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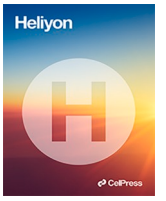

Research article

Promotion of mandibular distraction osteogenesis by parathyroid hormone via macrophage polarization induced through iNOS downregulation

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ABSTRACT

Objective: To investigate whether Parathyroid hormone (PTH) can promote mandibular distraction osteogenesis by regulating macrophage polarization and the underlying mechanisms of this phenomenon.

Methods: Forty-eight Rabbits were used to establish the mandibular distraction osteogenesis experimental model, randomly divided into 2 groups. Intermittent post-operative injections of 20 μg/kg PTH and normal saline were administered to the experimental and control groups, respectively. Regenerated new bone was examined using HE staining, osteoclast numbers were determined through tartrate-resistant acid phosphatase (TRAP) staining, and macrophage polarization markers arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS) expressions were elucidated using immunohistochemistry (IHC), the mRNA expression of CD206, CD11C, Arg1 and iNOS were detected using qPCR.

Results: The bone trabeculae in the experimental group were thicker, with a more homogeneous structure and more new osteoid than in the control group. In the area of distraction osteogenesis, the osteoclast count in the experimental group was higher than in the control group ($P < 0.05$). IHC results indicated differential expressions of Arg1 and iNOS in the experimental group compared to the control group ($P < 0.05$). Relative mRNA expressions of CD11c and iNOS were lower in the experimental group than in the control group ($P < 0.05$), whereas the expressions of CD206 and Arg1 mRNA were higher in the experimental group compared to the control group ($P < 0.05$).

Conclusion: Intermittent PTH injections increased macrophage quantity in the mandible generated by distraction osteogenesis, downregulated iNOS, upregulated Arg1, and promoted macrophage polarization from M1 to M2 phenotype, thereby promoting mandibular distraction osteogenesis.

1. Introduction

The promotion of bone regeneration in jaw distraction osteogenesis has been a key research topic over the past few decades.

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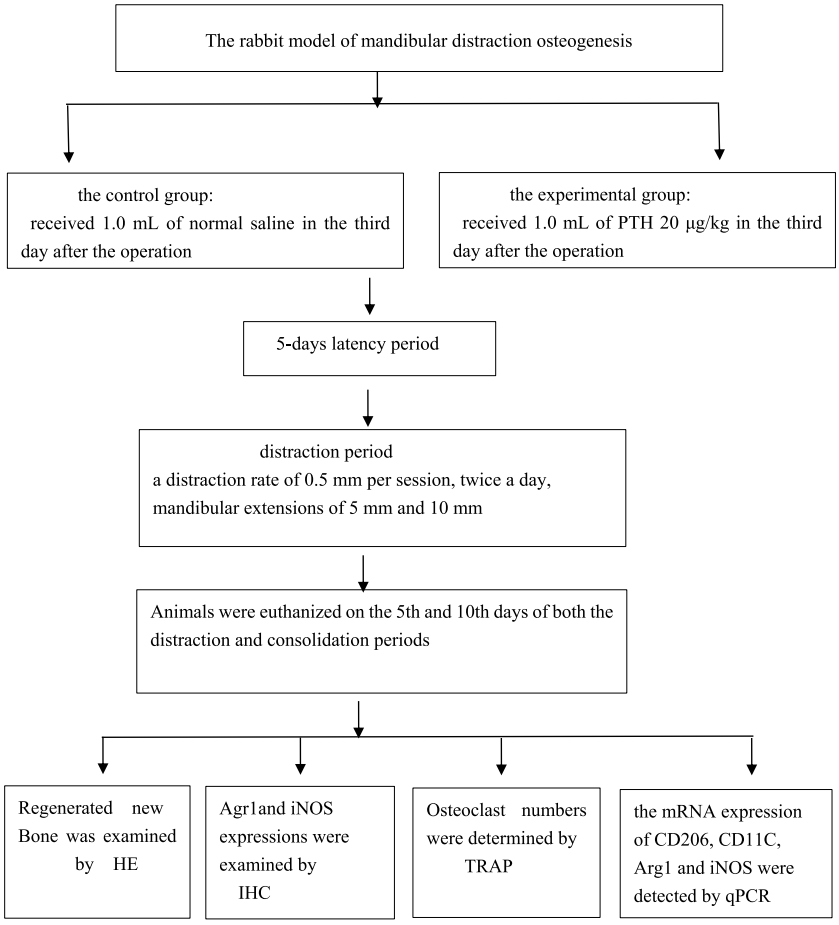
Parathyroid hormone (PTH) garnered attention for its dual regulatory effect on bone remodeling, making it a valuable tool in osteoporosis treatment and fracture healing. Despite its known benefits, the precise mechanism through which PTH regulates osteogenesis remains incompletely understood. Distraction osteogenesis involves a three-stage process: latency, distraction, and consolidation. During this process, the inflammatory environment triggered by surgical injury and the application of tensile force plays a crucial role. Various cells and cytokines become involved in stimulating bone formation and resorption activities. Neutrophils initiate the process by releasing chemokines (CCL2, CCL3, CCL4) that attract macrophages. These macrophages then polarize into different subtypes, M1 and M2, each with distinct functions in bone healing [1–3]. Bone remodeling starts with osteoclast-mediated bone resorption, forming bone resorption pits by removing old bone tissue, and osteoblasts form new bone in the bone resorption pits. Numerous studies have revealed that M1 macrophages promote osteoclastogenesis, the formation of bone-resorbing osteoclasts. This occurs through the secretion of pro-inflammatory factors like TNF-α, IL-6, and IL-1β. Conversely, M2 macrophages inhibit osteoclastogenesis by secreting anti-inflammatory factors such as IL-4 and IL-10 [4,5]. Studies have demonstrated that TNF-αknockout mice have reduced inflammatory bone resorption capacity compared with wild-type mice [6]. Gao XR et al. [7] postulated that IL-10 may inhibit osteoclast differentiation via maternally expressed gene 3 (MEG3)/STAT1/interferon regulatory factor 8 (IRF8), thereby preventing bone dissolution.

Some studies treated fractured bones in mice injected with PTH and found that PTH increased the number of bone macrophages, suggesting that PTH could also regulate the immune status of macrophages and play a promoting role in bone healing [8]. During the tissue healing process, macrophages transition from pro-inflammatory M1 phenotype to anti-inflammatory M2 phenotype, and these polarized macrophages promote tissue repair. Studies have shown that bone macrophages and inflammatory macrophages can affect the synthetic function of membrane osteoblasts, in which bone macrophages play a dominant role [9].

Recently, significant attention has been directed toward the role of osteal macrophages in PTH-induced osteosynthesis. Cho et al. [10] reported that daily injection of PTH for four weeks significantly increased the density of F4/80+bone macrophages in the subperiosteum and bone marrow of reconstructed tibia in mice, indicating that osteal macrophages can promote PTH-mediated osteosynthesis. Moreover, PTH can upregulate chemokines, recruiting macrophages and transforming their phenotype and

Table 1
Experimental flow chart.



function, thereby promoting osteogenesis [11]. Macrophage polarization enhances the removal of apoptotic MSCs and necrotic tissues, making PTH more effective in promoting bone synthesis [12]. In our previous studies [13–15], PTH was utilized to promote the regeneration of mandibular defects and mandibular distraction osteogenesis, and accelerate orthodontic tooth movement. The results demonstrated a significantly lower degree of inflammatory cell infiltration in the new bone tissues of the experimental group compared to the control group two weeks after PTH application. Additionally, TRAP staining revealed an increased quantity of osteoclasts in the mandibular fracture healing site and in the periodontal tissues on the compression side of the orthodontically treated teeth.

The actions of macrophages in distraction osteogenesis are as yet unclear. Based on the findings that PTH leads to an increased quantity of osteoblasts and macrophages, as well as a transformed macrophage phenotype during bone regeneration and remodeling, we designed this study to investigate whether parathyroid hormone can polarize macrophages to promote mandibular distraction osteogenesis and to explore the preliminary mechanisms through which PTH regulates macrophage polarization.

2. Materials and methods

2.1. Experimental animals and grouping

Forty-eight 6-month-old specific pathogen-free New Zealand white Rabbits (equal distribution of males and females), weighing 2.5 ± 0.5 kg were used in this study. The Rabbits were housed individually in a specific pathogen–free animal facility at a temperature of 22 ± 2 °C, a humidity of 50 ± 10 %, ventilation ≥14 times/h, and on a 12-h dark-light cycle. Each Rabbit was provided with 350 g standard pellets daily, along with free access to tap water. The Rabbits were randomly and equally divided into the experimental group and the control group. The experimental group received 1.0 mL of PTH 20 μg/kg (Tocris, Bristol, UK) injections every other day from the third day after the operation, while the control group received 1.0 mL of normal saline. Six animals from each group were sacrificed on the 5th and 10th day of the distraction period and the 5th and 10th day of the consolidation phase. This study was approved by the Animal Experiment Ethics Committee of Guizhou Medical University (the approval number:2000889). Experimental flow chart is shown in Table 1.

2.2. Establishment of the rabbit model of mandibular distraction osteogenesis

General anesthesia was induced using 3 % sodium pentobarbital administered through the auricular vein. After anesthesia, a 2.5 cm incision was made along the lower margin of the Rabbit mandible. The skin, subcutaneous tissue, muscle, and periosteum were dissected to expose the lateral aspect of the mandible. The mandibular body osteotomy was performed distal to the anterior teeth and mesial to the first molar, and a distractor (Ningbo Cibei Medical Treatment Appliance Co., Ltd., Zhejiang, People's Republic of China) was placed on the surface of the bone parallel to the lower edge of the mandible and fixed. The surgical area was washed with saline, and each layer of the incision was tightly sutured. After a 5-day latency period, the mandible was extended using a distraction rate of 0.5 mm per session, twice a day, totaling 1.0 mm per day. This distraction was conducted for periods of 5 and 10 days, resulting in mandibular extensions of 5 mm and 10 mm, respectively. Animals were euthanized on the 5th and 10th days of both the distraction and consolidation periods, and specimens were collected for research purposes. To prevent wound infections, all animals received daily intramuscular injections of 800,000 units of penicillin for three consecutive days post-surgery.

2.3. Acquisition of specimens of new bone tissue and histomorphologic observations

Six animals from each group were sacrificed on the 5th and 10th day of the distraction period, as well as the 5th and 10th day of the consolidation phase. Surgical-side mandible specimens were collected and investigated for gross morphology. Half of the specimens were fixed in 4 % paraformaldehyde for 24 h, decalcified in 10 % neutral EDTA for four weeks, and then embedded in paraffin. Continuous 5-μm histological sections were obtained from the distraction osteogenesis zone for HE staining. A TRAP kit (Beijing Solebio Biotechnology Co., Ltd.) was used for osteoclast staining according to the instructions. Osteoclast counts conducted in three randomly selected fields under 20× magnification. The other half specimens were preserved in RNALater solution for quantitative real-time PCR detection.

2.4. Detection of Agr1 and iNOS expression in the new bone tissues by IHC

The expression intensity of Arg1 and iNOS in paraffin sections was detected using the IHC experiment protocol (Beijing Biosynthesis Biotechnology Co., Ltd, Beijing, China). Image pro-plus 6.0 software was used to select at least three spots with the most positive cells in each section under a 200× field of view to measure the average integrated option density (IOD) value, determining the positive rate at different stages and between groups.

2.5. Arg1and iNOS mRNA expression analysis by qPCR

RNA later-preserved mandibular distraction osteogenesis zone samples were used for detecting Arg1, iNOS, CD206, and CD11c mRNA expression. Total RNA was extracted from bone tissue samples using Trizol reagent, and its concentration was measured using a nucleic acid quantifier. Reverse transcription was performed with the RevertAid First Strand cDNA Synthesis Kit. Real-time PCR was carried out using the PrimeScript RT Master Mix (Perpect Real Time) kit (TakaRa, Japan). The reaction conditions were as follows:

predenaturation at 95 °C for 30 s; and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Primer sequences are provided in Table 2. Relative quantitative 2-ΔΔCT was used to assess the expression of target genes with housekeeping genes as the control group.

2.6. Statistical analysis

Data were analyzed using SPSS 25.0 software (IBM Corp, Armonk, NY). All the measurement data followed the normal distribution and were expressed as the mean ± standard deviation ($\bar{x} \pm s$). Differences between experimental and control groups were analyzed using the *t*-test, the t' test if variance was not uniform. $P < 0.05$ was considered statistically significant, and histograms were drawn using Graphpad Prism.

3. Results

The surgery proceeded smoothly for all experimental animals. During the first three days post-operation, the animals ate more slowly and consumed less food than usual. By the fourth postoperative day, their eating habits had nearly returned to normal. The animals demonstrated good growth and steady weight gain. The skin incisions in the surgical area healed excellently, and no animals died throughout the entire experimental period. The new bone formation area of distraction osteogenesis is an inflammatory microenvironment in the early stage, dominated by M1 macrophages, which play a role in the stage. In our study, we found that the expressions of iNOS and CD11c (M1 macrophage markers) in the experimental group were lower than those in the control group. Over time, the M1 phenotype changes to M2. The quantity of osteoclasts/macrophages gradually decreased, the expressions of Arg1 and CD206 (M2 macrophage markers) in the experimental group were higher than those in the control group during the distraction period, indicating that osteogenesis was in the anabolism stage.

3.1. Morphological and imaging observation of distracted tissue

In this study, a Rabbit model of mandibular distraction osteogenesis was successfully established. Gross specimen investigation revealed enhanced bone generation in the distraction osteogenesis zone of the experimental group Fig. 1B and D compared to the control group Fig. 1A and C. X-ray examination revealed enhanced bone generation in the distraction osteogenesis zone of the experimental group compared to the control group on the 5th and 10th days of the distraction period (Fig. 2A and B) as well as the 5th and 10th days of the consolidation phase (Fig. 2C and D). Tissue morphology investigations indicated that the experimental group exhibited higher thickness of bone trabecula and increased osteoid content in the distraction osteogenesis zone in contrast to the control group.

3.2. Osteoclasts count

In TRAP staining, osteoclasts were identified by their red staining and exhibited a morphology extending in a direction consistent with the distraction, surrounding the new bone trabeculae. Osteoclast counts, we randomly selected three fields of view in each section and counted the number of osteoblasts by image at magnified ×20, revealed a gradual increase during the distraction period (Figs. 3–1A,1C), followed by a decrease in the consolidation phase in the control group (Figs. 3–2A,2C). The experimental group exhibited a significantly higher number of osteoclasts than the control group, with the highest counts recorded on the 5th day of the distraction period (Table 3,Figs. 3–1B). The difference was statistically significant on the 5th day of the distraction period and the 5th day of the consolidation phase ($P < 0.05$).

The difference was statistically significant on the 5th day of the distraction period and the 5th day of the consolidation phase ($P < 0.05$).

Table 2
Primer sequence.

Gene	Primer Sequence	
CD11c	Forward primer	AGGTCTGTGTTGAGTGTCGGG
	Reverse primer	GGTCAAGGGCAAGGTCAAAGG
CD206	Forward primer	TCAGTTCCAGTGCCGTCCAA
	Reverse primer	GGAAGGCACCCCCAAGCATA
Arg1	Forward primer	AACACTCCACTGACAACCACA
	Reverse primer	AGGGTCTACATCTCTCAAGCCA
iNOS	Forward primer	GCACGCCAAGAATGTGATGA
	Reverse primer	CACTACTCGTTCCAAGATGCCT
GAPDH	Forward primer	TCTGGCAAAGTGGATGTTGT
	Reverse primer	GTGGGTGGAATCATACTGGA

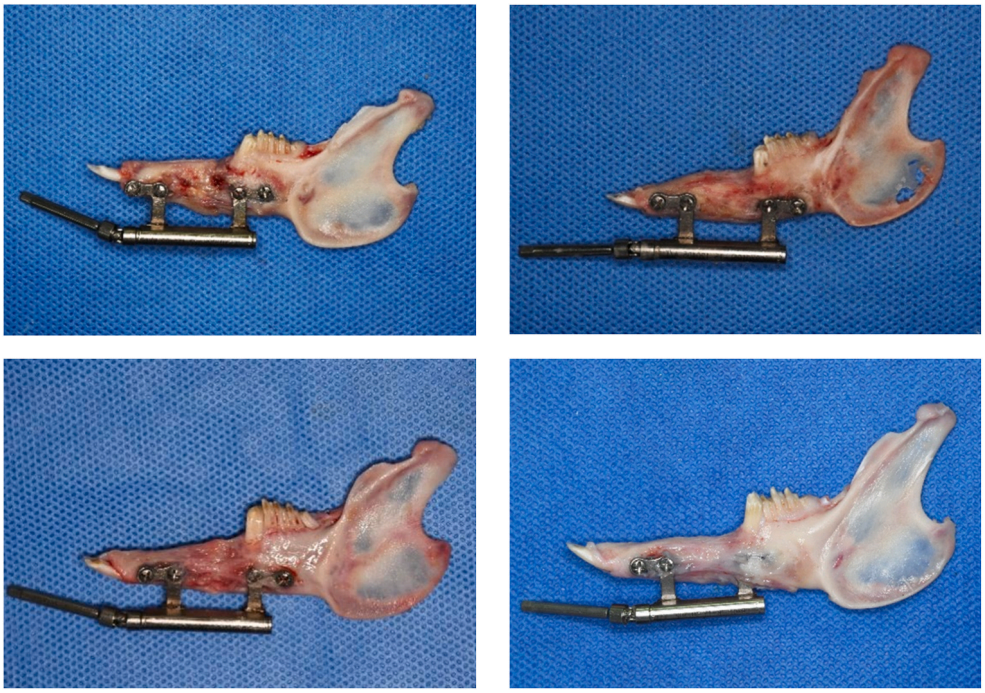


Fig. 1. Gross specimen investigation revealed enhanced bone generation in the distraction osteogenesis zone of the experimental group 1B and 1D compared to the control group 1A and 1C. 1D:The distraction gap featured new bone tissue, the surface of the distractor is covered with a large callus.
1A: 10th day of the distraction period, control group.
1B: 10th day of the distraction period, experimental group.
1C: 10th day of the consolidation phase, control group.
1D: 10th day of the consolidation phase, experimental group.

3.3. Immunohistochemistry (IHC)

3.3.1. IHC results of iNOS

iNOS, primarily expressed in the cytoplasm of macrophages, appeared brown-yellow. The average integrated optical density (IOD/Area) of iNOS in the distraction osteogenesis zone indicated that its expression intensity peaked on the 5th day of the distraction period (Figs. 4–1A,1B), gradually decreasing afterward. iNOS expression in the control group was significantly higher than in the experimental group on the 5th day, the 10th day of the distraction period (Figs. 4–1A,1C,1B, 1D), and the 5th day of the consolidation phase (Table 4, Figs. 4–2A,2B). The difference was statistically significant ($P < 0.05$).

In the distraction osteogenesis zone indicated that its expression intensity peaked on the 5th day of the distraction period, gradually decreasing afterward, the difference was statistically significant ($P < 0.05$).

3.3.2. IHC results of Arg1

Arg1 was mainly expressed in the cytoplasm of macrophages. The IHC results for Arg 1 mean integrated optical density value (IOD/Area) in the newly formed bone tissue indicated increased staining intensity in both the control and experimental groups during the distraction phase. This was followed by a gradual decline in the consolidation phase. The experimental group exhibited higher Arg1 expression than the control group on the 5th and 10th days of the distraction period and the 10th day of the consolidation phase (Figs. 5–1B,1D,1A,1C; 5-2D, 2C), with statistically significant differences ($P < 0.05$) (Table 5).

In the newly formed bone tissue indicated increased staining intensity in both the control and experimental groups during the distraction phase. This was followed by a gradual decline in the consolidation phase.

3.4. mRNA expression of macrophage-specific markers in distraction osteogenesis zone by qPCR

3.4.1. Relative mRNA expression of iNOS mRNA in the control group and the experimental group

The relative expression of iNOS mRNA peaked on the 5th day of the distraction period and then decreased. iNOS mRNA expression in the experimental group was consistently lower than that in the control group at each stage, with the difference being statistically significant ($P < 0.05$) (Table 6).

iNOS mRNA expression in the PTH group was consistently lower than that in the control group at each stage, with the difference

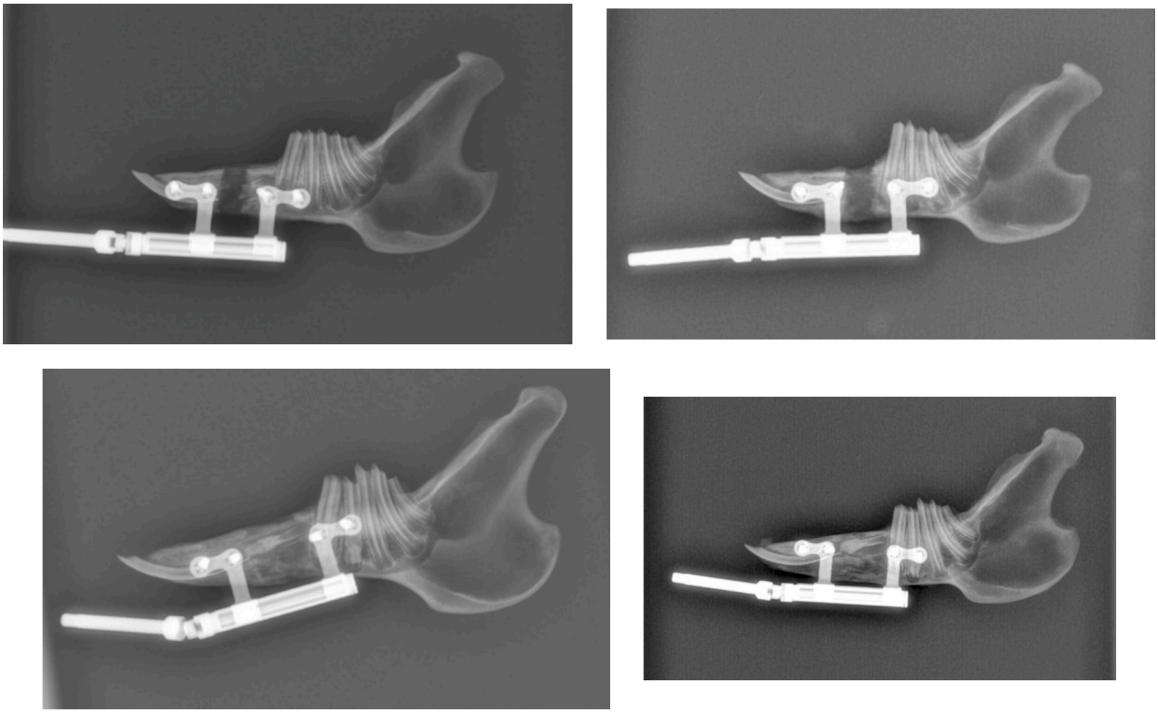


Fig. 2. Images of radiographic results: On days 5 (2A, 2B), and 10 (2C, 2D), gradual increases in bone density in the defect areas were evident for the control and experimental groups, there was a distinguishable difference between the 2 groups with respect to this bone density, and the increase was even more pronounced in the experimental group.
2A: 5th day of the distraction period, control group.
2B: 5th day of the distraction period, experimental group.
2C: 10th day of the consolidation phase, control group.
2D: 10th day of the consolidation phase, experimental group.

being statistically significant ($P < 0.05$).

3.4.2. Relative mRNA expression of Arg1 in the control group and the experimental group

The relative expression of Arg1 mRNA increased during the distraction period, reaching its peak on the 10th day of the distraction period and decreasing afterward. Arg1 mRNA expression in the experimental group was higher than that in the control group on the 5th and 10th day of the distraction period and the 5th day of the consolidation phase. The difference was statistically significant ($P < 0.05$) (Table 7).

The relative expression of Arg1 mRNA increased during the distraction period, reaching its peak on the 10th day of the distraction period and decreasing afterward, the difference was statistically significant ($P < 0.05$).

3.4.3. Relative expression of CD11c mRNA in the control group and the experimental group

CD11c mRNA expression peaked on the 5th day of the distraction period and then decreased. CD11c mRNA expression in the control group was significantly higher than in the experimental group on the 5th day, the 10th day of the distraction period, and the 5th day of the consolidation phase. The difference was statistically significant ($P < 0.05$ (Table 8).

CD11c mRNA expression in the control group was significantly higher than in the PTH group on the 5th day, the 10th day of the distraction period, and the 5th day of the consolidation phase, the difference was statistically significant ($P < 0.05$).

3.4.4. Relative expression of CD206 mRNA in the control group and the experimental group

CD206 mRNA expression in the control group increased during the stretch phase, reaching its peak on the 10th day of the distraction period and decreasing during the consolidation phase. In contrast, the experimental group exhibited peak CD206 mRNA expression on the 5th day of the distraction period, decreasing afterward. CD206 mRNA expression in the experimental group was significantly higher than that in the control group on the 5th day of the distraction period, the 5th day of the consolidation phase, and the 10th day, with the difference being statistically significant ($P < 0.05$) (Table 9).

CD206 mRNA expression in the PTH group was significantly higher than that in the control group on the 5th day of the distraction period, the 5th day of the consolidation phase, and the 10th day, with the difference being statistically significant ($P < 0.05$).

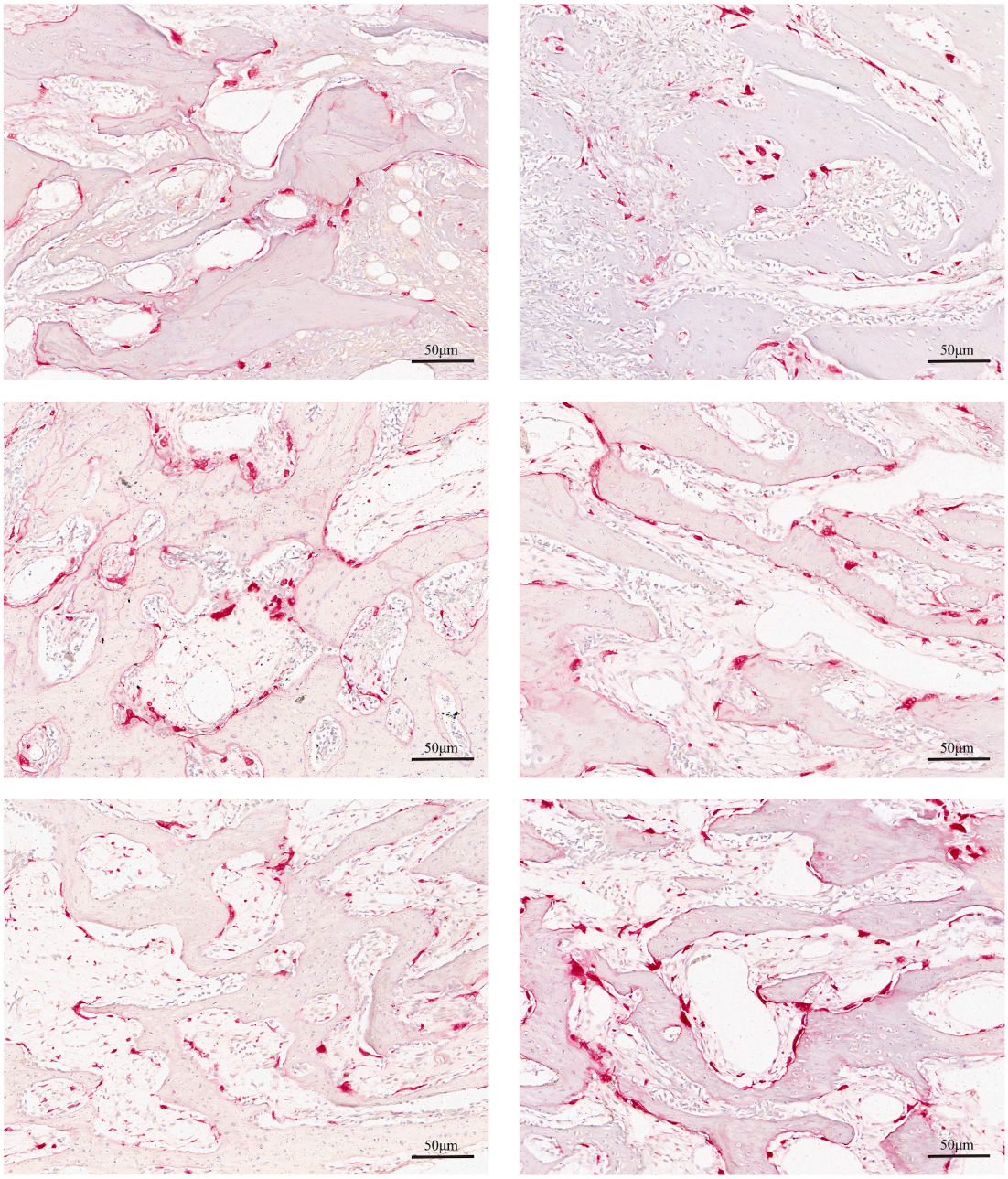


Fig. 3. Osteoclasts in the distraction area after operation in each group (phosphatase staining; magnification, × 20) Osteoclasts were identified by their red staining and exhibited a morphology extending in a direction consistent with the distraction, surrounding the new bone trabeculae. Osteoclast counts, revealed a gradual increase during the distraction period, followed by a decrease in the consolidation phase.
3-1A: 5th day of the distraction period, control group.
3-1B: 5th day of the distraction period, experimental group.
3-1C: 10th day of the distraction period, control group.
3-1D: 10th day of the distraction period, experimental group.
3-2A: 5th day of the consolidation phase, control group.
3-2B: 5th day of the consolidation phase, experimental group.
3-2C: 10th day of the consolidation phase, control group.
3-2D: 10th day of the consolidation phase, experimental group.

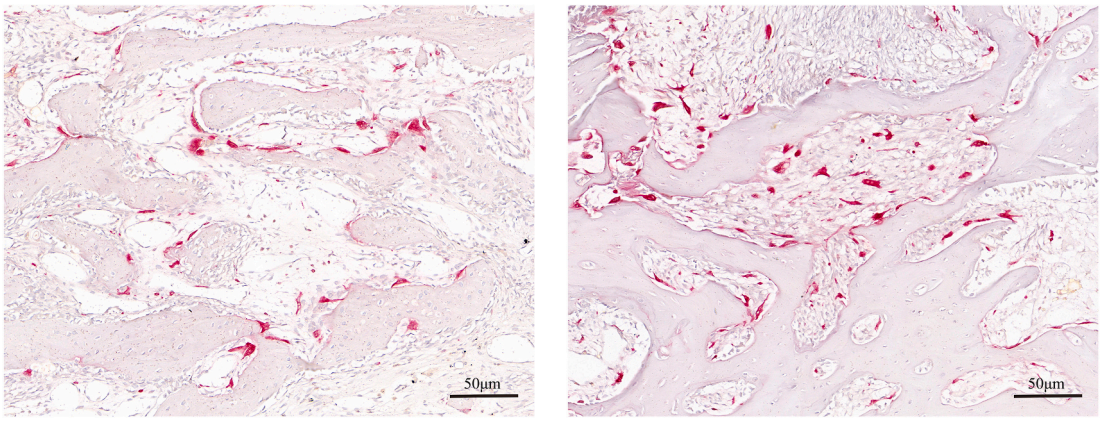


Fig. 3. (*continued*).

Table 3
Number of osteoclasts in the distraction area after operation ($\bar{x} \pm s$).

Time	Control group	Experimental group	F	*P*
Day 5 of distraction	41.444 ± 11.215	57.667 ± 8.573	11.885	0.003
Day 10 of distraction	50.889 ± 19.662	56.556 ± 5.812	0.687	0.419
Day 5 of consolidation	36.222 ± 17.598	50.000 ± 8.139	4.544	0.049
Day 10 of consolidation	28.778 ± 8.028	30.667 ± 9.862	0.199	0.662

4. Discussion

During the early stages of distraction osteogenesis, the osteogenesis process takes place within an inflammatory microenvironment involving various inflammatory cells and cytokines. Macrophages, as significant contributors to inflammatory factors, play an important role in tissue repair and regeneration. Previous studies have confirmed a close relationship between macrophages and early repair during the fracture healing process [16,17]. Osteoclasts, the only known tissue-resident macrophages with bone resorption capabilities, are multinucleated cells formed by the fusion of osteoclast precursors through the activation of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) [18,19]. Under the stimulation of RANKL, M2 macrophages can transform into osteoclasts [20]. Studies have also indicated that when different subtypes of macrophages are induced to differentiate into osteoclasts, M2 is more likely to generate osteoclasts [21,22]. Certain characteristics of M2 expressed by osteoclasts, such as the high expression of M2-specific markers CD163, CD206, and IL-10, suggest that osteoclasts may be formed by M2 fusion [23,24]. Nie et al. [25] reported that more M2 macrophages and osteoclasts appeared in the early stages of bone-inducing material implantation. *In vitro* experiments further confirmed that bone-inducing materials were more likely to form osteoclasts after promoting M2 polarization. It has been demonstrated that PTH can recruit macrophages [10] and participate in the bone repair process. PTH affects M-CSF production, potentially leading to increased macrophages and pro-anabolic effects on bone. Tang et al. [26]. reported that intermittent administration of different low doses of parathyroid hormone can further promote the healing process after mandibular ramus osteotomy. PTH can upregulate the expression of osteoprotegerin and reduce expression of RANKL, thus promoting new bone formation. *Bakr* et al. [24]. demonstrated that a single PTH injection increases osteoclastogenesis by the second week of the remodeling cycle in a stress fracture in vivo.

PTH has been used clinically to stimulate bone formation by enhancing the activities of osteoblasts and osteoclasts in a coordinated manner, although the precise underlying mechanisms remain unclear. In this study, an animal model of mandibular distraction osteogenesis was established to investigate the effects of intermittent PTH injections on macrophage polarization during distraction osteogenesis. The results from gross specimen analysis, imaging, and histomorphological studies indicated superior osteogenesis in the experimental group compared to the control group. TRAP staining revealed a higher quantity of osteoclasts in the experimental group, peaking on the 5th day of the distraction period, gradually decreasing afterward. This suggests that intermittent injection of PTH during distraction osteogenesis can increase the number of osteoclasts/macrophages and promote the bone remodeling. During the consolidation phase of distraction osteogenesis, the quantity of osteoclasts/macrophages gradually decreased, indicating that osteogenesis was in the anabolism stage.

The functional role of macrophages was initially established in supporting the maintenance of HSC niches and stimulating intramembranous bone formation in fracture sites. Macrophages have been classified into two main types: pro-inflammatory macrophages, known as M1 macrophages, characterized by CD11c and iNOS as the main secretory markers, and anti-inflammatory macrophages, termed M2 macrophages, identified by CD206 as the marker and Arg1 as the secretory marker. Arg1, secreted by M2 macrophages, competitively binds to the substrate arginine with iNOS, producing different metabolites and playing an important role in macrophage function. Studies [27]have demonstrated that iNOS and Arg1 play a key role in macrophage polarization, with

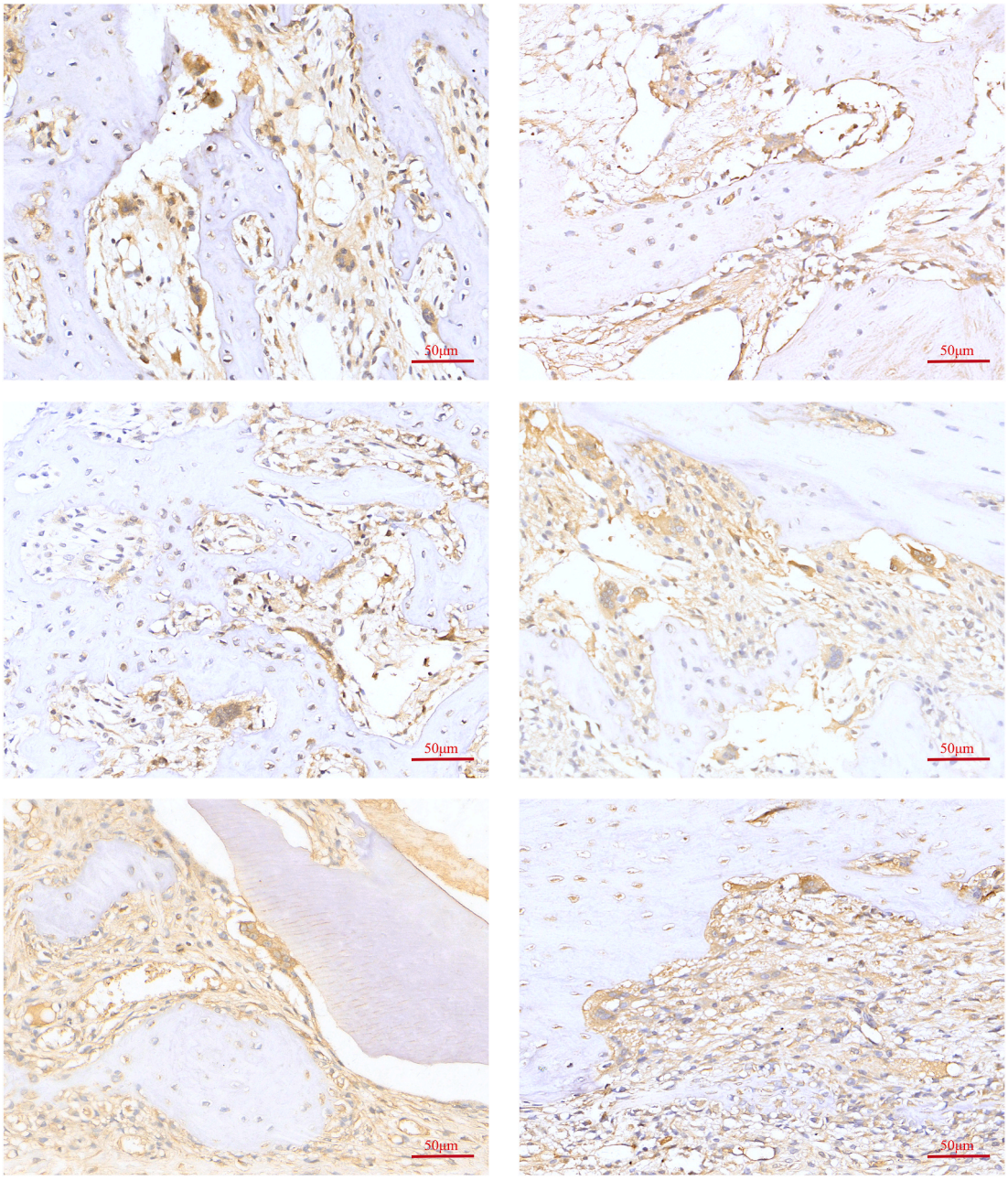


Fig. 4. The expression of iNOS in the distraction area in each group (Immunohistochemistry; magnification, × 40).
iNOS, primarily expressed in the cytoplasm of macrophages, appeared brown-yellow. On the 5th day of the distraction period, the number of macrophages with positive staining in the distraction area was large, with at least two cell nuclei in the cell, and some were spindle-shaped. On the 10th day of the distraction period, positive stained macrophagy multinucleated cells are located at the edge of the trabecular bone.
4-1A: 5th day of the distraction period control, group.
4-1B: 5th day of the distraction period, experimental group.
4-1C: 10th day of the distraction period, control group.
4-1D: 10th day of the distraction period, experimental group.
4-2A: 5th day of the consolidation phase, control group.
4-2B: 5th day of the consolidation phase, experimental group.
4-2C: 10th day of the consolidation phase, control group.
4-2D: 10th day of the consolidation phase, experimental group.

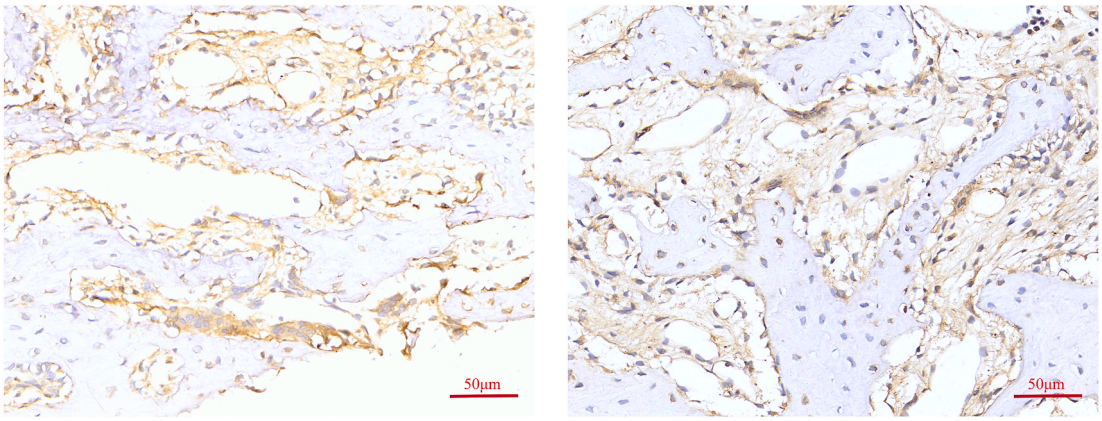


Fig. 4. (*continued*).

Table 4
The expression of iNOS in the distraction area in each group (IOD/Area $\bar{x} \pm s$).

Time	Control group	Experimental group	F	*P*
Day 5 of distraction	0.163 ± 0.040	0.122 ± 0.041	4.573	0.048
Day 10 of distraction	0.135 ± 0.043	0.089 ± 0.041	6.227	0.024
Day 5 of consolidation	0.124 ± 0.038	0.076 ± 0.045	5.049	0.039
Day 10 of consolidation	0.108 ± 0.020	0.075 ± 0.053	2.931	0.106

increased iNOS expression in M1 macrophages promoting nitric oxide (NO) production through a series of biochemical activities. NO can promote aerobic glycolysis of macrophages by inhibiting the tricarboxylic acid cycle, thereby promoting M1 polarization of macrophages. M2 macrophages, characterized by high Arg1 expression, which can compete with iNOS for the same substrate, inhibit NO-mediated inflammatory pathways, facilitating M2 macrophage generation. Indeed, the expressions of Arg1 and CD206 (M2 macrophage markers) in the experimental group were higher than those in the control group during the distraction period, while the expressions of iNOS and CD11c (M1 macrophage markers) in the experimental group were lower than those in the control group. This suggests that PTH may up-regulate the expression of Arg1 and down-regulate the expression of iNOS, promoting the polarization of macrophages from M1 to M2 phenotype and facilitating osteogenesis in the distraction osteogenesis zone. However, additional research is needed to explore the exact role of M2 macrophages and other macrophage phenotypes in the distraction osteogenesis healing cascade enhanced by PTH administration.

5. Conclusion

Intermittent injection of PTH increases the number of osteoclasts/macrophages, downregulates the expression of iNOS, and promotes macrophage polarization from M1 to M2 phenotype, thereby promoting osteogenesis in the distraction osteogenesis zone. However, additional research is needed to explore the exact role of M2 macrophages and other macrophage phenotypes in the distraction osteogenesis enhanced by PTH administration.

Ethics statement

The experimental protocol was reviewed and approved by the Animal Experimental Ethics Committee of Guizhou Medical University (the approval number:2000889).

Data availability

All data accessed and analyzed in this study are available in the article.

Funding

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CRediT authorship contribution statement

Dong-xiang Wang: Writing – original draft, Formal analysis, Data curation. **Zhi-shan Yang:** Methodology, Data curation. **Du-**

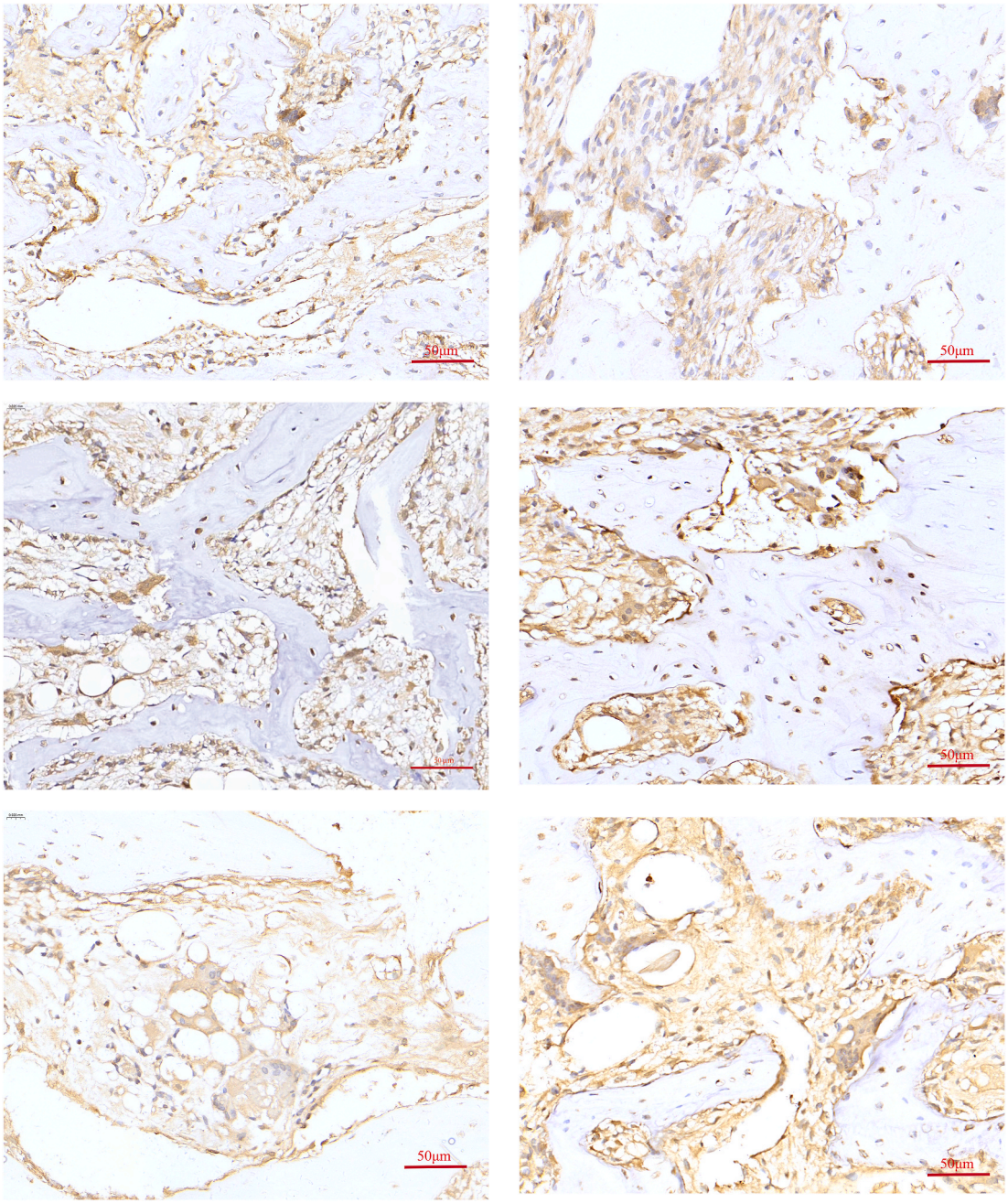

Fig. 5. The expression of Arg1 in the distraction area in each group (Immunohistochemistry; magnification, × 40).
Arg1, mostly brown-yellow, was mainly expressed in the cytoplasm of macrophages. On the 5th day of the distraction period, positive stained macrophages in the distraction area were small in number and smaller in morphology in each group. On the 10th day of the distraction period, the positive-stained macrophages had a larger shape.
5-1A: 5th day of the distraction period, control group.
5-1B: 5th day of the distraction period, experimental group.
5-1C: 10th day of the distraction period, control group.
5-1D: 10th day of the distraction period, experimental group.
5-2A: 5th day of the consolidation phase, control group.
5-2B: 5th day of the consolidation phase, experimental group.
5-2C: 10th day of the consolidation phase, control group.
5-2D: 10th day of the consolidation phase, experimental group.

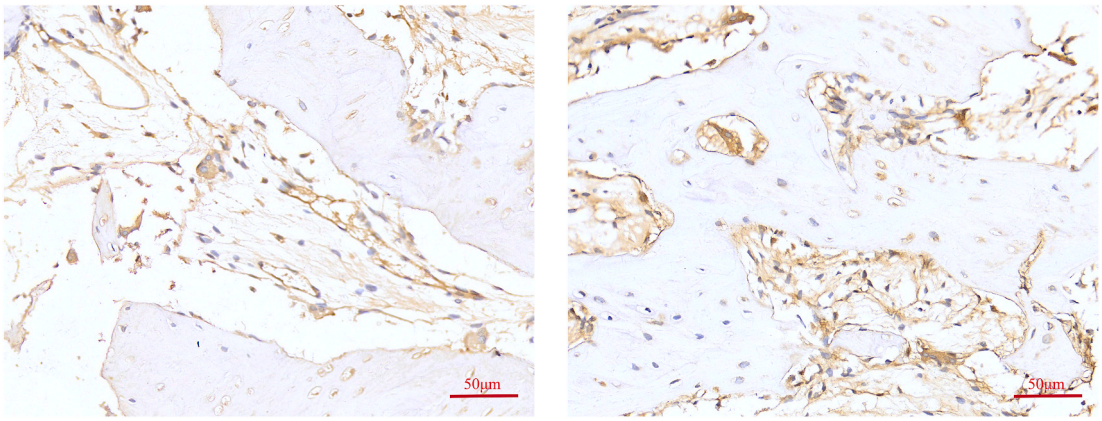

Fig. 5. (*continued*).

Table 5
The expression of Arg1 in the distraction area in each group (IOD/Area, $\bar{x} \pm s$).

Time	Control group	Experimental group	F	*P*
Day 5 of distraction	0.154 ± 0.040	0.192 ± 0.031	5.131	0.038
Day 10 of distraction	0.230 ± 0.036	0.272 ± 0.047	4.521	0.049
Day 5 of consolidation	0.168 ± 0.043	0.191 ± 0.085	0.476	0.500
Day 10 of consolidation	0.148 ± 0.011	0.182 ± 0.042	4.637	0.047

Table 6
Comparison of the relative mRNA expression of iNOS in the distraction area at different periods ($\bar{x} \pm s$).

Time	Control group	Experimental group	F	*P*
Day 5 of distraction	1.230 ± 0.051	0.474 ± 0.181	48.361	0.002
Day 10 of distraction	0.756 ± 0.129	0.347 ± 0.099	18.992	0.012
Day 5 of consolidation	0.645 ± 0.054	0.215 ± 0.040	122.403	0.000
Day 10 of consolidation	0.319 ± 0.066	0.062 ± 0.070	21.201	0.010

Table 7
Comparison of the relative mRNA expression of Arg1 in the distraction area at different periods ($\bar{x} \pm s$).

Time	Control group	Experimental group	F	*P*
Day 5 of distraction	0.978 ± 0.308	2.840 ± 0.736	16.374	0.016
Day 10 of distraction	1.711 ± 0.432	3.557 ± 0.472	24.949	0.008
Day 5 of consolidation	1.009 ± 0.278	2.219 ± 0.325	23.986	0.008
Day 10 of consolidation	0.340 ± 0.201	0.497 ± 0.250	0.716	0.445

Table 8
Comparison of the relative mRNA expression of CD11c in the distraction area at different periods ($\bar{x} \pm s$).

Time	Control group	Experimental group	F	*P*
Day 5 of distraction	2.005 ± 0.177	1.307 ± 0.294	12.447	0.024
Day 10 of distraction	1.940 ± 0.265	1.289 ± 0.249	9.598	0.036
Day 5 of consolidation	1.430 ± 0.266	0.792 ± 0.143	13.394	0.022
Day 10 of consolidation	0.561 ± 0.117	0.480 ± 0.117	0.705	0.449

chenhui Li: Formal analysis, Data curation. **Yong-di Li:** Formal analysis, Data curation. **Yu Wang:** Formal analysis. **You-li Chen:** Formal analysis. **Zheng-long Tang:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

Table 9
Comparison of the relative mRNA expression of CD206 in the distraction area at different periods ($\bar{x} \pm s$).

Time	Control group	Experimental group	F	*P*
Day 5 of distraction	2.337 ± 0.981	4.190 ± 0.473	8.669	0.002
Day 10 of distraction	3.159 ± 1.025	4.174 ± 0.658	2.123	0.219
Day 5 of consolidation	1.678 ± 0.140	3.161 ± 0.517	23.044	0.009
Day 10 of consolidation	0.631 ± 0.283	1.375 ± 0.270	10.874	0.030

influence the work reported in this paper.

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