Original Research *Feature article*

Active bone material containing modified recombinant human bone morphogenetic protein-2 induces bone regeneration in rabbit spina bifida

Xue-Cheng Sun[1,2*], Hu Wang[1,3*], Jian-Hui Li[1*], Li-Qiang Yin[4], Yu-Fang Yan[4], Xu Ma[1,3] and Hong-Fei Xia[1,3]

[1]Reproductive and Genetic Center, NHC Key Laboratory of Reproductive Health Engineering Technology Research (NRIFP), National Research Institute for Family Planning, Beijing 100081, China; [2]Medical Genetics, Zibo Maternal and Child Health Hospital, Zibo 255000, China; [3]Graduate Schools, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100730, China; [4]Yantai Zhenghai Bio-Tech Co., Ltd., Yantai 264006, China
*These authors contributed equally to this paper.
Corresponding authors: Xu Ma. Email: genetic@263.net.cn; Hong-Fei Xia. Email: hongfeixia@126.com

Impact Statement

Spina bifida is a common neural tube deformity. Traditionally, autologous bone grafting remains the "gold standard" for repairing bone defects. However, the source is limited and it is easy to cause secondary harm to the patient. These limit the widespread application of autologous bone grafting. Since it is difficult for a single material to meet the needs of repairing the structure and function of bone tissue, we urgently need an artificial composite bone repair material that can induce regeneration. In this study, we propose an active bone material and verify its efficacy and safety. This study is not only suitable for the screening of large-scale biomaterials, accelerating the research progress of regenerative repair products, but also conducive to the research on the mechanism of regeneration and repair of various materials.

Abstract

This study focuses on spina bifida, which is a high incidence among the current clinical manifestations of human birth defects. Because in the treatment of bone defects, the source of autologous bone is limited and it is easy to cause secondary injury to the patient. At the same time, since the bone tissue in animals needs to perform a variety of biological functions, its complex structure cannot be replaced by a single material. Therefore, in this study, we used Japanese white rabbits to establish an animal model similar to human congenital spina bifida. The established animal model is used to screen the best regenerative repair products for the treatment of congenital spondylolisthesis defects, and to evaluate the safety of regenerative repair products. The results show that bone morphogenetic protein (BMP)-2 combined with collagen material has a better regeneration effect than collagen material alone, and it did not negatively affect the health of animals. This study is not only suitable for the screening of large-scale biomaterials, accelerating the research progress of regenerative repair products, but also conducive to the research on the mechanism of regeneration and repair of various materials.

Keywords: Spina bifida, Japanese white rabbits, collagen material, bone morphogenetic protein-2, damage repair, active bone

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Introduction

Neural tube defects (NTDs) are a kind of congenital developmental malformations of the central nervous system that are quite common in children, which occur all over the world, with an average incidence of 1%.[1] Neural tube malformation is a serious birth defect caused by the incomplete or incomplete closure of the neural tube in early embryonic development, including anencephaly, encephalocele, craniospinal bifida, spine caused by unclosed tail (crack), and so on.[2] The development of the nervous system undergoes a series of complex biological processes, involving the proliferation, differentiation, apoptosis, autophagy of nerve cells and neurons, formation of nerve synapses, signal transmission and transduction, and formation of neural network nuclei. Apoptosis, as a special form of programmed death of biological cells, is subject to the participation and regulation of various effector cells in surrounding tissues. Once cell apoptosis is excessive, it can cause damage to the corresponding nerve cells and spinal cord tissue in the body, forming a common clinical spina bifida deformity.[3]

Due to physical or chemical factors,[4] spina bifida deformity can be divided into two types: dominant spina bifida and recessive spina bifida, and "dominant" and "recessive" refer to whether there are meningeal membranes or nerve tissues that bulge out of the spinal canal through spina bifida,

forming cystic lumps. Due to partial loss of the lamina, spinal bifida easily leads to scoliosis and myeloexpansion. It is often accompanied by nervous system dysfunction, causing dysfunction of the corresponding internal organs, such as urinary incontinence and clubfoot. Therefore, a good bone repair material is needed to fill the bone defect area.

As there are many diseases caused by bone defects, trauma, and so on, it has always been an urgent need to find a suitable bone repair material. Until a good bone replacement material is found, autologous bone grafting is still the "gold standard" for repairing bone defects. Although autologous bone grafts have a higher fusion efficiency than other grafts, they have a narrow source, limited bone mass, and can cause secondary injuries to the patients themselves after surgery.[5,6] Therefore, the clinical application of this method is limited to a certain extent. Since the bone tissue in animals needs to perform a variety of biological functions, its complex structure cannot be replaced by a single material. So, it is urgent to find a new type of artificial composite material that can induce regeneration.

At present, the most widely used material is collagen. Collagen can be used as a biological scaffold material. It is an organic component in bone tissue, so it has good biocompatibility. It can also be used as an attachment scaffold for osteoblasts to promote cell migration and differentiation and regulate cells growth; its disadvantage is that the mechanical strength is not enough, and it can be used together with hydroxyapatite[4] to improve the mechanical strength. On the basis of collagen materials, we can also add some osteoinductive or osteogenesis factors to make the repair effect of collagen materials more ideal. At present, the commonly used osteoinductive or osteogenic factors mainly include two types of cells and osteoinductive factors. Osteogenesis-inducing factor materials mainly include chemical factors such as dexamethasone, ascorbic acid, and insulin; bone morphogenetic proteins (BMPs);[2] transforming growth factor (TGF); and other growth factors, such as basic fibroblast growth factor (bFGF)[3] and insulin growth factor (IGF).[7]

In the research in the field of biomedicine, as experimental animals, rabbits are moderate in size, docile, and easy to operate; at the same time, they are cheap to buy and easy to feed; the most important thing is that there are many rabbits with cleft lip and palate and spina bifida modeling foundation. Therefore, rabbits were chosen as the model animal for studying spina bifida. Therefore, this study used rabbits as experimental animals to explore the bone induction and regeneration and repair effects of collagen scaffolds in animal models of spina bifida.

Materials and methods

Preparation of implant materials

Ordinary collagen scaffold material: mainly composed of inorganic hydroxyapatite and organic collagen. It is obtained by decellularizing and defatting bovine cancellous bone. Collagen is the main organic component of bone, and type I collagen and its cross-linked fibrous structure are the most abundant proteins in the extracellular matrix of bone. The collagen structure has an inductive effect on mineral deposition, and its surface contains sites for mineral deposition, which can effectively initiate and control the mineralization process, promote bone formation, and induce it into the implant. Mainly used for filling and repairing vertebral body defects.

Active bone material: a new type of bone repair material based on the above-mentioned common collagen scaffold material and compounded with human BMP. The traditional collagen carrier prevents the diffusion of BMP-2 components through the molecular structure of collagen molecules and maintains the concentration of BMP-2 around the host target cells. On one hand, it also requires that the pore size should not be too large, but the pore size is too small and is not conducive to the growth of osteoblasts. Technologically, it brings certain difficulties. On the other hand, using the current biomaterials as the carrier of BMP-2, the amount of BMP-2 is large. To this end, a fusion active repair factor with collagen-specific binding ability that can be used to activate collagen wound repair scaffolds was used in this study.

Collagen membrane: it is a collagen membrane made from bovine skin and processed by a special process, with a thickness of about 0.8 mm. It can guide cells to grow in, promote tissue regeneration and wound repair; at the same time, it has good histocompatibility and no immune rejection.

The common collagen scaffold materials, active bone materials, and collagen membrane we used in this study were purchased from Yantai Zhenghai Bio-Tech Co., Ltd. (Yantai, China). Among them, the active bone material has obtained the Chinese invention patent (authorization announcement number: CN 100355788C).

Groups and treatment

In this experiment, healthy, female Japanese white rabbits (JWRs), specific pathogen-free (SPF) grade, female, rabbits are about two months old and weigh about 2 kg, purchased from Beijing Huafukang Biotechnology Co., Ltd. (Beijing, China). All these animals are kept in an SPF barrier system. All experimental animals were housed in clean, dry, and ventilated spaces with free access to drinking water and food. The breeding temperature is controlled at 19–29°C, the relative humidity is 40–70%, and the noise is controlled at below 60 dB. The surgical modeling process of all experimental animals was completed under aseptic conditions. The NRIFP Ethics Committee approved this study.

During the operation, fasting JWRs for 10–12 h. Then, the rabbits need to be anesthetized. We choose serazine hydrochloride (concentration: 1–2 mg/kg) as an anesthetic, and the administration method is the ear vein injection. When the rabbit enters a deep anesthesia state, immobilize the rabbit; shave the hair on the back of the lumbosacral area and disinfect with iodophor; then cut the skin, remove the excess muscles on both sides, and expose the vertebral plate; remove the single vertebrae at the top of the lumbosacral area with rongeur board. Add an equal volume of block bone repair materials according to different groups, and then cover with a layer of collagen membrane and suture (Figure 1). Finally, all rabbits are treated with conventional antibiotic treatment

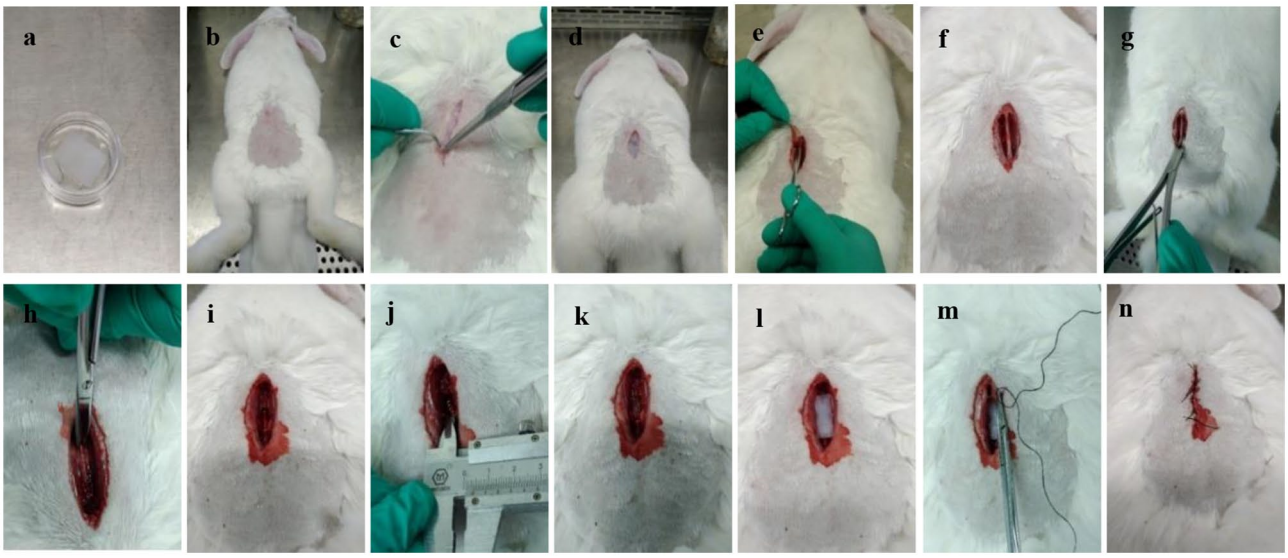

Figure 1. Surgical process. (a) Bone material and collagen membrane, (b to f) skin and muscle tissue at the surgical site are cut and removed, (g to j) expose the spinal cord, (k) add bone material, (l) add collagen membrane, and (m and n) suture the skin. (A color version of this figure is available in the online journal.)

(i.e. penicillin potassium: 4000 IU/kg/d). Antibiotics will be given for one week to avoid infection.

In this study, all JWRs were divided into four groups: (1) normal group: normal rabbits without any treatment; (2) control group: only remove the single lamina at the top of the lumbosacral area, and then suture it; (3) material group: remove the single lamina at the top of the lumbosacral area, then fill the defect area with an equal volume of ordinary collagen scaffold material, and finally suture; (4) BMP group: remove the single lamina at the top of the lumbosacral area, then use an equal volume of active bone material fills the defect area, and finally sutures. Each group has six rabbits.

JWRs were removed from each group three months after the operation. The rabbits were euthanized by injecting an overdose of serazine hydrochloride intravenously into the ear margin. Take out the vertebrae of the surgical site, place part of the vertebrae of three JWRs in 4% NaOH and 95% ethanol for 24 h, then take them out and put them in an oven to dry for making vertebrae models. Finally, take pictures and mark the appearance of the model. In addition, the remaining three JWRs vertebral bone tissues were used for histochemical testing. When making paraffin sections, first, the vertebral tissue containing the surgical site should be fixed in 4% paraformaldehyde for 24 h. Since calcium-containing bone tissue is not conducive to sectioning, decalcification with 10% ethylenediaminetetraacetic acid (EDTA) is also required for one month. The decalcified vertebral tissue was dehydrated and dipped in wax, and finally embedded in paraffin. When sectioning, we chose a rotary microtome (Leica RM2245, Leica, GmbH, Germany) to control the thickness of paraffin sections to be 6 μm. The cut wax slides are adhered to the glass slides for preservation. Sections were deparaffinized in xylene and rehydrated with graded alcohol prior to histochemical staining.

X-ray analysis

X-ray biopsy was used to roughly observe the bone repair of the defect in each group. X-ray analysis was carried out in Beijing Animal Hospital, using SOFTEX® M-60 X-ray machine (Kanagawa, Japan), the parameters were set to 80 kV voltage and 125 mA current, and the exposure time was 40 milliseconds (ms).

Hematoxylin and eosin staining

Hematoxylin and eosin (HE) staining was used to observe the general morphology of rabbit vertebral tissue. Hematoxylin is a basic dye that stains cell nuclei blue-violet. Eosin is an acid dye that stains the cytoplasm red. After staining, it was observed using a TE2000-U inverted phase contrast microscope (Nikon, Tokyo, Japan).

Sirius red stain

Sirius red staining is used to detect different collagen fibers, and Sirius red staining is performed according to the instructions of the commercial kit (Sembejia Biotechnology Co., Ltd., Nanjing, Jiangsu, China). Briefly, sections were first incubated with Sirius red staining solution for 1 h at room temperature, and then nuclei were counterstained with hematoxylin. Collagen type 1 turned bright orange in the stained sections. Image J was used to measure the proportion of positive signals in different fields of view. Each slice counts at least three fields of view.

Periodic acid–Schiff staining

Periodic acid–Schiff (PAS) staining is used to evaluate glycogen concentration, and PAS staining is performed according to the instructions of the commercial kit (Sembejia Biotechnology Co., Ltd.). Briefly, sections were first incubated with periodate solution protected from light for 5 min. It was then incubated with Schiff's reagent for 20 min. Finally, the nuclei were counterstained with hematoxylin. The stained cartilage structures turned purple or red. Sections were observed using a differential interference contrast (DIC) optical microscope (DMIL LED, Leica Co., Ltd., Germany). Capture at least three fields of view per slice.

Bone-specific alkaline phosphatase assay

Bone-specific alkaline phosphatase (ALP) is a common osteoblast marker and is generally used to detect the number and activity of osteoblasts. We used calcium cobalt staining to detect the expression of ALP. Experiments were performed according to a commercial kit (KeyGEN BioTECH Co., Ltd, Nanjing, Jiangsu, China). Briefly, sections were incubated with ALP solution for 2 min at room temperature, then with cobalt nitrate solution for 5 min, and finally counterstained with eosin. Stained sections were visualized with a Leica DMIL LED microscope (Leica Co., Ltd.). Osteoblasts will be stained black. Capture at least three fields of view per slice.

Detection of apoptosis assay by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining was performed according to the kit's instructions. It can mark broken DNA. Nuclei were counterstained with hematoxylin after staining. The sections were observed with an inverted microscope (Leica Co., Ltd.), and three fields of view were randomly selected for each section to take pictures. TUNEL-positive cells and total cells were counted with Image J, and their ratios were calculated. Each slice counts at least three fields of view.

Immunohistochemical staining

Immunohistochemical staining is mainly used to mark the expression of a certain protein in tissue. The operation steps can be briefly summarized as heat-induced epitope repair, blocking, primary antibody incubation, secondary antibody incubation, diaminobenzidine tetrahydrochloride (DAB) color development and hematoxylin counterstaining of nuclei. We choose 10% goat serum as the blocking solution. We chose horseradish peroxidasie (HRP)–labeled goat antirabbit immunoglobulin G (IgG) (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) as the secondary antibody. The stained sections were observed with an inverted microscope (Leica Co., Ltd.). At least three fields of view were randomly selected for each slice, and the mean optical density (MOD) of the positive signal was analyzed using Image J software. The MOD ratio of the signal is expressed as the ratio of the MOD of the signal to the total signal.

The primary antibodies we used in this experiment include the following: anti-BMP-2 polyclonal antibody (1:1000; Abcam, ab6285, Cambridge, UK); anti-Runx2 polyclonal antibody (1:1000; ABclonal, A2851, Wuhan, China); anti-Vegf polyclonal antibody (1:1000; ABclonal, A0280); and anti-Hif-1α polyclonal antibody (1:1000; ABclonal, A11945).

Serum bone Gla protein analysis

Three months after the operation, three JWRs were randomly selected from each group. Approximately 3.5 mL of blood was collected through ear vein sampling. Some blood is used for direct testing, and the rest is used for serum separation. The blood and serum collected are used to determine the rabbit's blood routine, liver function, kidney function, and serum bone Gla protein (BGP). Use LH 750 automatic blood analyzer (Beckman Coulter Inc., Brea, CA, USA) for blood routine examination. Use DXC 800 automated biochemical analyzer (Beckman Coulter Inc.) for blood biochemical test.

Statistical analysis

In this study, statistical results were expressed as mean value ± standard deviation (SD). We considered this difference to be statistically significant when $P < 0.05$. When pairwise comparisons were made, we used Student's *t*-test for data analysis, such as differences in trabecular bone and mineral density between the injured side and the normal side. Other results in this study were calculated using one-way analysis of variance (ANOVA).

Results

Morphometric analysis of vertebral bone tissue

First, compare the injured side and the normal side of the vertebrae of each group of JWRs by general observation (Figure 2(A) and (B)). We can see that the bone defect area in the control group was hardly repaired, and the bone defect area in the material group was slightly repaired, while the BMP group had the best repair effect. The degree of repair is close to normal bone. The X-ray detection results were used to calculate the repair ratio of bone defects in each group. The results are shown in the Figure 2(C).

In order to further evaluate the degree of bone repair in each group of spina bifida defect areas, the X-ray scan results were used to calculate bone density, which was positively correlated with brightness. The calculation results show that compared with the normal group, the brightness of the control group and the material group is the lowest, and the bone defect is hardly repaired effectively. The BMP group had better repairing effects in the bone defect area, and the bone density values were not significantly different compared with the normal group.

HE staining to detect the morphological structure of the surgical site

The results of HE staining showed that after surgical treatment and feeding for three months, compared with the normal group, there was no obvious bone repair in the control group and the material group three months after the operation. In the control group, almost no bone fibers were seen in the damaged area; while only a small amount of bone fibers were observed in the damaged area of the material group. Correspondingly, a large number of bone trabeculae and fibrous tissue were found in the bone repair area of the BMP group, which had been connected with the osteogenic tissue, and only had a small amount of hollow structure. The BMP group had the least cavitation structure and formed the most uniform bone tissue (Figure 2(D)). These results showed that the BMP group showed a good repair effect, and the repair effect of the BMP group was better than that of the collagen material alone.

The effect of the combination of collagen matrix and BMP-2 on osteoblasts, collagen, and sugar related to bone formation

Sirius red staining is used to detect different collagen fibers, and type 1 collagen can be stained bright orange. According

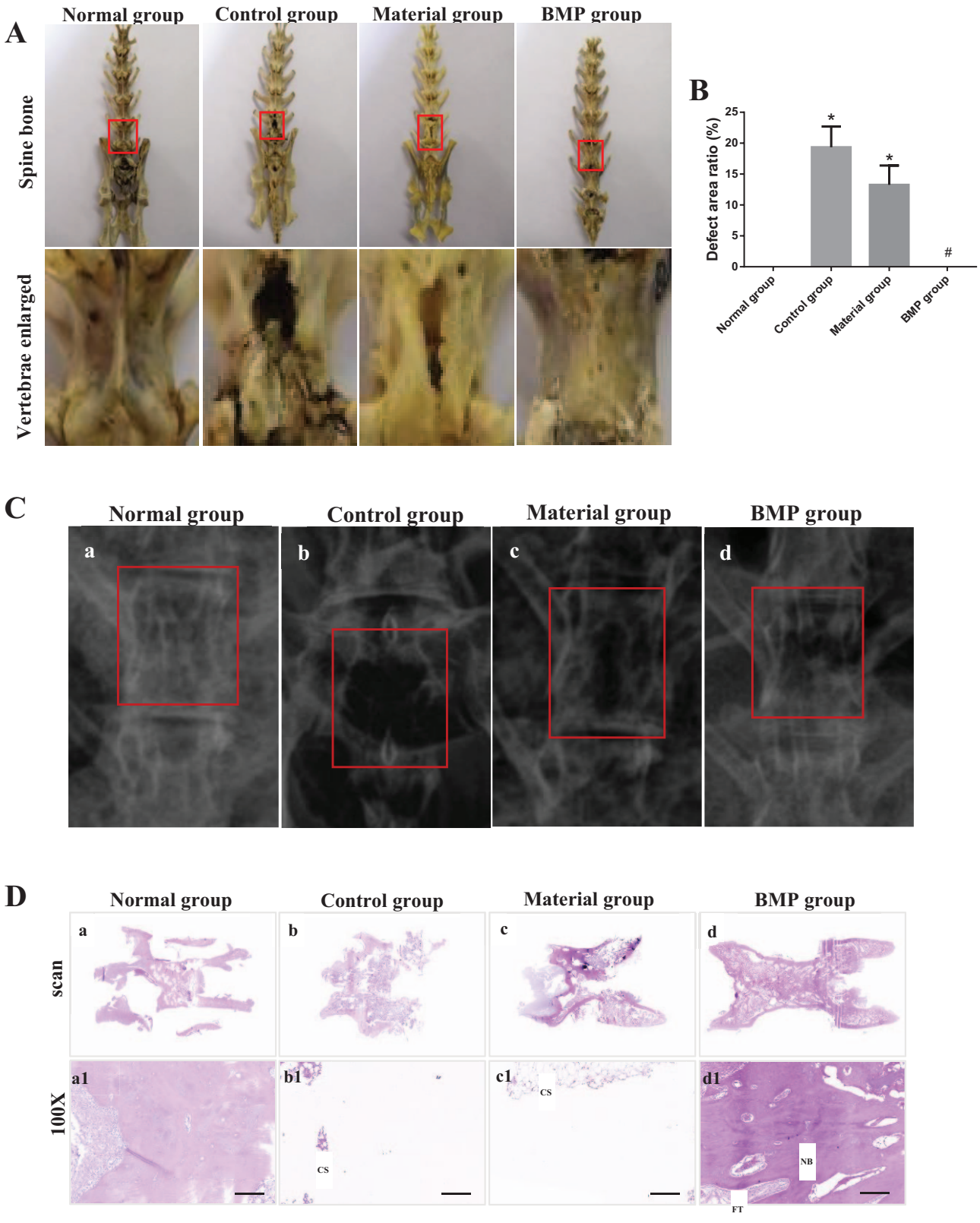

Figure 2. Gross observation of vertebrae morphology and structure. (A) The general appearance of the vertebrae three months after surgery. The red box and red plus sign assist in displaying the recovery of the transplanted area. (B) The statistics of bone defect area ratio, each group was compared with the control group, and the difference was denoted by * (*$P < 0.05$). (C) X-ray analysis of vertebral bodies was performed three months after surgery. (D) HE staining results – (a to d) and (a1 to d1) HE staining results three months after surgery. (a) and (a1) Normal group; (b) and (b1) control group; (c) and (c1) material group; (d) and (d1) BMP group. (a) to (d) Tissue scan results after HE staining. (a1) to (d1) The result of HE staining after 100× magnification. Scale bar is 100 μm. (A color version of this figure is available in the online journal.)
CS: cavitation structure; FT: fibrous tissue; NB: new bone.

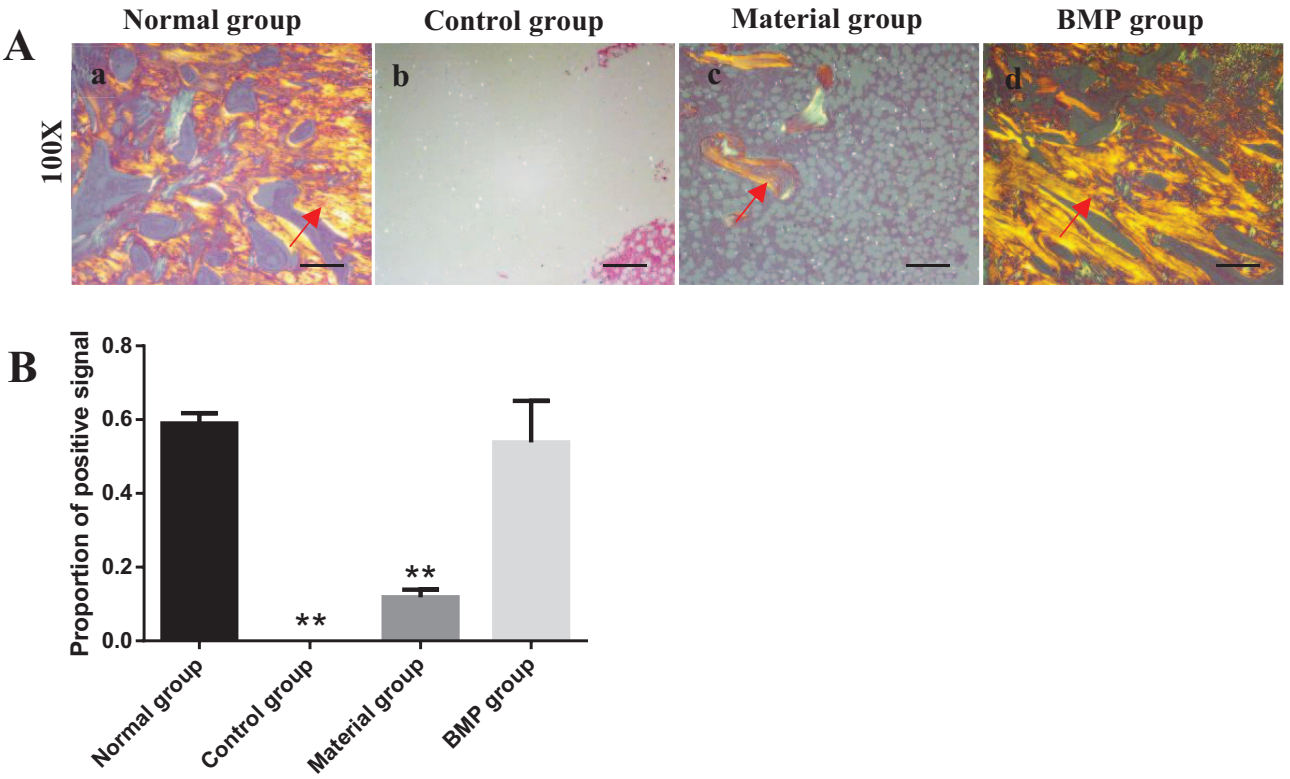

Figure 3. Sirius red staining results (100×). (A) (a) to (d) Sirius red staining results of each group three months after surgery. (B) The percentage of type 1 collagen three months after surgery. All groups were compared with the normal group, and the statistical difference was denoted by *. Scale bar is 100 μm. (A color version of this figure is available in the online journal.)
**$P < 0.01$.

to the staining results, we can see that type 1 collagen is widely and uniformly distributed in normal vertebrae. Compared with the normal group, almost no type 1 collagen was observed in the defect area of the control group. In the defect area of the common material group, a small amount of type 1 collagen was observed due to the formation of a little bone tissue. When the collagen matrix was implanted into BMP, a large amount of type 1 collagen was observed in the original defect area. The statistical results are shown in Figure 3. These results suggest that the use of active bone material fused to BMP-2 induces the formation of type 1 collagen more efficiently than the use of ordinary collagen material alone.

In normal cartilage matrix, carbohydrates are an important component, and PAS staining can be used to detect the content and location of carbohydrates. The staining results showed that normal vertebrae do not have chondrocytes due to lack of osteogenesis. The control group also had no distinct red or purple areas. The trabecular borders of the common material group were red or purple because of a small amount of new bone. In the BMP group, a large purple-red area appeared in the trabecular bone due to a large amount of cartilage formation (Figure 4(A)). These results indicate that combining the collagen matrix with BMP can promote the growth of chondrocytes better than using only the collagen matrix.

ALP staining is used to detect osteoblasts, which can be stained black. The results showed that there were a few positive signals in normal vertebrae, suggesting that there were a few osteoblasts in normal bone tissue. The control group had very little positive signal due to very little bone tissue formation. Both the material group and the BMP group had black signals, and the BMP group had the most positive signals (Figure 4(B)). These results suggest that the active bone material combined with BMP-2 promotes the formation of osteoblasts better than the common material alone.

Analysis of apoptosis in bone defects

TUNEL staining can detect cell apoptosis at the surgical site. Since TUNEL can mark broken DNA, the nuclei of apoptotic cells will be stained brown. It can be seen from the results that since there is almost no new bone formation in the bone defect position of the control group, statistics cannot be performed, and the apoptosis rate is statistically zero. At the same time, the apoptosis rate of the material group was higher than that of the normal group, while the apoptosis rate of the BMP group was lower than that of the normal group. The difference is significant (Figure 5). This indicates that the defect recovery effect of BMP group is better than material group.

Use immunohistochemistry to detect the expression of four cytokines

The expression of BMP-2 is shown in Figure 6(A) and (B). At three months after operation, part of BMP-2 was expressed in osteocytes of normal group. Since there was almost no new bone formation in the defect area of the control group, only very little BMP-2 expression was observed. In the material group, both osteoblasts and osteoclasts at the edge of the

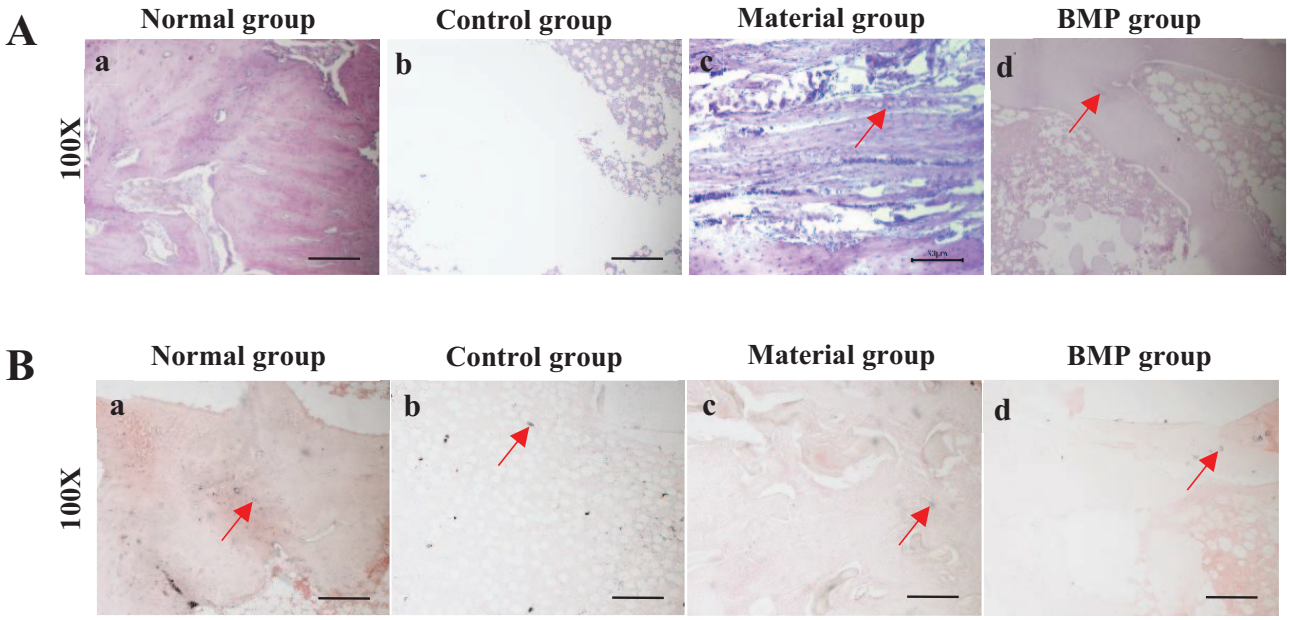

Figure 4. Expression of glycogen and alkaline phosphatase in vertebral tissue. (A) PAS staining results (100×). (a) to (d) PAS staining results three months of each group after surgery. (B) ALP staining results (100×). (a) to (d) ALP staining results three months of each group after surgery. Mark the area of the positive signal with a red arrow. Scale bar is 100 μm. (A color version of this figure is available in the online journal.)

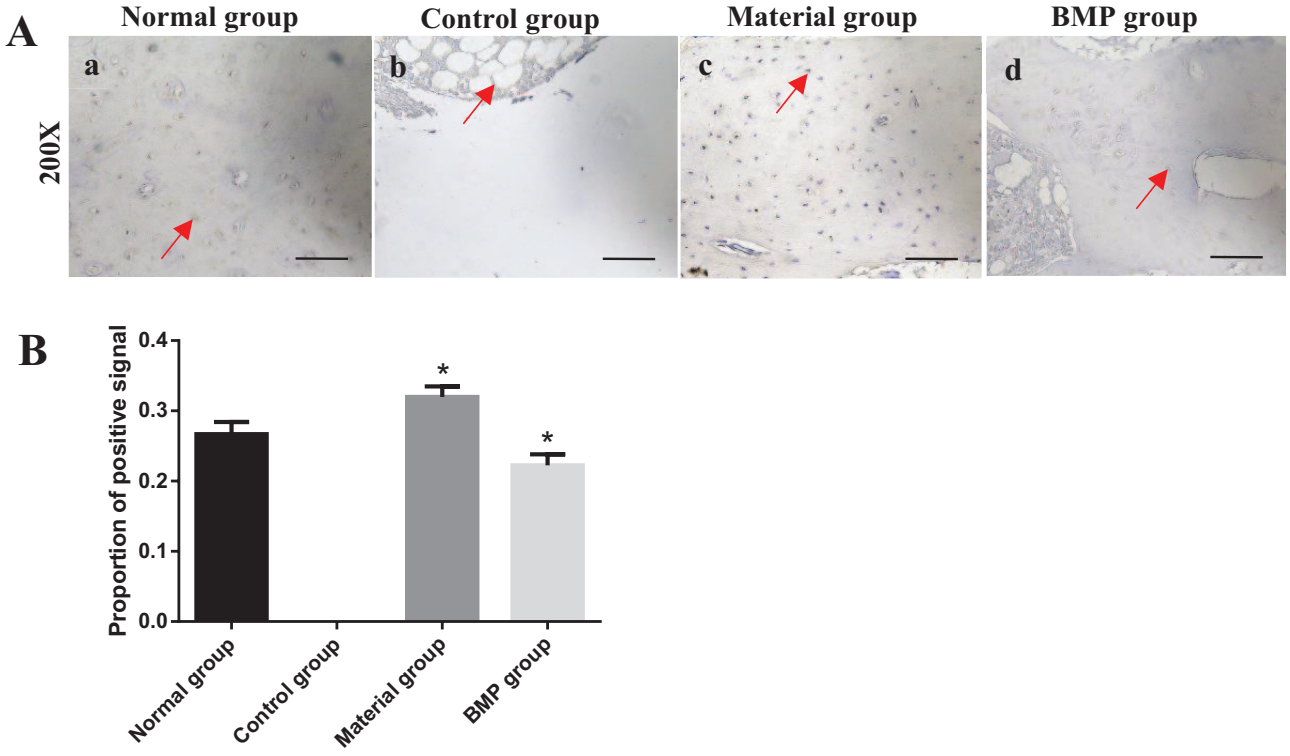

Figure 5. TUNEL results (200×). (A) (a) to (d) TUNEL staining results three months after surgery. The areas with positive signals are marked with red arrows. (B) The percentage of positive signal in each group at three months. Scale bar is 50 μm.
Represents the statistical difference between each group and the normal group ($P < 0.05$).

trabecular bone had a certain expression of BMP-2, but the expression was slightly lower than that in the normal group. The BMP group had the highest expression level because it carried BMP-2 itself. The above differences are all significant.

The expression of Runx2 is shown in Figure 6(C) and (D). As a cytokine related to osteogenesis, Runx2 has the highest expression in the material group. We speculate that the BMP group has relatively perfect osteogenesis, so the expression level of Runx2 is similar to that of the normal group. However, only a small amount of expression was observed in the control group because the complete skeleton was not formed.

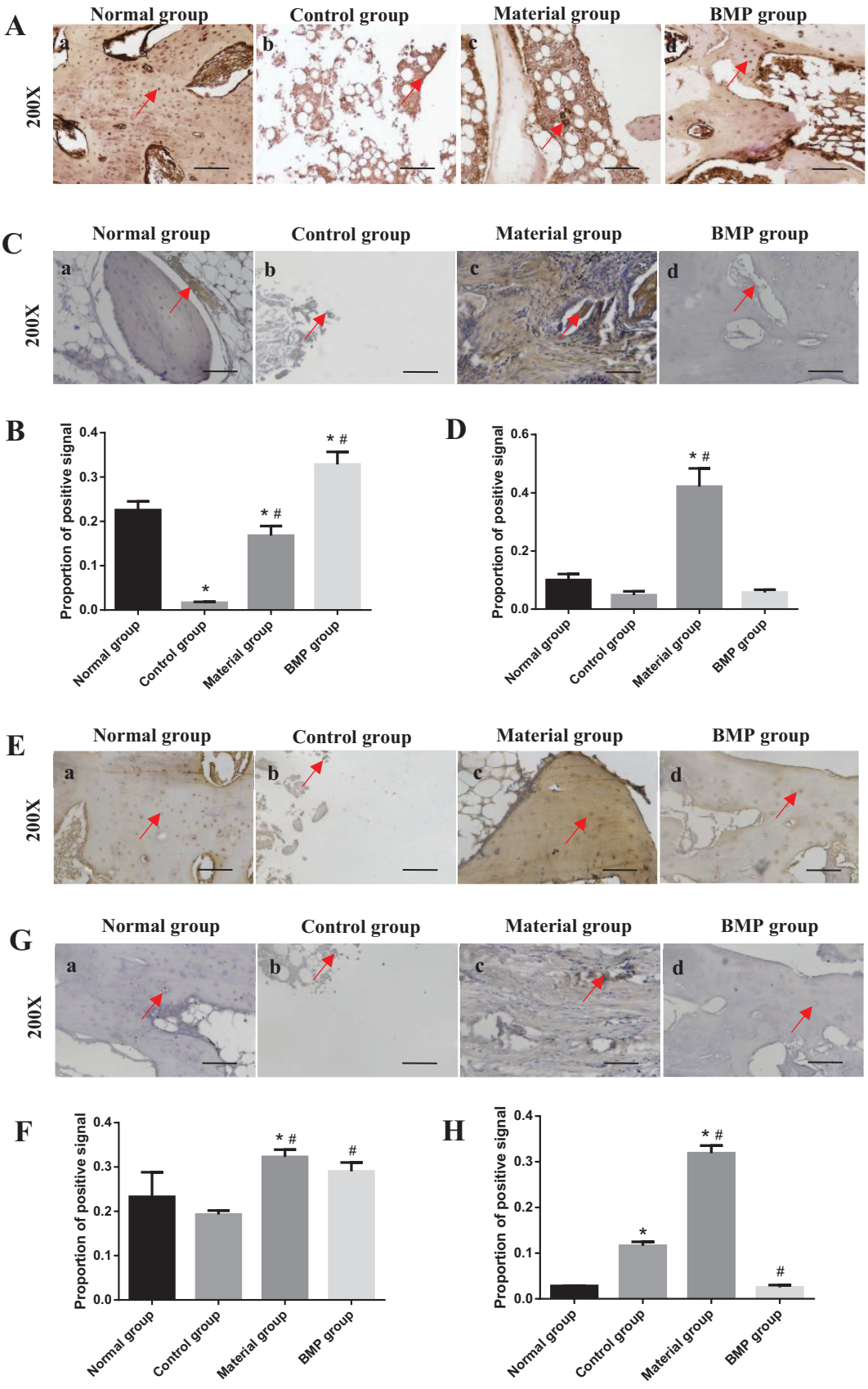

Figure 6. IHC test results in vertebral tissue three months after surgery. (A and B) BMP-2; (C and D) Runx2; (E and F) Vegf; (G and H) Hif-1α. (A, C, E and G) The picture of IHC test result (200×), the areas with positive signals are marked with red arrows. (B, D, F and H) The percentage of positive signal in each group at three months. Scale bar is 50 μm. (A color version of this figure is available in the online journal.)
Represents the statistical difference between each group and the normal group ($P < 0.05$).
#Represents the statistical difference between each group and the control group (#$P < 0.05$).

The expression of Vegf is shown in Figure 6(E) and (F). Vascular endothelial growth factor is associated with angiogenesis, and we also observed its highest expression level in the material group, indicating that there is more vigorous angiogenesis in this group.

The expression of Hif-1α is shown in Figure 6(G) and (H). The expression of hypoxia-inducible factor-1 is similar to the Runx2. We also believe that the bone repair of BMP group has been basically completed.

The result of serum BGP analysis

The detection result of BGP is shown in Figure 7. It can be seen from the results that the content of BGP in the BMP group was comparable to that in the normal group and slightly higher than that in the control and material groups. This suggests that the former has more vigorous osteoblast activity than the latter.

Assessment of postoperative health status of rabbits

Use blood routine (Table 1), liver function (Table 2), and kidney function (Table 3) to evaluate the health of the rabbits at three months after surgery, and obtain the data of each experimental group with the normal group and the control group data for comparison. The blood routine results three months after the operation showed that the standard deviation of red blood cell distribution width (RDW-SD) value and the mean corpuscular volume (MCV) value of the material group were higher than those of the normal group, and the difference was significant. While the mean corpuscular hemoglobin concentration (MCHC) value was higher. The normal group was significantly reduced. In addition, the mean corpuscular volume (MCV) value in the BMP group was higher than that

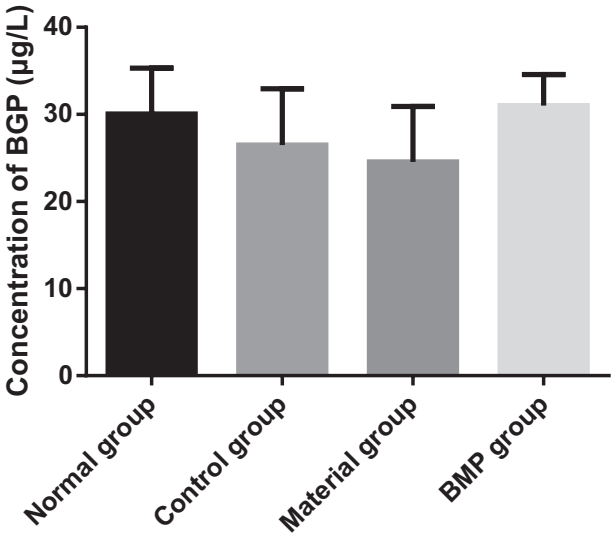

Figure 7. Serum BGP concentration three months after surgery.

Table 1. Blood routine test results three months after surgery.

Detection index	Unit	Normal group	Control group	Material group	BMP group
RBC	10^{12}/L	5.987 ± 0.329	5.537 ± 0.133	5.190 ± 0.512	5.243 ± 0.207
HCT	%	38.433 ± 1.668	37.767 ± 0.531	37.500 ± 3.184	38.900 ± 2.325
RDW-CV	10^9/L	13.167 ± 0.591	14.700 ± 1.720	15.767 ± 0.704	13.733 ± 1.320
RDW-SD	%	29.600 ± 2.061	35.467 ± 2.980	39.467 ± 0.602**	35.667 ± 2.719
MCV	fL	64.300 ± 1.745	68.300 ± 2.197	70.567 ± 1.960*	72.833 ± 2.106**
HBG	g/L	122.333 ± 2.494	118.333 ± 3.091	112.667 ± 9.104	117.000 ± 5.354
MCH	pg	20.433 ± 0.785	21.400 ± 0.942	21.167 ± 0.403	22.000 ± 0.589
MCHC	g/L	318.000 ± 7.118	313.000 ± 4.320	300.000 ± 4.320*	302.333 ± 4.784*
WBC	10^9/L	8.617 ± 1.030	8.710 ± 1.715	8.107 ± 0.923	8.900 ± 1.979
LYM#	10^9/L	3.091 ± 0.699	2.539 ± 0.282	3.093 ± 0.458	2.646 ± 0.085
LYM %	%	35.647 ± 5.742	29.827 ± 3.667	38.047 ± 2.402	30.943 ± 5.500
NEUT#	10^9/L	4.763 ± 0.576	5.367 ± 1.241	4.343 ± 0.513	5.424 ± 1.590
NEUT %	%	55.390 ± 4.150	61.110 ± 2.679	53.633 ± 3.176	60.007 ± 4.461
MONO#	10^9/L	0.542 ± 0.102	0.620 ± 0.200	0.439 ± 0.097	0.532 ± 0.242
MONO %	%	6.417 ± 1.600	6.903 ± 1.116	5.433 ± 1.228	5.703 ± 1.286
EO#	10^9/L	0.172 ± 0.024	0.161 ± 0.022	0.200 ± 0.031	0.262 ± 0.086
EO %	%	1.993 ± 0.100	1.893 ± 0.244	2.503 ± 0.505	2.903 ± 0.426
BASO	10^9/L	0.048 ± 0.015	0.023 ± 0.010	0.031 ± 0.013	0.036 ± 0.012
BASO %	%	0.553 ± 0.117	0.267 ± 0.090	0.383 ± 0.153	0.443 ± 0.202
PLT	10^9/L	150.000 ± 4.899	193.333 ± 16.214	175.333 ± 26.662	180.000 ± 19.799
PDW	%	15.900 ± 0.490	15.700 ± 0.141	16.167 ± 0.403	15.800 ± 0.432
MPV	fL	7.167 ± 0.411	6.767 ± 0.287	7.000 ± 0.497	6.700 ± 0.510
PLCR	%	13.700 ± 3.374	11.033 ± 2.330	14.133 ± 2.500	11.100 ± 2.406
PCT	%	0.035 ± 0.004	0.043 ± 0.005	0.043 ± 0.005	0.077 ± 0.052
CRP	mg/L	5.193 ± 0.754	9.113 ± 2.169	6.290 ± 7.220	9.220 ± 5.252

BMP: bone morphogenetic protein; RBC: red blood cell; HCT: hematocrit; RDW-CV: red blood cell volume distribution width; RDW-SD: standard red blood cell distribution width; MCV: mean corpuscular volume; HBG: hemoglobin; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cell; LYM: lymphocyte; NEUT: neutrophil granulocyte; MONO: monocyte; EO: eosinophil; BASO: basophil; PLT: platelet; PDW: platelet distribution width; MPV: mean platelet volume; PLCR: platelet-large cell ratio; PCT: procalcitonin; CRP: C-reactive protein.
Mean and SD values were calculated for each group.
Represents the statistical difference between each group and the normal group ($P < 0.05$; **$P < 0.01$).

Table 2. Liver function test results in blood three months after surgery.

Detection index	Unit	Normal group	Control group	Material group	BMP group
ALT	IU/L	43.400 ± 2.174	48.933 ± 18.570	22.700 ± 5.356#	44.500 ± 4.869
AST	IU/L	21.833 ± 0.205	27.367 ± 3.886	22.633 ± 2.969	25.833 ± 5.949
ALP	IU/L	55.933 ± 26.423	77.433 ± 21.389	89.967 ± 13.061	125.067 ± 10.135
TP	g/L	52.733 ± 1.391	66.700 ± 4.742	59.567 ± 2.613	63.900 ± 8.799
ALB	g/L	32.567 ± 2.904	45.500 ± 2.269***	40.733 ± 2.330*	41.067 ± 2.117*
GLB	g/L	20.167 ± 4.282	21.200 ± 4.411	18.833 ± 3.838	22.833 ± 7.069
A/G		1.710 ± 0.445	2.253 ± 0.542	2.253 ± 0.527	1.937 ± 0.454
TBIL	Umol/L	9.323 ± 0.751	12.380 ± 1.422*	11.113 ± 0.774	11.450 ± 1.384
DBIL	Umol/L	5.257 ± 0.968	5.740 ± 1.128	4.497 ± 0.450	4.340 ± 0.868
IBIL	Umol/L	4.067 ± 1.625	6.640 ± 1.013	6.617 ± 0.416	7.110 ± 0.529*

BMP: bone morphogenetic protein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; TP: total protein; ALB: albumin; GLB: globulin; TBIL: total bilirubin; DBIL: bilirubin direct; IBIL: indirect bilirubin.
Mean and SD values were calculated for each group.
Represents the statistical difference between each group and the normal group ($P < 0.05$; ***$P < 0.001$).

Table 3. Renal function results test in blood three months after surgery.

Detection index	Unit	Normal group	Control group	Material group	BMP group
BUN	mmol/L	5.197 ± 0.649	4.447 ± 0.616	4.627 ± 0.956	6.647 ± 1.459
CR	mmol/L	65.333 ± 6.928	119.433 ± 3.584*	95.690 ± 5.046	106.590 ± 27.378
UA	mmol/L	29.667 ± 0.047	31.633 ± 2.734	29.700 ± 0.000	30.133 ± 0.613

BMP: bone morphogenetic protein; BUN: blood urea nitrogen; CR: creatinine; UA: uric acid.
Mean and SD values were calculated for each group.
Represents the statistical difference between each group and the normal group ($P < 0.05$).

in the normal group, while the MCHC was lower in the BMP group three months after surgery. There were significant differences between the two groups.

The liver function test results showed that for alanine aminotransferase (ALT), the material group had a significant decrease compared with the control group. For albumin (ALB), the control group, material group and BMP group all have a significant increase compared with the normal group. For indirect bilirubin (IBIL), the BMP group was significantly higher than the normal group.

Renal function test results showed that the creatinine (CR) value of the control group was significantly higher than that of the normal group, but there was no significant difference between the material group and BMP group. This shows that after modeling spina bifida of JWRs and repairing them with bone repair materials and active bone materials for three months, the liver and kidney functions of JWRs are less affected, and the safety of various repair materials can be guaranteed.

Discussion

Among the common central nervous system diseases in humans, spina bifida is one of the most common diseases and one of the most difficult to treat. Many researchers are also working on related research. Related treatments involve many disciplines to ensure the best treatment results and provide patients with the best treatment (quality of life).[8,9] Developmental abnormalities of the central nervous system can lead to NTDs and serious complications. There are two forms of NTD, spina bifida dominant and spina bifida recessive. Overt spina bifida can be divided into open spina bifida and closed spina bifida according to whether it is accompanied by exposed nerve tissue. Spina bifida occulta, however, usually has no abnormal appearance and can only be detected by imaging studies.

The recombinant human bone morphogenetic protein (rhBMP-2) is a polyglycan protein with a relative molecular mass of approximately 32×10^3. rhBMP-2 is similar to natural BMP-2 in the process of inducing osteogenesis, but rhBMP-2 induces osteogenesis earlier than natural BMP-2, and also has different effects on the amount of osteogenesis, the formation of blood vessels and bone marrow-like tissue, and so on. It is obviously better than natural BMP-2.[9] Up to now, BMP-2 has achieved a series of research results in the repair of bone defects. Taking rabbits as an example, researchers have used the method of loading BMP-2 with biomaterials to complete the repair of rabbit skull defects,[10] and it was found that BMP-2-loaded composite scaffolds could significantly increase the amount of bone formation; BMP-2-loaded biomaterial group had more consistent bone regeneration in bone defects, while no BMP-2-loaded group, bone regeneration mainly occurred in the edge of the bone defect gradually develops toward the center of the defect.[11] Other researchers combined Adv-BMP-2-transfected autologous bone marrow mononuclear stem cells (BMSCs) with natural coral to repair the 12 mm defect of the lower edge of the orbital bone in rabbits. After 16 weeks, gross observation and micro-computed tomography (CT) confirmed that the rabbit bone defect was healed well, and the bone mineral density was also good enough. Bone formation was significantly higher than the BMSCs combined with natural coral group and pure natural coral group.[12] In addition, BMP-2 loaded biomaterials have also been used to repair radial and femoral injuries, knee cartilage injuries, and spinal fusion,[13–16] with better repair results.

BMP-2 is a cytokine that can promote the differentiation of mesenchymal stem cells into osteoblasts. Although BMP-2 has a high activity in osteoblasts, when it is implanted in the body alone, it is easily diffused and degraded, thereby impairing its function. In a previous study, some researchers placed the scaffold material in a BMP-2 solution to allow it to adsorb. Then, it was found that the adsorbed BMP-2 was rapidly released in a short time.[17] In fact, however, the excessively rapid release of BMP-2 is unfavorable for its sufficient promotion of osteogenesis.[18] Furthermore, in clinical practice, BMP-2 is transported to the appropriate site of osteogenesis through a carrier and released in situ. Therefore, in this study, the "active bone material" we used was collagen material fused with BMP-2. This new material can slowly release BMP-2 on the basis of traditional collagen materials. This ensures that the regeneration of bone defects can be effectively promoted for a long period of time. This technology has been authorized by China's national patent.

The research results also show that although ordinary collagen materials can also play a certain repairing effect. However, the repair effect is obviously inferior to that of active bone material. Serum test results also indicated that the active bone material did not cause an inflammatory response in animals, proving its good histocompatibility. Therefore, this material is expected to be used for clinical treatment of bone tissue regeneration in the future.

In conclusion, our research has found that the use of active bone materials or the combination of collagen matrix can significantly improve the repairing effect of JWRs spina bifida defect without causing negative effects on the body such as inflammation. The clinical application provides a valuable reference.

AUTHORS' CONTRIBUTIONS

X-CS, XM, and H-FX designed the study. X-CS, HW, and J-HL were responsible for the *vivo* surgery. X-CS and HW were responsible for *in vitro* experiments. X-CS, HW, and HFX prepared the manuscript. Y-FY and L-QY provided the bone repair materials and collagen membranes. X-CS, HW, J-HL and H-FX were responsible for revising the manuscript critically for important intellectual content. X-CS, HW, and J-HL contributed equally to this paper. All authors read and approved the final manuscript.

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ORCID ID

Hu Wang https://orcid.org/0000-0002-7802-9167

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