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Original Article

Expression of bone morphogenetic protein 2 in rabbit radial defect site with different lengths

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Abstract: Background: It has been studied that the distribution of bone morphogenetic protein 2 is regular under bone defect situation. Objective: To observe the expression of bone morphogenetic protein 2 in rabbit radial defect site with different lengths. Methods: Forty-eight New Zealand rabbits were divided into two groups randomly. 0.5 cm bone defect and 3.0 cm bone defect were made by wire saw at the middle part of radius bone after anaesthesia. Results and conclusions: Western blot results showed that in the 0.5 cm bone defect group, the expression of bone morphogenetic protein 2 of the tissues in the bone defect site was increased gradually at 1, 3, 4 weeks after operation, and the expression in each defect group was increased when compared with that immediately after injury ($P<0.05$). In the 3.0 cm bone defect group, the expression of bone morphogenetic protein 2 of tissues in bone defect site was increased gradually and reached to its peak at 3 weeks after the operation ($P<0.05$). The peak value in the 3.0 cm bone defect group was significantly higher than that in 0.5 cm bone defect group ($P<0.05$). The peak value was maintained in high level. The comparison of bone callus formation showed that the bone callus formation of 3.0 cm bone defect group was less than that of the 0.5 cm bone defect group at 3 and 4 weeks after operation ($P<0.05$). The results indicate that expression of the bone morphogenetic protein 2 in 3.0 cm bone defect site is increased significantly, but the expression level cannot make the bone defect heal itself.

Keywords: Tissue construction, bone tissue construction, bone defect, bone length, bone healing, rabbit

Introduction

Bone defect is the continuous bone deletion caused by trauma, infection, tumors, and congenital malformations; especially when the bone defect is too large, inappropriate treatments often lead to bone nonunion, limited functional recovery of the affected limb and even amputation. Therefore, how to effectively promote the healing of fresh and old bone defect, and for different organisms, what is the length of the bone defect which can achieve self-healing through the body's own repair have been hot research topics [1, 2]. Although continuous efforts of researchers have clearly confirmed that too long the bone defect, local blood circulation disorders, abnormal activity of bone defect ends, and excessive inflammation in the bone defect sites can lead to the non-healing of bone defects [3, 4], currently there are few research regarding systemic and local changes in human pathophysiology under the condition of bone defect [5], and there are few studies on the pathophysiology of bone defects, directly resulting in inadequate theoretical basis for the treatment of bone defects in clinical.

Bone morphogenetic protein-2 is a kind of bioactive protein from the bone and bone-derived cells; in the absence of bone injury, it is only remained at a low expression level to maintain normal physiological activities of the organism; but when there are bone defects, its expression is significantly increased and it participates in the repair of surrounding tis-

sues under the stimulation of bone necrosis, bone damage and inflammations of surrounding tissues. It is not only the paracrine product of bone metabolism but also a specific bone growth factor; its target cells are the perivascular mesenchymal cells with potential differentiation. Its main osteogenic role is to induce mesenchymal cells to differentiate into osteoblasts and chondrocytes, and to produce new bone [6]. Bone morphogenetic proteins of different races share a high homology, with the ability of interracial osteoinduction; the dose-response of bone morphogenetic protein was positively correlated [7], but there is a significant difference in the dose and time for osteoinduction among different races of animals. It exists in all the fracture healing process and plays an extremely important role in the repair process of fractures, and it is the known cytokine with the strongest ability of ectopic osteogenesis, having a strong capacity to induce the formation of bone tissue, so it has been widely used to promote the healing of fractures and bone defects. Although previous studies have also confirmed that in the state of bone defects, there is a certain law for distribution and generation of bone morphogenetic protein-2 [8], there are few studies regarding the relationship between the length of bone defects and the expression of bone morphogenetic protein-2.

Healing of bone injury is a complex pathophysiological process, which involves numerous biological factors and responses; and in various stages of the healing of bone damage, pathophysiological changes are different; therefore a comprehensive and complete study of all physiological processes is almost impossible, and the process is not completely understood in present [9].

This study chose bone morphogenetic protein-2 (which plays an important role in the healing process of the fracture and can represent the process of fracture healing) as the study subject, through the animal models with large lengths of bone defect and the comparison with short length of bone defect, to observe the relationship between the expression of bone morphogenetic protein-2 and bone healing, attempting to explain the relationship between the secretion of bone morphogenetic protein-2 and bone healing from the perspective of molecular biology in the longer bone defect and to explore physiopathological changes in long bone defect, thereby laying the theoretical foundation for the nonunion treatment caused by bone defects.

Material and methods

Design

A randomized controlled animal experiment. Time and place: Form April to November in 2011, it was completed Experimental Animal Center attached to Xinjiang Medical University.

Materials

48 4-month-old New Zealand white rabbits with male and female each for half, weighing 2.0-2.5 kg, were provided by Experimental Animal Center attached to Xinjiang Medical University. The Animal license number was SYSK (new) 2011-0004. Experimental animal treatment complied with "guidance on treatment of experimental animals". Which was issued by Ministry of Science and Technology People's Republic of China [10].

Experimental method

Grouping, preparation of rabbit model with bone defects and specimen collection: 48 rabbits were randomly divided into two groups. 24 rabbits were in 0.5 cm bone defect group, and the rest was in 3.0 cm bone defect group. After using 30-35 mg/kg sodium pentobarbital in all experimental animals for anesthesia, hair cut off were done at the damaged area. Strong iodine was used for disinfection. Sterile towels and sheets were laid in damage zone. Left dorsal forearm 3 cm incision layer-cut were used in all the animals, interferring the surrounding tissue as little as possible until revealed the periosteal tissue and middle of radial. Retained the osteotomy site of the periosteum, 0.5 and 3.0 cm bone defects were produced according to the wire saw in the middle radius of rabbits [11]. After injuries, routine and sutured incision were done. All of the above operations were complete by the same injury doctor. The animals were fed with sub-cage separately after injury, without activity limitations. 80×10^4 U penicillin injection was given to the injury rabbits twice every day in order to prevent from infection. When modeling (immediately), tis-

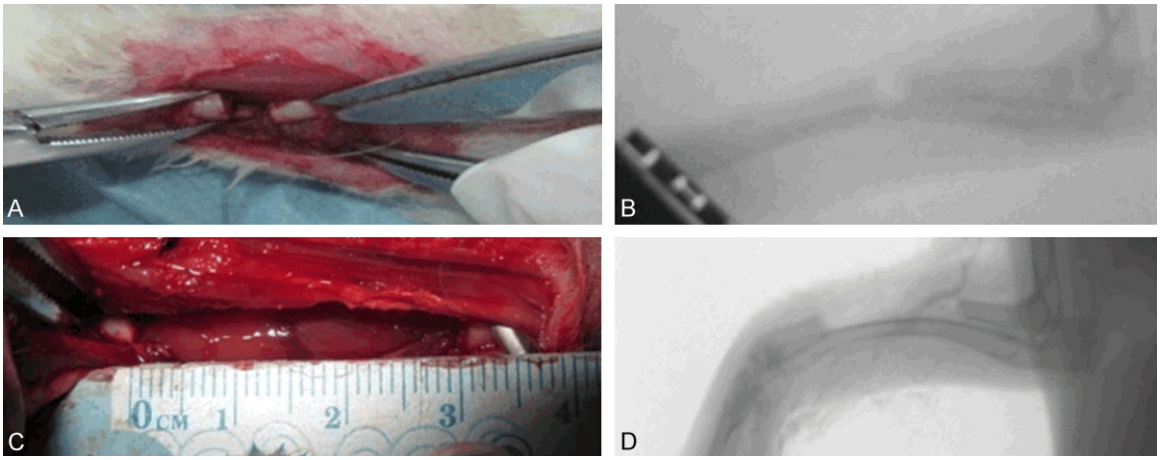

Figure 1. X-ray film and general observation of rabbit radial defects of 3.0 and 0.5 cm in the middle radius. A: Immediate lesion morphology of the bone defect of 0.5 cm; B: X-ray film of the left forearm in the 0.5 cm bone defect; C: General observation of the defect of 3.0 cm in the modeling surgery; D: X-ray film of the left forearm in the 0.3 cm bone defect. Note: Both the X-ray detection and general observation indicated that rabbit radial defects of 3.0 and 0.5 cm in the middle radius were successfully established.

sues surrounding bone defects were randomly selected from eight animals. Eight animals were randomly sacrificed 1, 3, 4 weeks after modeling injury. X-ray of bone defect part were taken in order to get the general healing condition of fracture. Western blot detection and semi-quantitative were taken on the filling tissue from bone defect area.

Imaging defection and general observation of rabbit bone defect area: X-ray imagine were taken immediately from eight random selected animals after the injury of bone defect site and sacrificed animals 1, 3, 4 weeks after injury. X-ray imagine of bone defect site and general observation were taken. Fracture healing condition was observed in rabbits (**Figure 1**).

Western blot analysis of the expression of bone morphogenetic protein 2 from the filling tissues in rabbit defected bone site: Experiments were carried out according to the previous literature step [12]. Protein extraction, concentration measurement, SDS-PAGE electrophoresis, transferred membrane, plus an anti-anti-rabbit bone morphogenetic protein-2 polyclonal antibody (1:200), secondary antibody horseradish peroxidase-labeled goat anti-rabbit antibody (1:10 000), washing the membrane with TBST, ECL color, X-ray exposure film had been done accordingly. Quantity one image software were used to compare the absorbance values from the bone morphogenetic protein-2 electrophoretic bands detected by analysis system. Take glyceraldehyde 3-phosphate dehydrogenase (GAPDH, glyceraldehyde-3-phosphate dehydrogenase) as an internal reference, relative content of partial bone morphogenetic protein 2 were determined by the ratio of absorbance values between bone morphogenetic protein-2 absorbance values and GAPDH absorbance values.

The main outcome measure: Qualitative detection and semi-quantitative test results of expression of bone morphogenetic protein 2 in rabbit radial defect site; the result of Lane-Sandhu X-ray score of bone healing in bone defect site attached to **Table 1** [13]; the result of general observation at different points of time in the bone defect site.

Statistical analysis

Measurement data were described as $\bar{x} \pm s$; SPSS 12.0 statistical software was used for data analysis; mean differences between groups were compared using replicated measured analysis of variance; $P<0.05$ was considered statistically significant.

Results

Experimental analysis of the number of animals

In the follow-up study, four of forty-eight rabbits died: two rabbits died 5 d after injury in 3.0 cm

Table 1. Scoring system of the Lane-Sandhu X-ray of bone union

Classification	Criteria	Lane-Sandhu X-ray score
Bone formation	Without bone formation	0
	Bone formation accounted for 25% of bone defects	1
	Bone formation accounted for 50% of bone defects	2
	Bone formation accounted for 75% of bone defects	3
	Bone formation accounted for 100% of bone defects	4
Bone union	Fracture line was clear	0
	Part of the fracture line presented	2
	Fracture line disappeared	4
Bone shaping	No bone shaping	0
	Marrow cavity formation	2
	Cortical bone shaping	4

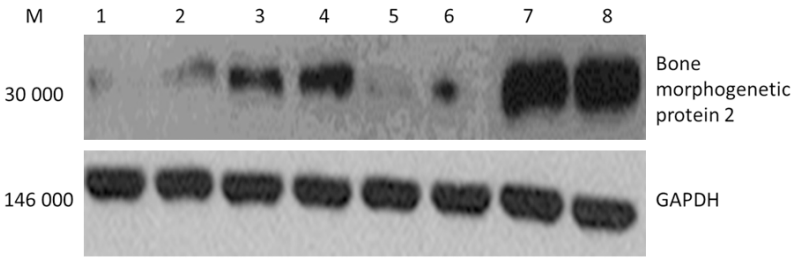

Figure 2. Expression of the bone morphogenetic protein 2 in the tissues with bone defects of 0.5 and 3.0 cm at different time points. Note: In the groups of 0.5 cm and 3.0 cm bone defects: Bone morphogenetic protein-2 protein positively expressed at the time points of damage immediately, 1st, 3rd, 4th week after damage, and the expression of bone morphogenetic protein 2 showed a gradually increased trend. 3.0 cm bone defect groups; 1, 2, 3, 4 represented for damage immediately in 0.5 cm group, and 5, 6, 7, and 8 represented for damage at immediately, the 1st, 3rd, 4th week after the injury.

bone defect group; one rabbits died 10 d after injury in 3.0 cm bone defect group, two rabbits died 14 d after injury in 0.5 cm bone defect group. The dead animals were all given to re-fill in order to ensure that the number of experimental specimens. Finally, 48 rabbits were enrolled in the final analysis.

*The expression of bone morphogenetic protein 2 in rabbit defect bone (**Figure 2; Table 2**)*

As was shown in **Figure 2** and **Table 2**, in the 0.5 cm bone defect group, the expression of bone morphogenetic protein 2 reached its lowest point at injury time ($P<0.05$). Compared with the injury time, it increased gradually 1, 3, 4 weeks after injury time ($P<0.05$). In the 3.0 cm bone defect group, the expression of bone morphogenetic protein 2 reached its lowest point at experimental animal injury time (modeling time) ($P<0.05$). 1, 3, 4 weeks after injury, the expression gradually increased ($P<0.05$). Variance analysis of repeated measurements among the groups showed that the expression of bone morphogenetic protein 2 among the groups showed significant differences ($P<0.05$) except 3, 4 weeks after injury time.

General morphology of rabbit defect bone

0.5 cm bone defect group: 1 week after injury: Bone defect zone was filled with soft tissue hematoma which was dark brown and semi-gel state. Soft tissue around bone defect zone was edema, the unusual activity was significantly obvious between the ends of the defect bone. Three weeks after the injury: bone defect area was filled with dark brown, quality and tough fibrous tissue. They were ill-defined with the surrounding tissue and their surface were uneven. Bone defect soft tissue edema was reduced compared with previous. The abnormal activity was reduced between the ends of the defect bone. Four weeks after the injury: bone defects were filled with a lot of dark gray, hard, callus-like substance. Their surface were uneven, and they were easy to dissect from surrounding tissue. Callus-like substance were proliferated, and the connection of bone ends was relatively strong.

3.0 cm bone defect groups: 1 week after injury: the performance was consistent with the 0.5

Table 2. Weatern blot semi-quantitative results of expression of bone morphogenetic protein 2 at different time points in the 0.5 cm and 3.0 cm bone defects group ($\bar{x}$±s, n=8)

Group	0.5 cm bone defect group			
	damage immediately	1st week after the injury	3rd week after the injury	4th week after the injury
Bone morphogenetic protein 2	206.2±18.3	242.8±21.5	403.3±38.3	495.6±40.7
GAPDH	1513.3±147.6	1499.2±135.3	1508.2±145.7	1497.7±133.7
Bone morphogenetic protein 2/GAPDH	0.1±0.0	0.2±0.0	0.3±0.0	0.3±0.0
[a]*P*		<0.05	<0.05	<0.05
[b]*P*	<0.05	<0.05	<0.05	<0.05
Group	3.0 cm bone defect group			
	damage immediately	1st week after the injury	3rd week after the injury	4th week after the injury
Bone morphogenetic protein 2	236.4±20.8	268.4±24.2	527.9±49.2	553.1±50.2
GAPDH	1534.1±147.9	1555.2±150.4	1509.2±143.5	1515.2±145.9
Bone morphogenetic protein 2/GAPDH	0.2±0.0	0.2±0.0	0.3±0.0	0.4±0.0
[a]*P*		<0.05	<0.05	<0.05
[b]*P*	<0.05	<0.05	>0.05	>0.05

Note: Between the group of immediately damage and each group after damage, [a]P<0.05; among different time points in the same group, [b]P<0.05. Absorbance was positively correlated with the expression levels of protein, the higher the value; the higher the value, the higher the expression of the target protein.

cm bone defect group. Three weeks after the injury: Bone defect zone was filled with soft, dark brown tissues. However, the volume of tissue was less, and soft tissue edema were significant. Unusual activity between the ends of the defect bone existed. Four weeks after the injury: bone defects were partly filled with hard and dark gray fibrous tissues. There was almost no callus formed at bone ends. Callus-like substance at the surface of bone stump was not obvious. There were still unusual activity between bone ends, which was slightly better than before.

Results of X-ray examination of bone defected zone in bone defected rabbits and the formation of callus

With the extension of injury time, the defects were gradually healed in 0.5 cm defect group, but the defects cannot be self-healed in 3.0 cm bone defect group (**Figure 3**).

Callus formation conditions in bone defect rabbits with different lengths

3 and 4 weeks after injury, the results of callus X-ray score in 0.5 cm bone defect group were (7.78±0.59) points and (9.75±0.72) points. In 3.0 cm bone defect group, they were (1.89±0.17) points and (1.92±0.13) points. In 0.5 cm bone defect group, the generation capacity of callus increased significantly (P<0.05). In 3.0 cm and 0.5 cm bone defect group, the formation of callus were significantly improved three weeks after injury. However, the amount of callus formation in 3.0 cm bone defect group was significantly less than that in 0.5 cm bone defect group.

Discussion

Bone defect is the continuous bone deletion caused by trauma, infection, tumors, and congenital malformations; for the shorter bone defects, after conventional treatments, such as reset, fixation and functional exercise, bone defects can generally get a good healing. But if the defect is too large, the situation is very thorny. Inappropriate treatments often lead to old bone defects, resulting in bone nonunion, limited functional recovery of the affected limb and even amputation, which may bring a great loss and burden to the patients and society. Therefore, how to effectively promote the healing of bone defects has long been a hot spot for researchers [14, 15].

Bone defects and fractures share the same healing process, which is affected by various factors. Healing of bone defects in appearance mainly manifests as early callus formation and the continuous and dynamic remodeling repair of callus lately. Bone healing histologically

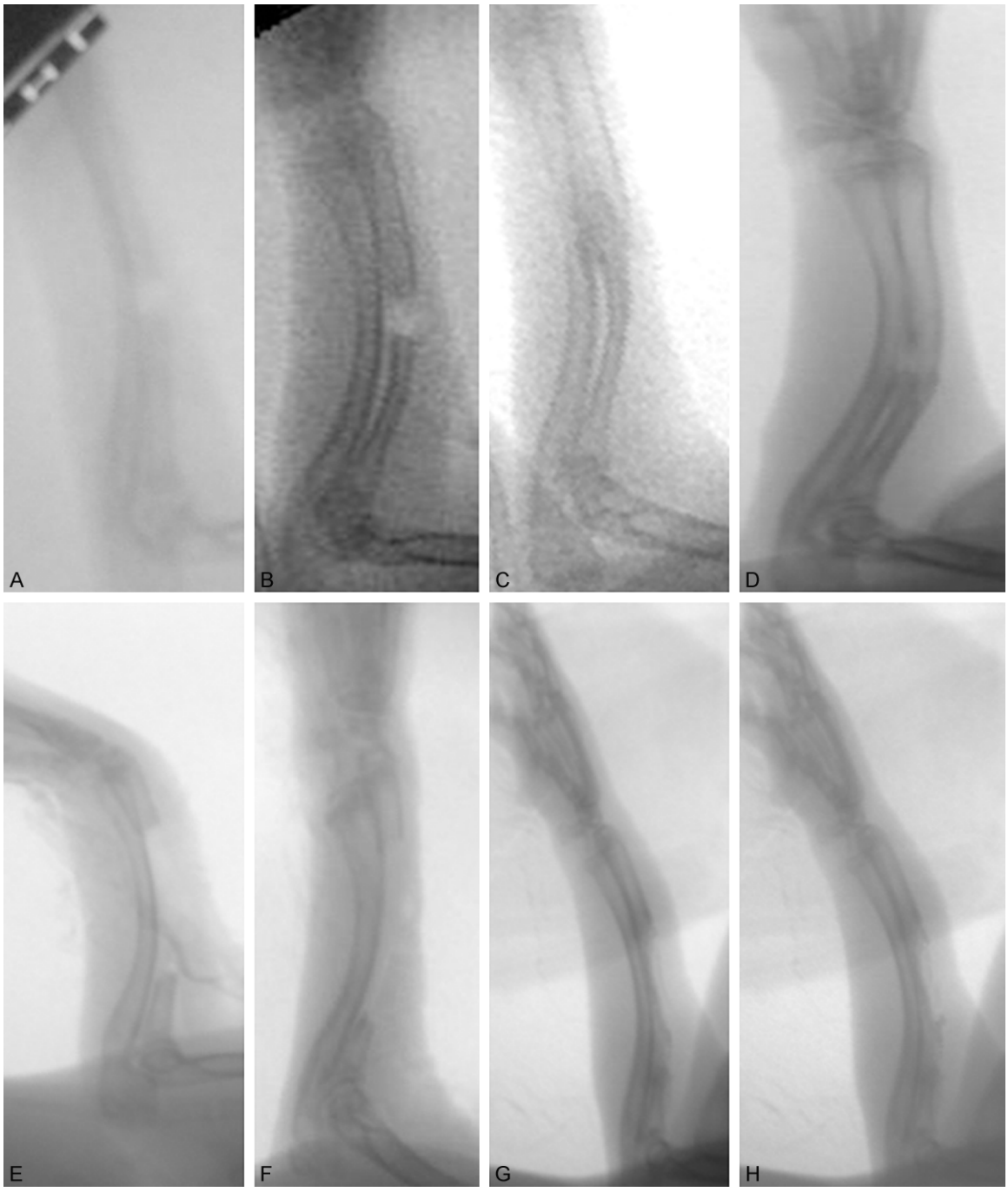

Figure 3. X-ray results of rabbits radial bone defects of 0.5 cm and 3.0 cm. A-D: The X-ray results of 0.5 cm bone defect group at the time points of damage immediately, 1st, 3rd, 4th week after damage; E-H: The X-ray results of 3.0 cm bone defect group at the time points of damage immediately, 1st, 3rd, 4th week after damage. Note: With the extension of injury time, the defects in 0.5 cm bone defect group were gradually healed, while defects in 3.0 cm bone defect group cannot be healed.

mainly manifests as successively experienced hematoma and inflammation, the original cartilage callus formation, mature lamellar bone formation and bone plate reconstruction and remodeling [16]. From the perspective of molecular biology, it is considered that fracture healing is the dynamic evolution mediated by cells and cell-matrix and the bone healing process regulated by various factors in the body and local microenvironment, involving changes of

the expression of a lot of bone growth promoting factors [17]. It is a slow process of bone remodeling, which requires at least four processes, including cell recruitment, regulation of factors, osteoinduction and osteoconduction [18, 19]. This process is also affected by many factors such as the local blood supply, movement mechanics and surrounding soft tissue conditions. When the bone defects are not too large, the healing of bone defects is substantially equivalent to the normal process of bone healing after reset. When the degree of bone defect is great, or it is accompanied by severe soft tissue defects, the bone defect often cannot be effectively healed, leading to the termination of bone healing [20].

Bone morphogenetic protein-2 is a very important biological factor in the healing of bone defects. From the perspective of molecular biology, the healing of fractures and bone defects is the process mediated by a series of cytokines [21, 22]; in all cytokines, the bone morphogenetic protein-2 in the bone morphogenetic protein family is the most important biological factor affecting fracture healing [23], with an efficient osteoinductive activity. In the early fracture healing, bone morphogenetic protein-2 can induce bone formation, recruit and differentiate undifferentiated mesenchymal cells and bone cell lines, maintain the unique osteoblast phenotype, increase osteoblast markers, and promote the calcification of extracellular matrix [24]. In the latter part of bone formation, bone morphogenetic protein-2 can also be used as an osteoclast differentiation factor to directly or indirectly stimulate osteoclast differentiation together with other osteoclast differentiation factors, involved in bone reconstruction. Its effects on bone are not only throughout the process of bone metabolism, but also significant.

When the bone defect is too large, it can cause bone nonunion and result in corresponding changes in the pathophysiology. Although the bone has the ability to repair itself, the capacity is limited; and coupled with complex injuries such as too large the length of the defect, local infection, soft tissue defects and inactivation, poor systemic conditions, pathological changes of other organs, it often leads to the unhealed fresh bone defect [25, 26]. In these factors of nonunion, the length of the defect is often an important factor. Johnson et al. Johnson et al [27, 28] noted that, when the length of the bone defect is longer than three or four times of the diameter of backbone (the length of unrepair itself), it can often lead to the bone nonunion caused by bone defects. The average diameter of radius of New Zealand white rabbits is about 4 or 5 mm, so based on the above theory, when the rabbit radial bone defect is longer than 2.0 cm, it will be possible to give rise to bone nonunion because of bone defects [29]. In other words, in this state, the repair response of the body to a greater degree of bone defect is different from that to a smaller extent; but what factors led to the different repair response? And in this pathological state, how the human body reacted? The solvation of the above problems will contribute to those cases of bone nonunion caused by a wide range of bone defects in clinical. Referring to the above theory and combining the ideas of articles, the experiment designed the bone defects of 3.0 cm and 0.5 cm in the two experimental groups respectively to ensure that the bone nonunion caused by excessive bone defects was successfully copied, in order to study the changes in molecular biology of the body in this state. Because the bone morphogenetic protein-2 is a more important factor in all of the biological factors and it can represent the changes in the fracture healing process, so we selected the bone morphogenetic protein-2 as the subject to study the pathophysiological changes of the body in this state, providing theoretical basis for the early and effective treatments of the bone nonunion caused by longer bone defects.

The experimental results suggested that in the 0.5 cm bone defect group which was produced at the middle part of radius bone, compared with the injury time, the expression level of bone morphogenetic protein 2 in part of bone defect site were increased obviously 1, 3, 4 weeks after injury and the expression level gradually increased after 1, 3, 4 weeks with time shift; in the 3.0 cm bone defect group which was produced at the middle part of radius bone, compared with the injury time (represent the normal condition), the expression level of bone morphogenetic protein 2 in part of bone defect site were increased obviously 1, 3, 4 weeks after injury and the expression level gradually increased after 1, 3, 4 weeks with time shift; But at this time the expression level

of the bone morphogenetic protein 2 basically reached the peak three weeks after the injury and it did not increase gradually with time went by after injury. This was different from that in the 0.5 cm bone defect group. It was predicted that in a larger distance of bone defect, although the length of the bone defect increased significantly, the body's capabilities of bone morphogenetic protein-2 secretion reached its saturation point. The body's reaction was completely different compared with that in the small distance bone defect group. At the time points of damage immediately (on behalf of the normal state) and one week after injury, the content of bone morphogenetic protein-2 in 3.0 cm bone defect group was slightly higher than that in 0.5 cm bone defect group, without statistically significant differences; it implied that in the 1st week after injury, the changes of defect length cannot significantly change the expression of bone morphogenetic protein 2. In the 3rd week after damage, the content of bone morphogenetic protein-2 in 3.0 cm bone defect group was higher than that in 0.5 cm bone defect group, indicating that with the increasing length of the bone defect, bone morphogenetic protein-2 expression levels will be gradually increased; In the 4th week after damage , the content of bone morphogenetic protein-2 in 3.0 cm bone defect group was slightly higher than that in the 0.5 cm group, but the difference was not statistically significant, indicating that although the expression of bone morphogenetic protein 2 would increase with the increasing length of the defect, when it reached the maximum secretion of the body, the expression of bone morphogenetic protein-2 secretion will reach a stable level.

The X-ray score results of the bone callus in bone defects showed that, the X-ray scores of the 3rd and 4th week after injury in 0.5 cm bone defect group was significantly more than those in 3.0 cm bone defect group, implying that when the length of the defect was too large, the ability of the body to repair itself cannot afford the repair of larger defects, although the expression of the bone morphogenetic protein 2 increased significantly. This suggested that in the treatment of bone defects with larger lengths, the simple use of bone morphogenetic protein-2 protein may be ineffective [30, 31]; it may be due to that the content of the bone morphogenetic protein-2 had not yet reached a sufficient concentration to promote the ossification in bone defect sites; because the general observation of bone defect sites showed the formation of fibrous and hematoma tissues in the sites of bone defects, which was consistent with the early performance of the short bone defects; but the bone defects had not been healed, what would lead to the above still need further studies.

It was found that the expression of bone morphogenetic protein 2 increased with the increasing length of the bone defect, and it reached the maximum at a certain larger length and was likely to remain at that level; for the greater degree of bone defects, the simple use of small doses of bone morphogenetic protein-2 may be invalid for the treatment of bone defects. But because of the small number of test specimens and the inadequate observation time, the establishment of the above theory still requires more and more in-depth research.

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Disclosure of conflict of interest

None.

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