure 2A** and **2C**). As a comparison, the cartilage from the diseased

model had an eroded surface structure, where it was difficult to identify the layers, and the cells also aggregated as well (**Figure 2B** and **2D**).

Clinical score demonstrated the effectiveness of bone setting in treating the disease. After 4 weeks of treatment, the cartilage from the control group had a normal morphology, and a smooth surface, with no damage on the cartilage observed. As a comparison, the cartilage from the disease model had a rough and damaged surface, with cracks observable on the cartilage surface. The DS treatment also had certain degree of efficacy on the treatment - although rough surface and cracks were also observed. The score of OA was illustrated in **Table 1**.

Microscope characterization of HE staining in different groups of rats confirmed the efficacy of bone setting in treating the disease. For the cartilage from naïve rats, the microscope observation of stained cartilage showed a complete, smooth structure. The cells within the cartilage tissue had a well-organized, layered structure (**Figure 3A**). On the contrast, the cartilage from the diseased model group had a rough surface; additionally, and the cells had a disrupted structure and the cartilage had certain degree of damage (**Figure 3B**). The treatment with the sticker improved the disease symptom: The cartilage had an improved surface smoothness and a more clear structure and an increased number of cartilage cells (**Figure 3C**). DS treatment also improved the disease symptom as well, although surface roughness and disorganized cells were still observable (**Figure 3D**). Markin score was employed to assess the treatment efficacy. The disease model group had a significantly higher score than naïve group ($P < 0.05$). The sticker and DS group had a significantly lower score than the disease model group ($P < 0.05$); There was also statistic difference between the sticker and DS group ($P < 0.05$) (**Table 2**).

Bone setting can modulate this VEGF expression in the disease: Very few VEGF positive cells were observable in the control group (**Figure 4A**). A huge population of VEGF positive cells were observable in the disease model and DS group (**Figure 4B**); upon sticker treatment, the population of VEGF positive cells decreased (**Figure 4C**). Similarly, the DS treatment also down-regulated the amount of VEGF positive cells in the tissue (**Figure 4D**). Statistically, the disease model had a higher population of positive cells than the naïve group ($P < 0.05$). The sticker group had a much lower population of VEGF positive cells compared to disease model group ($P < 0.05$). Statistical difference was also observed between the DS and sticker group ($P < 0.05$) (**Table 3**). These data indicated that the bone setting was effective in regulating the level of VEGF cells in OA rats. And the potency of bone setting was similar to DS treatment.

Conclusion

This work studied the impact of a conventional Chinese bone setting on knee osteoarthritis disease. The study started with establishing the disease model and confirm this establishment by morphological observation and immunohistochemical staining. To test the impact of the bone setting on the disease treatment, the morphology, immunohistochemical staining, and clinical score of rats with different treatments were compared. The studies in work demonstrated the bone setting was effective in treating the disease. VEGF expression tests demonstrated the bone setting can modulate the expression of this protein, which partially revealed the mechanism in this treatment. The study can help us understand the working mechanism of this medicine, which has been employed for hundreds of years in history.

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Disclosure of conflict of interest

None.

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