External axial Stress Induces Rabbit Tibia Fracture Healing via Stimulating the Expression of VEGF, CD34, BMP-2 and TGF-β1

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Title page

External axial stress induces rabbit tibia fracture healing via stimulating the expression of VEGF, CD34, BMP-2 and TGF-β1

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Abstract

Background

It is well acknowledged that stress stimulation can promote the healing rate of fracture. However, there were few literature that described the relationship between stress stimulation at the fracture site and the molecular mechanism in fracture healing process. The aim of this study was to investigate whether external axial stress, provided by the stimulator, postoperatively, can accelerate the union of rabbit tibial fracture, and to reveal the possible mechanism.

Methods

Seventy-two New Zealand rabbit tibial fracture models were established and randomly divided into two groups. Rabbits in experiment group (n=36) were subjected to external axial stress stimulation for 30 minutes every two days from the eighth day postoperative, but rabbits in control group received no stress stimulation. All rabbits were treated with external plaster for limb immobilization after operation. Lane-Sandhu X-ray evaluation system was used to evaluate the bone healing process at 2, 4, 6 and 8 weeks postoperative. Specimens were harvested for immunohistochemical test and semi quantitative analysis. They were applied to evaluate the expression of vascular endothelial growth factor (VEGF), CD34, bone morphogenetic protein-2 (BMP-2) and transforming growth factor-β1 (TGF-β1) at postoperative 2, 4, 6 and 8 weeks, respectively.

Results

The mean Lane-Sandhu X-ray score of experiment group was significantly higher than control group at 4, 6 and 8 weeks ($P<0.05$, $P<0.01$ and $P<0.01$， respectively). More callus and better calcification were observed in the experiment group. The expression of VEGF ($P<0.01$, $P<0.05$, $P<0.01$, $P<0.01$, respectively) and BMP-2 ($P<0.05$, $P<0.05$, $P<0.01$, $P<0.01$, respectively) at each phase in the experiment group was significantly higher than the control group. The expression of CD34 ($P<0.01$ and $P<0.01$, respectively) and TGF-β1($P<0.01$, $P<0.01$, respectively) in experimental group was higher than control group at 2 and 4 weeks.

Conclusions

Postoperative external axial stress stimulation can facilitate the expression of VEGF, CD34, BMP-2 and TGF-β1, and stimulate the vascularization and ossification of the fracture site, therefore, accelerate the fracture union.

Key words Fracture union, external axial stress, VEGF, TGF-β1, CD34, BMP-2.

Background

There is a growing number of lower extremity fractures in recent decades due to car accidents and high energy injury. It is still a challenge for orthopedic surgeons regarding how to facilitate bone union and reduce delayed union and nonunion. It is well acknowledged that stress stimulation can promote the healing rate of fracture [1]. The size and quantity of bone unit is fitted with mechanic distribution of its own, which, called Wolff law, was described by Wolff [2]. The process of fracture healing is related to a series of cytokines, such as VEGF, TGF-β1 and BMP-2, which play an important role in the vascular regeneration, callus formation and callus remodeling [3-5]. However, there were few literature that described the relationship between stress stimulation at the fracture site and the molecular mechanism in fracture healing process. With the advancement of biological technology, there are many suitable methods to explore the mechanism of fracture healing [6-10]. The External Axial Stress Stimulator (EASS), which creates axial stress, can provide loading sharing at human tibia and heel and facilitate fracture healing, has been reported by scholars [11]. However, the molecular mechanism of the fracture healing by EASS is still unclear. The aim of the study was to explore whether axial stress stimulation can facilitate union of the fracture site and reveal the possible mechanism.

Materials and methods

Animals and instruments

Seventy-two skeletal mature female New Zealand rabbits, averaged 3 kilos and 7 months old, were subjected to tibia fracture model after feeding for a week. The rabbits were processed strictly according to protocol made by ethics committee department in our hospital. The EASS was designed by ourselves independently (the patent number CN1803117, Fig 1), which included rapping hammer, control unit, and bracket. The rapping hammer simulated the physiologic stress load animals received when walking, thus generated effective micro-strain to promote fracture healing. Stress ranged from 1 to 50 N, and the frequency was 0.5 to 3Hz. Rapping time was 5 to 35 seconds, and time interval was 3 to 19 seconds.

The rabbits, given general anesthesia by pentobarbital sodium by intravenous injection, were subjected to water deprivation for 6 hours, and abrosia for 12hours preoperative. After sterilization, the incisions were made in front of the right anteromedial tibia. The surrounding soft tissues were segregated and fretsaws were used to form one millimeter

fracture space. Anatomical reduction and ideal positioning for the implant were promoted before the osteotomies was created in all samples. The incisions were sutured after normal saline washing. Once the operation finished, plasters were applied to limit excessive movement of the operated limbs. These rabbits were randomly and equally divided into two groups, including the experiment and control group, and were injected with penicillin for three days postoperative. In experiment group, heels of operated limbs were subjected to stress stimulation from the eighth day postoperative. Before rapping, the rabbits were injected with ketamine. The rabbits were positioned on the platform and the operated limbs were fixed at the same horizon of the rapping hammer in order to make sure the direction of the stress was vertical to the fracture line. The ipsilateral knees were restricted and the heels were positioned against the rapping hammer to standardize the stress stimulation (Fig 2). Some ketamine was added to alleviate the pain during the process. The stimulation was given once two days, thirty minutes each time with fifteen N, one Hz, five seconds rapping and three seconds interval. After rapping, the operated limbs were stabilized with plaster again. Rabbits in the control group received no stress stimulation but external plaster fixation only.

After operation, complications, such as incision infection, were recorded. X-ray of the tibia was performed at second week, fourth week, sixth week, and eighth week postoperative. Bone healing was measured by Lane-Sandhu scoring system [12]. Histological evaluation of both groups were performed at second, fourth, sixth, and eights week postoperative. Specimens were harvested from the operated limbs and fixed in 4% paraformaldehyde for twenty-four hours, followed by ethylenediamine tetraacetic acid (EDTA) decalcification, ethyl alcohol dehydration and paraffin embedding. Eventually, the slices were dyed with hematoxylin and eosin. New bone growth and angiogenesis at the fracture site were observed with the use of light microscope and compared with software of ImagePro Plus 6.

The tissue slices were placed at indoor temperature for sixty minutes for dewaxing, followed by immunohistochemisty dye, and the absorption values were measured to evaluate the expression of CD34, BMP2, TGF-β1, and VEGF. Statistics obtained from Lane-Sandhu evaluation was calculated with SPSS 16 software. CD34, BMP2, TGF-β1, and VEGF were analyzed by medical image analysis system. $P<0.05$ was considered to be significant.

Results

All the rabbits were given normal food intake. No symptom of incision infection was observed. Fracture site in the control group was substituted by only few callus, compared with the experiment group with more new bone in-growth and bone callus in the eighth week postoperative (Fig 3).

At the second week postoperative, fracture lines were obvious in both groups. At the fourth week postoperative, bone callus was formed in both groups and the experiment group showed better callus formation than the control group. In the sixth week postoperative, the experiment group had dense callus, the fracture line was fuzzy, and the osteotomy gap nearly disappeared, however, the control group had less callus and the fracture was still visible. In the eighth week postoperative, fracture line disappeared and fracture healed well in both groups. Bone callus in experiment group formed densely and more than that in control group (Fig 4). Significantly higher Lane-Sandhu score was showed in the experiment group than the control group at the fourth, sixth, and eighth week postoperative ($P<0.05$, $P<0.01$ and $P<0.01$，respectively. Table 1).

Table 1: Lane-sandhu's X-ray score of both groups at each phase postoperatively.

Group	The second week	The fourth week	The sixth week	The eighth week
Control group	1.33 ± 0.22	3.67 ± 0.15	5.67 ± 0.44	7.08 ± 0.32
Experiment group	1.67 ± 0.37	5.33 ± 0.54[a]	7.00 ± 0.82[b]	9.67 ± 0.47[c]

Note: compared with the control group,[a]$P<0.05$;compared with control group,[b]$P<0.01$; compared with control group,[c]$P<0.01$.

At the second week, hematoma formed at fracture site, lots of inflammatory cells aggregated, and capillaries and fibroblasts proliferated in both groups. At the fourth week, primary bone callus formed in both groups, more fibrocytes were observed in the experiment group. At the sixth week, more bone trabecula structures were observed in the experiment group than the control group. At the eighth week, cartilage tissue had transformed into mature bone trabecula in both groups, but more mature bone callus was observed in the experiment group, with better mineralization and denser collagen tissue (Fig 5).

CD 34 staining in the experiment group showed significantly dyed than the control group at the second and fourth week ($P<0.01$ and $P<0.01$, respectively), but no significant difference was observed at the sixth and eighth week (Table 2, Fig 6). There was significant difference regarding the VEGF ($P<0.01$, $P<0.05$, $P<0.01$, $P<0.01$, respectively)(Table 2, Fig 7) and BMP-2 ($P<0.05$, $P<0.05$, $P<0.01$, $P<0.01$, respectively)(Table 2, Fig 8) staining between the two groups at each period, and the experiment group showed deeper colored. TGF-β1 staining showed significantly dyed in the experiment group than the control group at the second and fourth weeks ($P<0.01$, $P<0.01$, respectively), but no significant difference was found at the sixth and eight weeks (Table 2, Fig 9).

Table 2: Comparisons of CD34, VEGF, TGF-β1 and BMP-2 Immunohistochemical staining absorbance values between control group and experimental group.

Group	The second week	The fourth week	The sixth week	The eighth week
CD34				
Control group	84.79 ±7.35	252.65 ± 14.49	103.75 ± 5.95	81.76 ± 5.40
Experiment group	192.82 ± 8.65[a]	451.69 ± 40.94[b]	106.91 ± 6.78	82.46 ± 6.02
VEGF				
Control group	132.17 ± 7.47	319.18 ± 12.09	221.27 ± 13.02	184.31 ± 9.48
Experiment group	278.63 ± 9.62[a]	532.50 ± 13.50[c]	350.40 ± 15.07[a]	267.00 ± 22.55[a]
TGF-β1				
Control group	154.70 ± 8.26	249.41 ± 12.03	146.64 ± 10.06	110.66 ± 1.60
Experiment group	250.87 ± 6.62[a]	433.78 ± 3.41[a]	150.91 ± 4.40	115.09 ± 0.78
BMP-2				
Control group	126.12 ± 2.57	194.67 ± 5.29	296.10 ± 12.99	199.08 ± 10.19

Experiment group	134.52 ± 2.00[d]	211.90 ± 7.78[e]	571.79 ± 7.97[a]	260.36 ± 7.15[a]

Note: compared control group with experimental group, [a] $P<0.01$, [b] $P<0.01$, [c] $P<0.05$, [d] $P<0.05$, [e] $P<0.05$; VEGF: vascular endothelial growth factor; TGF-β1: transforming growth factor; BMP-2: bone morphogenetic protein-2

Discussion

Bone healing is an extremely complex course that has involved many cells and bone growth factors. After cells collecting, factor regulating, bone induction and bone conduction, bone healing will undergo blood organization, callus formation and callus transformation, which are affected by blood supply at fracture part, biomechanical environment and nutrition condition [13]. In recent decades, many researches indicated that moderate stress stimulation could promote bone healing. On the one hand, mechanical stress can indirectly stimulate osteoblasts formation, facilitate the proliferation and differentiation of osteoblasts and the formation of extracellular matrix [14,15]. On the other hand, mechanical stress can also promote cell factors release to improve skeleton vascularization and mineralizaion. Presently, some studies showed that shear stress and rotation stress hindered the differentiation of mesenchymal cells, inhibited the revascularization of fracture part, and increased the fibrous tissues [16-18]. In Pauwcls' study [19], hydrostatic stress could promote formation of cartilage. Carter DR et al. [20,21] had set up ossification model and suggested that the transformation from cartilage to bone depended on the cyclic stress stimulation after fracture progression. Skerry et al. [22] thought that dynamic stress could facilitate mineralization and sedimentation of bone stroma, and increased the blood flow. Therefore, there is an intimate connection between stress environment and bone tissue. Present study had got the conclusion that axial stress could augment dendritic tuber among the connection of bone cells, transform the bone cytoskeletion, change the form of bone cells, and increase the cell matrix [23,24]. Under axial stress stimulation, osteoblasts regulated the multiplication of osteoclasts by expressing macrophage colony stimulating factor (CSF) and nuclear factor κB activation/osteoprotegerin (OPG). In our study, we found that the expression of VEGF, TGF-β1, BMP-2 and CD34 in fracture site increased variously after exposed to intermittent rapping stress. The up-regulated expression of these factors indicated that bone callus increased and bone healing was accelerated, which could be verified by radiography. In a word, mechanical stress load could facilitate the activation of osteoblasts, induce osteoblasts

to release a series of growth factors, and regulate the function of osteoblasts and osteoclasts by signal transmitting.

The growth of vascular in fracture site is the prerequisite of fracture healing [25]. VEGF, an important signal protein to guide the vascular growth, is an unique growth factor [26]. Mohanti et al. [27] revealed that VEGF appeared at the beginning eight hours after fracture at the fracture site, then declined slightly and returned to a high level of expression, which maintained for 72 hours to 3 weeks. After that, the expression level decreased gradually with the progression of fracture healing. $CD34^+$ cells could differentiate into the cells required for blood vessel regeneration and ultimately assist in the repairing of vascular vessels [28]. In the present study, we found that VEGF and CD34 expressed in both groups and reached the summit at the fourth week postoperative. In radiography, bone callus in experiment group at the fourth week was significantly more than that in control group. The approach at the fourth week to the eighth week regarding Lane-sandhu's X-ray score meant that the change of molecule was earlier than the change in radiography.

In this study, the VEGF expression could happen at any point and the cells that could express the VEGF were various in the progression of fracture healing [29]. From the first to second week of fracture healing, VEGF can be found in mesenchymal stem cells (MSCs) of fracture part. The present study showed that under axial stimulation, VEGF expression could get to higher level at early time, which led to the increasing of vascular endothelial cells and promoted fracture healing [30]. At the fourth week, fibrelike texture could be seen in primary callus after HE staining, and the expression of VEGF was also high, which indicated that the new vascular formation began and the vascular tissues could deliver the nutrition and cells to the area. In addition, we speculated that CD34 began to mobilize and promote the new vascularization. These cells could guide MSCs to the fracture site by vascular transportation. Then the MSCs would differentiate into cartilage or osteoblasts, even some endothelial progenitor cells signaled by CD34, participating in repairing the vascular tissues [31,32]. Consequently, significantly more $CD34^+$ cells were detected at the fourth week in experiment group than the control group. So we inferred that axial stress could help $CD34^+$ cells facilitating MSCs' transferring to fracture part in vitro and that $CD34^+$ cells accelerated vascularization to promote bone healing by inducing them transferring to vascular endothelial cells.

BMP-2 that can induce osteoblast is the most important cell factor in regulating bone healing progression [33]. In Carolin Schwarz' study, they found that BMP-2 began to increase from the second week postoperative after compressive stress stimulation [34]. Johnson et al reported that BMP-2 could promote and induce fracture healing for the treatment of nonunion of femoral fracture [35]. In the present study, at the second and fourth week postoperative, the expression of BMP-2 was low in both groups because vasucularization in fracture site had not happened for cells and factors transporting. At the sixth week, the expression of BMP-2 reached the peak in experiment group so they could activate osteoblasts and promote proliferation and differentiation of osteoblasts [36,37]. Moreover, vascularization was much faster with stress stimulation, which could supply more MSCs and stimulate the expression of BMP-2. At the eighth week, BMP-2 started to decrease.

TGF-β1 can inhibit osteoclasts and promote osteoblasts, induce MSCs transforming to adipocytes, regulate the differentiation and the growth of cartilage. TGF-β1 can also help synthesizing the type I collagen and osteopontin by recruiting osteoblasts, and facilitating the synthesis and enrichment of stroma [38-40]. Few literature reported whether axial stress stimulation might regulate the expression of TGF-β1. In the present, the expression of TGF-β1 showed an upward trend in both groups, besides, the expression of TGF-β1 was significantly higher in experiment group which meant axial stress stimulation could obviously enhance the expression of TGF-β1. However, more exploration should be established to reveal the mechanism of the promoted expression of TGF-β1.

Conclusions

Postoperative external axial stress stimulation can facilitate the expression of VEGF, CD34, BMP-2 and TGF-β1, and stimulate the vascularization and ossification of the fracture site, therefore, accelerate the fracture union

List of Abbreviations

VEGF: vascular endothelial growth factor

BMP-2: bone morphogenetic protein-2

TGF-β1: transforming growth factor-β1

EASS: The External Axial Stress Stimulator

EDTA: ethylenediamine tetraacetic acid

CSF: colony stimulating factor

OPG: osteoprotegerin

MSCs: mesenchymal stem cells

Declarations

Ethics approval and consent to participate

Ethics approval and consent to participate was obtained from Ethics Committee of Xiamen university and all participants.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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Writing – review & editing: Lianshui Huang, Xiaoshan Zhang, Zemao Huang, Weike Cheng.

Lianshui Huang and Weike Cheng retrieved literature, conceived of the study, and participated in its design and coordination and drafted the manuscript. Xiaoshan Zhang, Zemao Huang, Lei Wang and Wei Xie retrieved data, participated in design and drafting of themanuscript. Weike Cheng and Zhenqi Ding supervised design, coordination, and drafting of the manuscript, and revised the final edition of the manuscript for important professional content. All authors read and approved the final manuscript.

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Figure legend

Figure 1: External Axial Stress Stimulator (EASS)

Figure 2: (a) After sterilization, the incision was made in front of the anteromedial tibia. Anatomical reduction and ideal positioning for the implant was promoted before the osteotomies created in all samples. (b) A bone gap, around 1 mm, was created to establish fracture models. (c) Plaster for external fixation. (d) The rabbits were positioned on the platform and the operated limbs were fixed at the same horizon of the rapping hammer in order to obtain the stress vertical to the fracture line.

Figure 3: Fracture site in (a)the control group was substituted by only few callus, compared with (b) the experiment group with more new bone in-growth and bone callus in the eighth week postoperatively.

Figure 4: At the second week postoperatively, fracture lines were obvious in both groups. (a) control group, (e) experiment group; At the fourth week postoperatively, bone callus was formed in both groups and (f) the experiment group showed better callus formation than (b) the control group. In the sixth week postoperatively, (g) the experiment group had dense callus, the fracture line was fuzzy, and the osteotomy gap nearly disappeared, while (c) the control group had less callus and the fracture was still visible. In the eighth week postoperatively, fracture line disappeared and fracture healed well in both groups. Bone callus in (h) experiment group formed densely and more than that in (d) control group.

Figure 5: Results of hematoxylin and eosin staining at each time point (200×). In the second week, hematoma in fracture site formed, lots of inflammatory cells aggregated, and capillaries and fibroblasts proliferated in both groups. In the fourth week, primary bone callus formed in both groups, more fibrocytes were observed in the experiment group. In the sixth week, more bone trabecula structures were observed in the experiment group than the control group. In the eighth week, cartilage tissue had transformed into mature bone trabecula in both groups, while more mature bone callus was observed in the experiment group, with better mineralization and denser collagen tissue.

Figure 6: Immunohistochemistry observation of CD34 staining (200×). CD 34 staining in the experiment group showed significantly dyed than the control group at the second and fourth week, but no significant difference was observed at the sixth and eighth week.

Figure 7: Immunohistochemistry observation of VEGF staining (200×). VEGF staining in the experiment group showed significantly deeper colored than the control group at each period.

Figure 8: Immunohistochemistry observation of BMP-2 staining (200×). BMP-2 staining in the experiment group showed significantly deeper colored than the control group at each period.

Figure 9: Immunohistochemistry observation of TGF-β1 staining (200×). TGF-β1 staining showed significantly dyed in the experiment group than the control group at the second and fourth weeks, but no significant difference was found at the sixth and eight weeks.

Figures

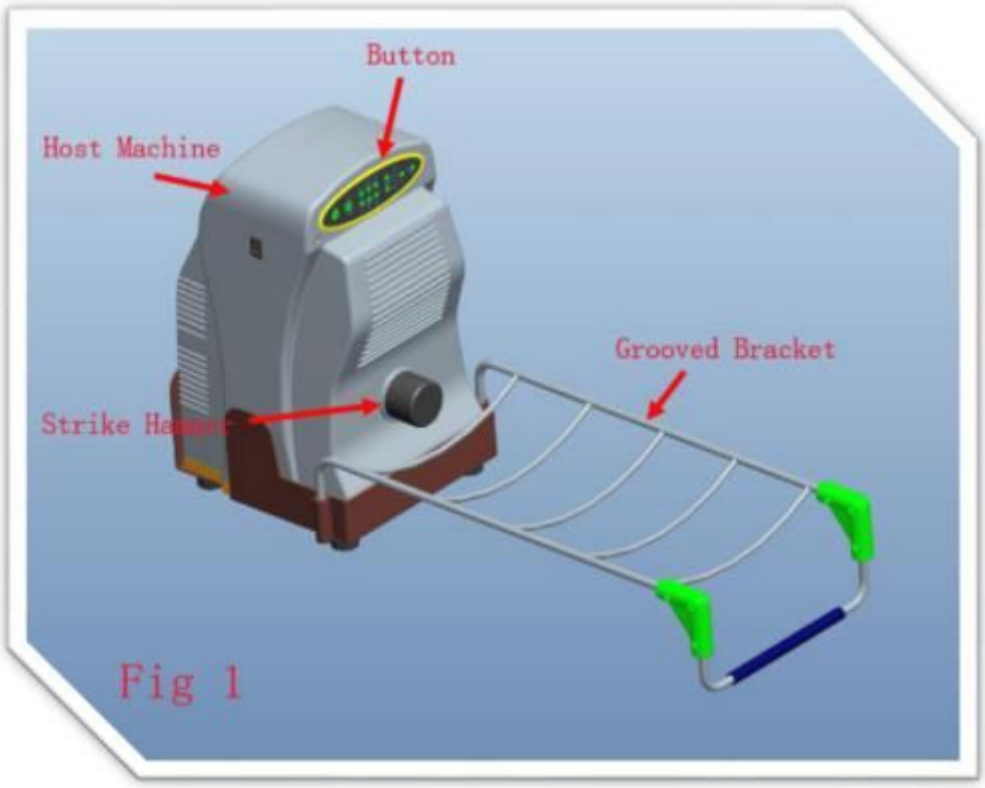


Figure 1

ExternalAxial StressStimulator(EASS)

Figure 2

(a) After sterilization, the incision was made in front of the anteromedial tibia. Anatomical reduction and ideal positioning for the implant was promoted before the osteotomies created in all samples. (b) A bone gap, around 1 mm, was created to establish fracture models. (c) Plaster for external fixation. (d) The rabbits were positioned on the platform and the operated limbs were fixed at the same horizon of the rapping hammer in ordertoobtainthestressverticaltothefractureline.

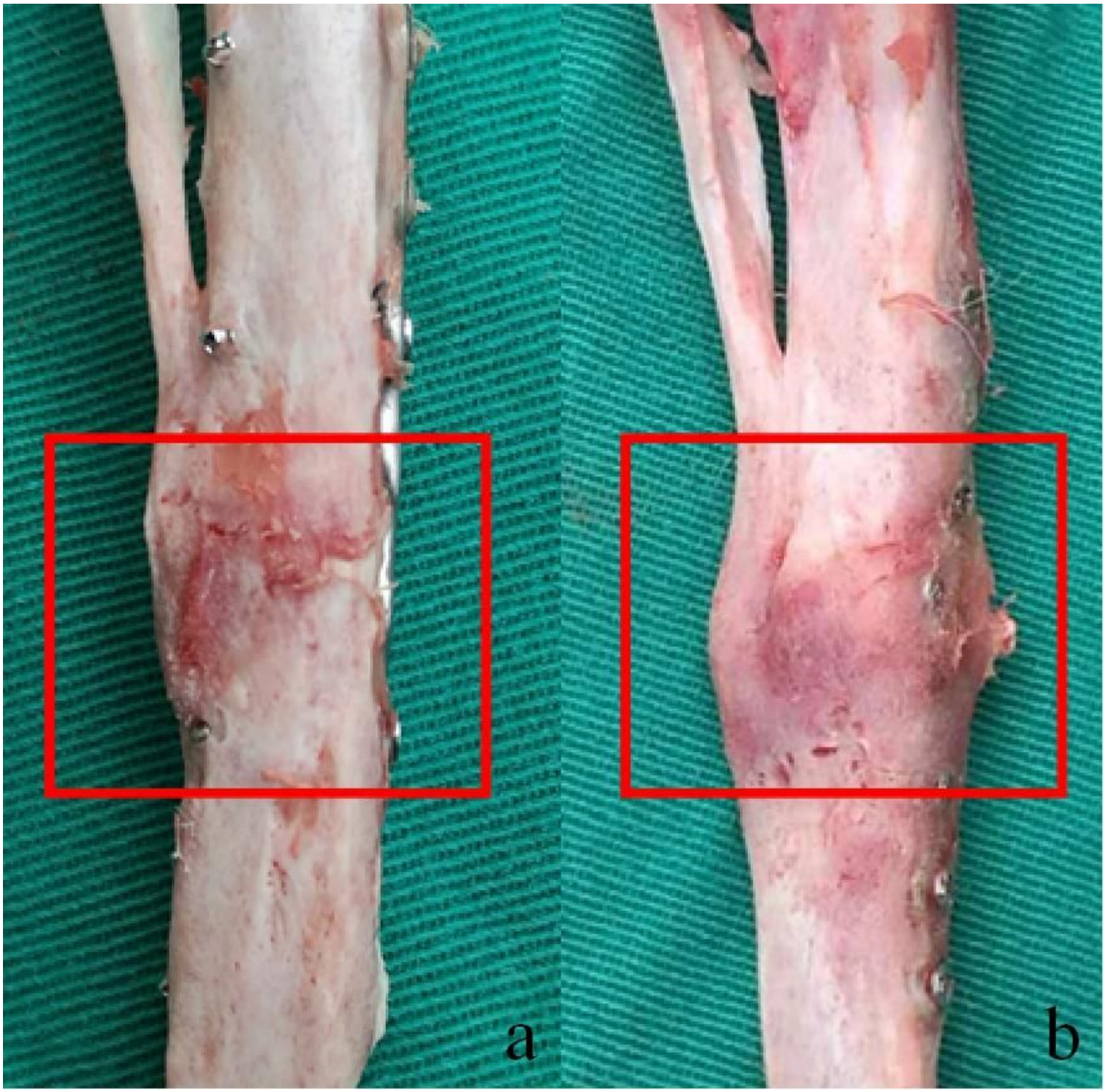
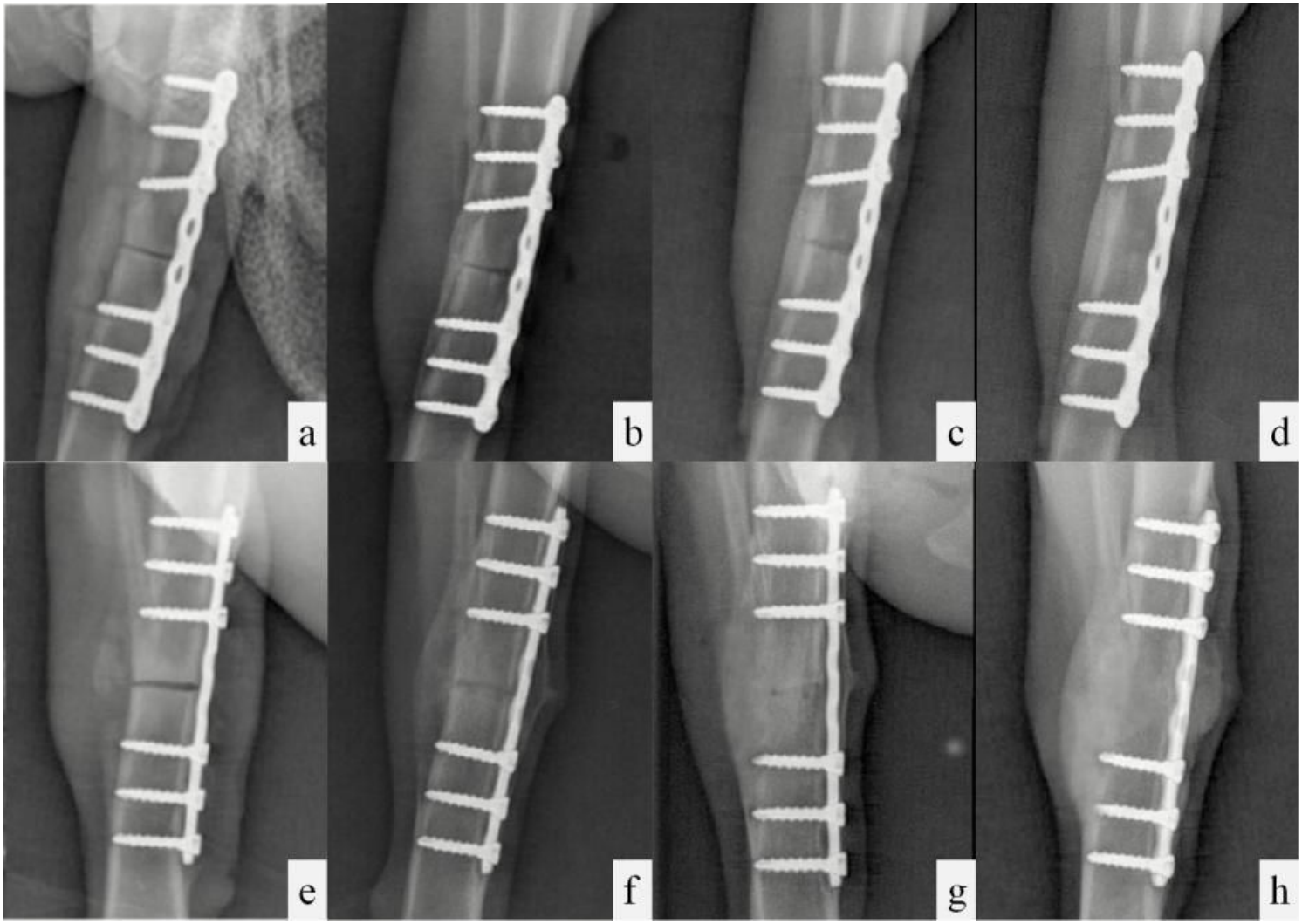


Figure 3

Fracture site in (a)the control group was substituted by only few callus, compared with (b) the experiment group with more new bone in-growth and bone callus in the eighth weekpostoperatively.



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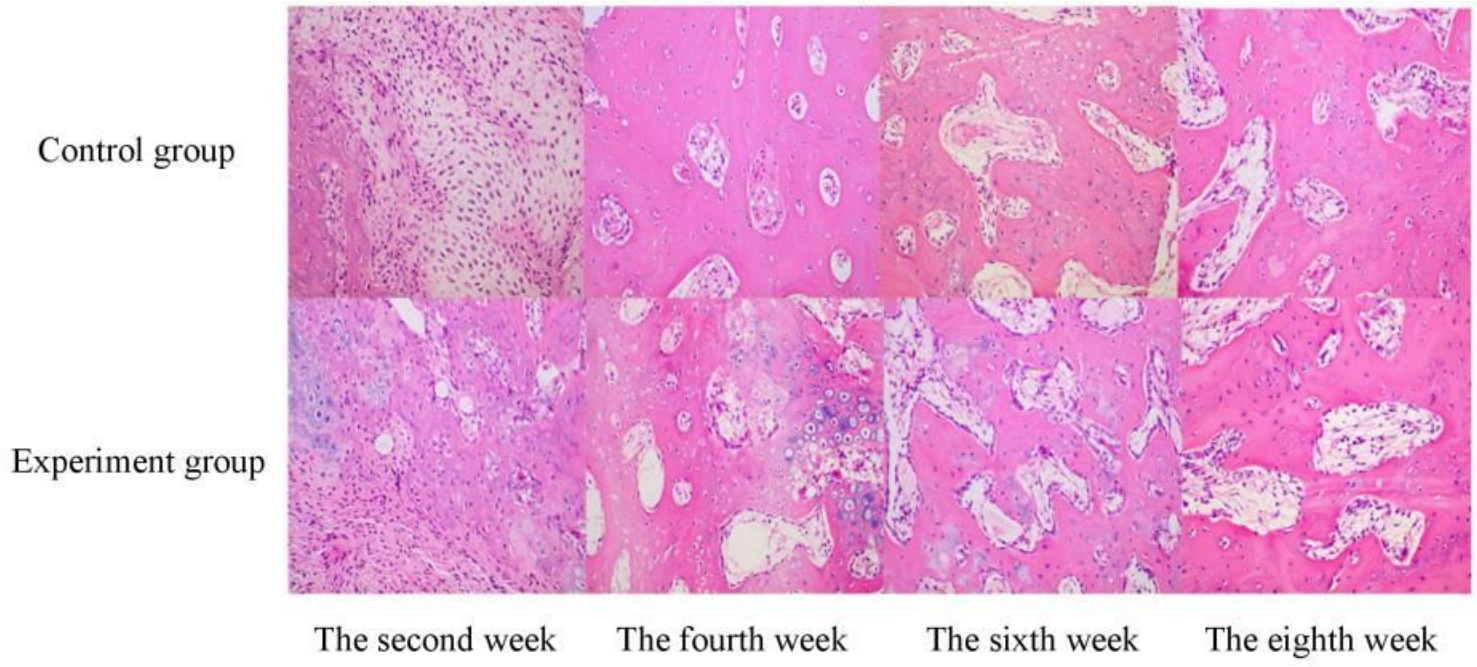

Figure 5

Results of hematoxylin and eosin staining at each time point (200×). In the second week, hematoma in fracture site formed, lots of inflammatory cells aggregated, and capillaries and fibroblasts proliferated in both groups. In the fourth week, primary bone callus formed in both groups, more fibrocytes were observed in the experiment group. In the sixth week, more bone trabecula structures were observed in the experiment group than the control group. In the eighth week, cartilage tissue had transformed into mature bone trabecula in both groups, while more mature bone callus was observed in the experiment group,withbettermineralizationanddensercollagentissue.

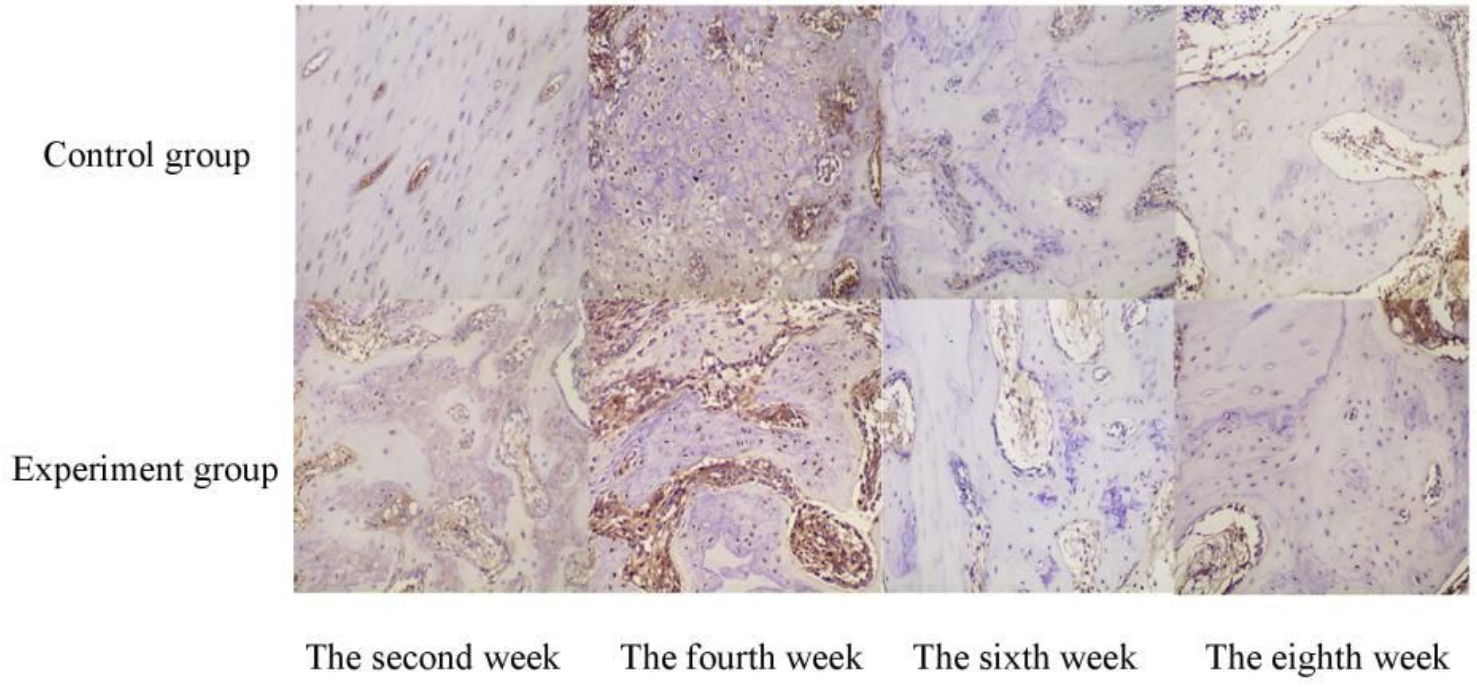

Figure 6

Immunohistochemistry observation of CD34 staining (200×). CD 34 staining in the experiment group showed significantly dyed than the control group at the second and fourth week, but no significant difference was observed at the sixth and eighth week.

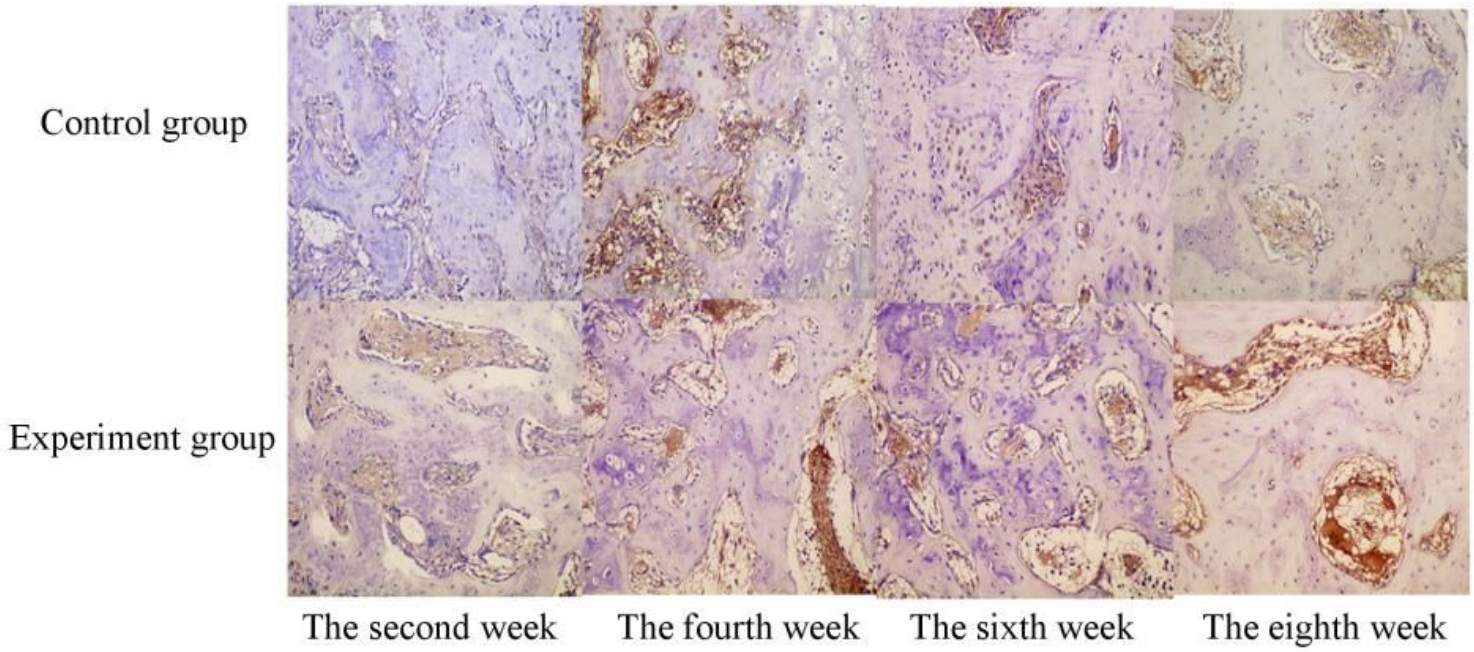

Figure 7

Immunohistochemistry observation of VEGF staining (200×). VEGF staining in the experiment group showed significantly deeper colored than the control group at each period.

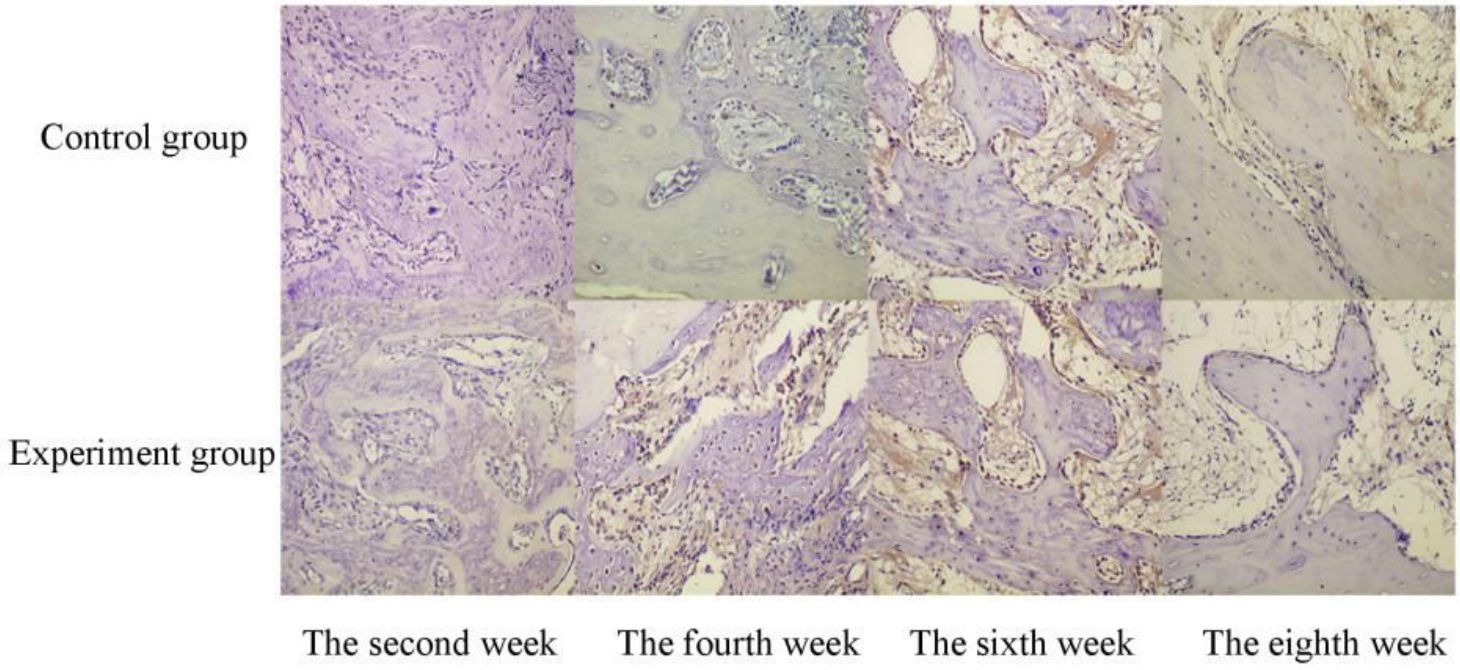

Figure 8

Immunohistochemistry observation of BMP-2 staining (200×). BMP-2 staining in the experiment group showed significantly deeper colored than the control group at each period.

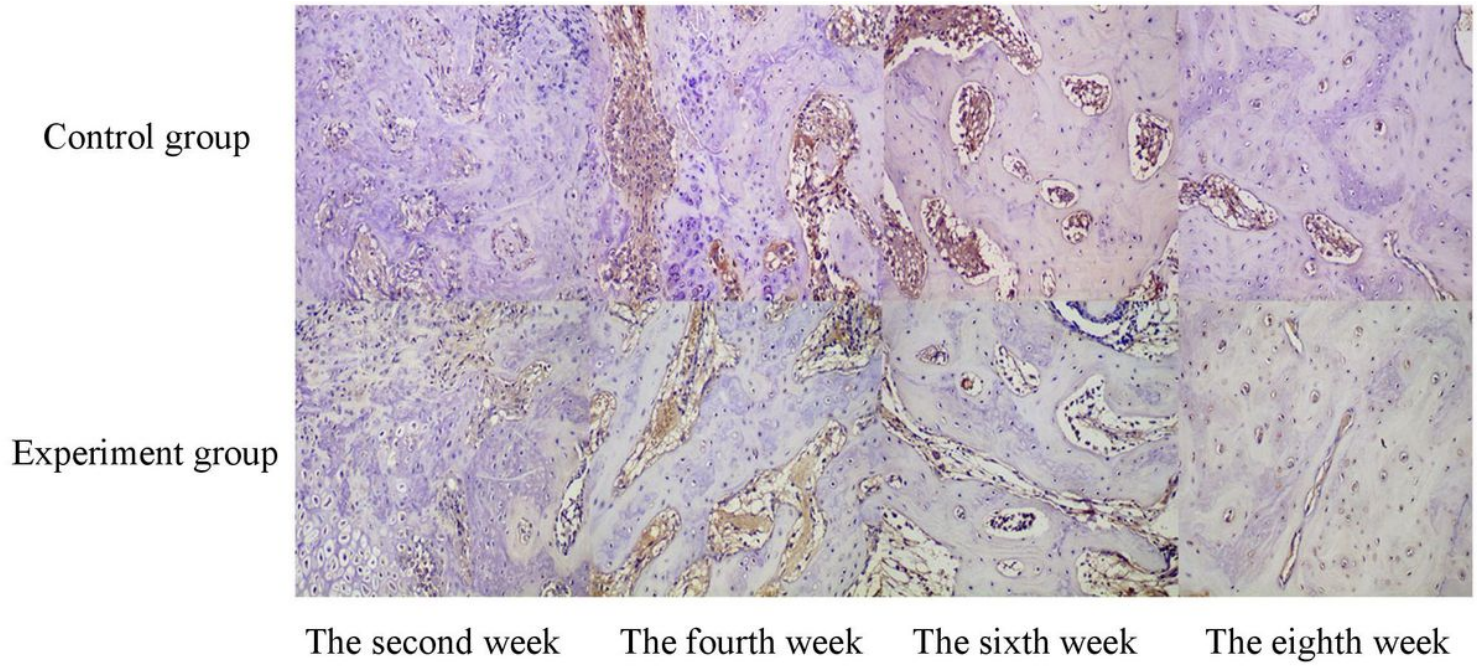

Figure 9

Immunohistochemistry observation of TGF-β1 staining (200×). TGF-β1 staining showed significantly dyed in the experiment group than the control group at the second and fourthweeks,butnosignificantdifferencewasfoundatthesixthandeightweeks.