

# Bone collagen particles combined with hUC-MSCs to repair alveolar cleft in rabbits

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## Research

**Keywords:** alveolar cleft, Bone collagen particle, hUC-MSCs (human umbilical cord mesenchymal stem cells), micro-focus computerized tomography (micro-CT)

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# Abstract

**Background:** Alveolar cleft is a kind of cleft lip and palate, which seriously affects the physical and mental health of patients. In this study, a similar model of human alveolar cleft phenotype was established in rabbits to evaluate the effect of bone collagen particles combined with human umbilical cord mesenchymal stem cells (hUC-MSCs) on the repair of alveolar cleft.

**Materials & Methods :** In this study, 24 adult Japanese white rabbits (JWRs) were selected and randomly divided into 4 groups. Including normal group, control group, materials group and MSCs group. The model of alveolar cleft was established by removing the incisors on the left side of the upper jaw. The normal group did not receive any treatment. In the control group, the incisors were removed and sutured directly. In the material group, the incisor were removed, then filled with bone collagen particles, and finally sutured. In the MSCs group, the incisors were first removed, then filled with bone collagen granules incubated by hUC-MSCs, and then stitched. Blood biochemical analysis was performed 3 months after the operation. Skull tissues were collected for gross observation, and micro-focus computerized tomography (micro-CT) analysis. Paraffin sections were prepared for histological and immunohistochemical staining.

**Results:** The bone collagen particles and hUC-MSCs are not biotoxic and can promote alveolar regeneration. Bone collagen particles combined with hUC-MSCs were much better than those used alone in inducing bone repair and regeneration.

**Conclusions:** hUC-MSCs can be used as a bone regeneration inducer combined with bone materials for bone regeneration and repair.

**Keywords:** alveolar cleft, Bone collagen particle, hUC-MSCs (human umbilical cord mesenchymal stem cells), micro-focus computerized tomography (micro-CT)

## 1. Background

Alveolar cleft is common in clinic, which not only affects the normal eruption of teeth and the development of jaws, but also affects the physical and mental health of patients. Therefore, it is very important to establish an animal model of alveolar cleft that is similar to human alveolar cleft disease and can be duplicated. This method can provide a theoretical basis for the occurrence and development of the disease, and also provide a good scientific research foundation for the repair of alveolar cleft.

The most commonly used model animals are primates (1,2), sheep(3), canine animals(4), felids(5), rodents and rabbits(6,7). Rabbits have the advantages of short growth cycle, easy feeding and low cost. Moreover, compared with other animals, rabbits are of moderate size, gentle temperament and easier to operate. Studies in our laboratory have also proved that rabbits can be used to establish animal models of alveolar cleft (8). Therefore, rabbits were selected as experimental animals to establish the animal model of alveolar cleft.

Alveolar cleft caused only by facial muscles can be corrected or repaired by surgical suture, but there is still no effective treatment for alveolar cleft caused by bone defect. At present, the treatment methods of alveolar cleft can be divided into distraction osteogenesis and bone grafting.

Traction osteogenesis refers to the technology of bone correction or repair by applying physical traction force in a specific direction and frequency to partially or completely detached biological tissue so that the gap is gradually replaced by new bone. Binquer et al. used traction osteogenesis to correct alveolar cleft (9). Although this method avoids the immunogenicity of the foreign implanted tissue, the procedure is complicated, requires a long treatment period, and requires two operations to place and remove the retractor. Still not a good treatment.

Bone materials commonly used in bone grafting include autogenous bone, allogeneic bone, allogeneic bone and tissue-engineered bone. Boyne et al. have repaired oronasal fistulas by inserting a small amount of cancellous bone from the autogenous ilium into the fractures (10). This method is considered to be the gold standard for the clinical repair of alveolar clefts (11,12). Nevertheless, autologous bone grafts are bound to cause donor trauma and deformity, which an ideal repair should avoid. Nique et al. once used allograft bone to treat alveolar cleft, and postoperative imaging showed that the tooth successfully erupted and grew into the graft bone, but this process required a long time (13). El Deeb et al. filled hydroxyapatite in the crack of the alveolar cleft and found no tooth eruption (14). Allogeneic bone allograft or artificial bone, although can avoid donor area deformity, but there is a risk of immune rejection and transmission of disease. With the development of tissue engineering technology, the application of tissue engineering bone to repair alveolar fractures is no longer a problem. Currently, the most widely used scaffold materials are collagen, hydroxyapatite and so on (15,16). Because of the complex structure and function of bone tissue, it is difficult for a single material to meet the demand. It is commonly used to repair alveolar clefts by combining scaffold materials with factors or stem cells that induce bone regeneration. Compared with factors, stem cells have the advantages of low cost and easy access. Common stem cells are bone marrow mesenchymal stem cells (17), umbilical cord mesenchymal stem cells (18,19), embryonic stem cells and so on. Compared with stem cells derived from bone marrow, stem cells derived from umbilical cord have the advantages of low immunogenicity, rapid proliferation, wide availability and no ethical concerns (19,20). Chronic spinal cord injury in dogs has been treated with hUC-MSCs combined with collagen scaffolds (21).

In this study, bone collagen granules prepared by decellularization and degreasing of bovine cancellous bone were used as scaffold materials. The main ingredients are collagen and hydroxyapatite. It not only preserves its natural three-dimensional porous structure, but also reduces its immunogenicity. On this basis, composite human umbilical cord derived mesenchymal stem cells were used to repair alveolar fissure.

The purpose of this study was to investigate the feasibility and effectiveness of bone collagen particles inoculated with hUC-MSCs in the rabbit model of alveolar cleft. The results indicate that hUC-MSCs

combined with bone collagen particles may be a reliable alternative therapy for the repair of alveolar bone defects.

## 2. Results

### 2.1 Blood analysis:

Blood routine (Table 1&4), liver function (Table 2&5), renal function (Table 3&6) and BGP of each group were measured at 3 and 6 months after the surgery, and the values of each group were compared with those of the normal group. The blood routine results showed that NEUT content increased and LYM content decreased in the control group at 3 months, and they all tended to be normal at 6 months. The content of CRP, EO and NEUT in the material group increased at 3 months, while the content of LYM decreased, and all of them tended to be normal at 6 months. CRP content in MSCs group decreased at 6 months, and other indicators showed no significant abnormality. Liver function results showed that ALT and AST levels in the control group were higher than those in the normal group at 3 and 6 months. The content of ALT and AST in the material group was higher than that in the normal group at 3 months and normal at 6 months. No significant abnormalities were found in the MSCs group. Renal function results showed that CR levels in the control group, the materials group and the MSCs group increased significantly at 3 months and tended to normal at 6 months. BUN content in the control group and MSCs group increased significantly at 3 months and tended to be normal at 6 months. BGP results showed that at 3 months, the control group was lower than the normal group, while the materials group and the MSCs group were not significantly different from the normal group (Figure 2a). At 6 months, the content of BGP in the material group decreased, while that in the MSCs group increased even higher than that in the normal group (Figure 2b). To sum up, there was a certain inflammatory response in both the control group and the material group at 3 months, which increased the load on the liver and kidney. The MSCs group only increased the load of the kidney, but had no significant effect on the liver. At 6 months, the indicators of each group were basically normal. Serum BGP was continuously secreted in MSCs group and was higher than normal group. We hypothesized that hUC-MSCs could prolong bone repair time. This indicated that neither bone collagen granule material nor hUC-MSCs were toxic, and that hUC-MSCs could reduce inflammatory reactivity.

### 2.2 Imaging analysis:

*General observation (Figure 3).* The appearance of the skull was observed from both vertical and horizontal angles, with the surgical position in the red box. From a vertical point of view, asymmetry of the left and right maxillary bone was observed in both the control group and the material group, while no significant asymmetry was observed in the MSCs group. From a horizontal point of view, the repair effect of both materials was better at 6 months than at 3 months. The volume of new bone in the material group and the MSCs group increased significantly, while the surgical location of the control group did not change significantly in the two periods.

*Micro-CT imaging.* The internal images of the normal side and the operative side of each group were compared at 3 or 6 months for preliminary analysis (Figure 4 A&B). The red box is the surgical site. Accurate analysis of bone density and percentage of trabecular bone in each group (figure 4 C). Internal images from both angles showed no significant repair of the operative side in the control group during the two periods. A small amount of new bone tissue was found on the surgical side of the material group. A significant amount of new bone tissue was found on the surgical side of the MSCs group 6 months after surgery. The results showed that the percentage of bone trabeculae was the highest in the MSCs group, followed by the material group and the lowest in the control group (figure 4 Ca& Cc). Bone mineral density (BMD) of control group, material group and MSCs group was not significantly different at 3 months (figure 4 Cb). BMD of MSCs group was significantly higher than that of the other two groups at 6 months (figure 4 Cd). Therefore, the osteogenic ability of bone collagen particles combined with hUC-MSCs was significantly better than that of bone collagen particles alone.

## 2.3 Histological analysis:

*HE staining* HE staining showed no significant bone repair in the control group. There are only a few scattered bones. At 3 months after surgery, a large amount of bone fibers and a small amount of bone marrow and trabeculae were observed in the damaged area of the material group (Figure 5c1). At 6 months after surgery, trabeculae, a large amount of cavitation structure and a small amount of bone marrow were observed in the damaged area of the material group. At 3 months after the surgery, no cavitation structures were found in the damaged area of the BMP group, and a large number of bone trabeculae and fibrous tissues appeared (Figure 5d1). At 6 months after the surgery, there was still a large amount of bone trabeculae and fibrous tissue in the damaged area, and new bone tissue appeared (Figure 5h1). The results also showed that the osteogenic ability of bone collagen particles combined with hUC-MSCs was significantly better than that of bone collagen particles alone.

*Sirius red staining:* The Sirius red staining results showed that the orange color was darker at 6 months after the surgery than at 3 months after the surgery (Figure 6A). The statistical results showed that the percentage of type 1 collagen in the normal group was about 60%, that in the BMP group was about 40%, and that in the control group and the material group was extremely low. At 6 months after the operation, the percentage of type 1 collagen in the normal group and the BMP group remained basically unchanged, while the percentage of type 1 collagen in the material group increased to 40%, while the percentage in the control group remained extremely low. Compared with the control group and the material group, the BMP group had the highest bone trabecular content and almost all of them were bright orange. The results showed that the effect of collagen particles combined with hUC-MSCs on inducing collagen type 1 was stronger than that of collagen particles alone.

*PAS staining* Comparing the PAS staining results of the two periods, we found that the control group contained a large number of cavitation structures, and no obvious red or fuchsia areas were observed (Figure 7b&f). At 3 months after surgery, red or fuchsia was observed at the edge of trabecular bone in the

material group (Figure 7c), and decreased significantly at 6 months after surgery (Figure 7g). In the BMP group, red or fuchsia was darker and more intense at 6 months than at 3 months. The new bone tissue of the BMP group showed large red areas (Figure 7h). The results showed that collagen granule combined with hUC-MSCs could promote the generation of chondrocytes better than collagen granule alone.

*ALP* The results of ALP staining showed that the black color was obvious at 3 months after the surgery, and almost disappeared at 6 months after the operation. The black area was most obvious in the normal group at 3 months (Figure 8a), and no significant black area was observed at 6 months (Figure 8e). No significant black was observed in the control group (Figure 8b&f). The black areas in the material group and the MSCs group were mainly concentrated at the edge of the new trabecular bone at 3 months (Figure 8c&d). The black areas indicate the presence of osteoblasts. Therefore, it can be speculated that the number of osteoblasts at 3 months after collagen granule implantation is significantly higher than that at 6 months after implantation.

*IHC (BMP2)* Immunohistochemical results showed no significant expression of bmp-2 in normal group and control group (Figure 9a,b,e&f). In the materials group and the MSCs group, bmp-2 was mainly concentrated in osteocytes and osteoclasts at the edge of trabecular bone (Figure 9c,d,g&h). The expression level of bmp-2 in the material group and the MSCs group at 3 months after surgery was significantly higher than that at 6 months after surgery. The expression level of bmp-2 was the highest in MSCs group. The results showed that the ability of active collagen particles combined with hUC-MSCs to induce the generation of BMP2 was better than that of bone collagen particles alone.

## 2.4 Proliferation and apoptosis analysis

Ki67 (Figure 11B) and TUNEL (Figure 10B) methods were used to detect cell proliferation and apoptosis in each group at 3 and 6 months after surgery. TUNEL assay showed that the apoptosis rate of the control group was similar to that of the normal group at 3 months after surgery. The apoptosis rate of the material group was lower than that of the normal group. The apoptosis rate of MSCs group was significantly higher than that of normal group. The immunohistochemical test results of Ki67 showed that the proliferation rate of the control group and the material group was significantly lower than that of the normal group at 3 months after the surgery. The proliferation rate of the MSCs group was similar to that of the normal group. At 6 months after the surgery, there was no significant change in the proliferation rate of the control group, which was still lower than that of the normal group. The proliferation rate of the material group increased obviously but did not exceed that of the normal group. The proliferation rate of the MSCs group was still close to that of the normal group.

Comprehensive analysis showed that the apoptosis rate and proliferation rate of the normal group remained unchanged. The control group showed high apoptosis rate and low proliferation rate in both periods. In the material group, the apoptosis rate and proliferation rate were significantly increased. The MSCs group showed that the proliferation rate remained unchanged while the apoptosis rate decreased

significantly. Therefore, it can be speculated that the combination of bone collagen particles with MSCs can further promote cell proliferation and apoptosis, thus promoting bone regeneration.

### 3. Discussion

hUC-MSCs were taken from neonatal umbilical cords, which are medical waste. Therefore, it has the advantages of abundant source, low cost, no harm to donors and no ethical issues involved. This makes it an ideal candidate for the potential of stem cells in medical applications (22). Although hUC-MSCs have good bone inductivity, they are easy to be absorbed and degraded in vivo. Therefore, an ideal scaffold material is needed to enable hUC-MSCs to better act on bone injury sites [20]. The combination of scaffold material and hUC-MSCs can slow down the degradation rate of hUC-MSCs, thus prolonging the bone repair time.

Bone collagen particles used in this study are heterogenous bone made from bovine cancellous bone after degreasing and decellularization. Bone matrix particles after decalcification and deproteinization are mainly composed of hydroxyapatite and collagen. The material has high strength, strong bone conductivity and good biological safety. The bone matrix retains a natural three-dimensional network that facilitates cell implantation and growth. After degreasing and decellularization, the allogeneic bone can effectively reduce its immunogenicity (23). Heterogenous bone matrix is more widely derived than allograft bone, and the degradation and absorption time is shorter, all of which meet the requirements of ideal carrier.

The purpose of this study was to evaluate the effect of bone collagen particles combined with hUC-MSCs on the repair of alveolar bone defects. The results of the blood test showed that the bone collagen particles and hUC-MSCs were non-toxic, and that the hUC-MSCs could reduce the inflammatory response. Gross observation of the skull model and Micro CT scan results suggested that the effect of bone collagen particles combined with hUC-MSCs on bone regeneration and repair was stronger than that of bone collagen particles alone. Tissue staining results also showed that the bone collagen particles combined with hUC-MSCs had significantly increased trabecular bone formation rate. The number of osteoblasts and chondrocytes was also significantly increased. The expression levels of collagen 1 and bmp-2 in the MSCs group were also significantly higher than those in the material group. The results of cell proliferation and apoptosis suggested that the combination of bone collagen particles with hUC-MSCs could further promote cell proliferation and apoptosis, thus promoting bone regeneration.

Bone induction refers to the induction of connective tissue adjacent to bone graft by certain factors in bone materials. By affecting undifferentiated bone progenitor cells and promoting their differentiation and proliferation, they eventually become osteoblasts and promote the formation of new bone. The alveolar cleft model established in this study is a hole formed by pulling out the incisor [8]. After removing the incisors, the damage to the inner wall of the bone around the incisors was not obvious except for the root of the incisors. Therefore, the osteogenic induction ability of different positions was not uniform after the addition of bone collagen particles. Although there was still a certain gap between the newly

generated bone from bone collagen particles combined with hUC-MSCs and the normal incisor, it was enough to prove that hUC-MSCs could be used as bone generation inducer combined with bone materials for bone regeneration and repair. Therefore, future studies can start from how to improve the bone inductance of hUC-MSCs to different degrees of injury.

## **4. Conclusions**

First, we established the animal model of alveolar cleft based on the existing experience. The bone collagen particles combined with hUC-MSCs was then implanted into the rabbits. Bone regeneration and repair in each group were observed at 3 and 6 months after implantation. The bone collagen particles combined with hUC-MSCs are far better than the bone collagen particles alone in inducing bone repair and regeneration. This experiment provides a new method for the repair of alveolar cleft.

## **5. Materials And Methods**

### **5.1 Isolation & culture of hUC-MSCs**

HUC-MSCs were isolated from Wharton's jelly. In this study, tissue block adherent culture was used to isolate hUC-MSCs. The blood vessels in the umbilical cord were first removed and the tissue cut into about 1 cubic millimeter pieces. Finally, the tissue blocks were evenly spread on the bottom of the petri dish for primary culture. HUC-MSCs within five generations were collected to incubate bone collagen particles.

### **5.2 Preparation of implant materials**

Bone collagen granules are prepared from bovine trabeculae and retain their natural three-dimensional porous structure. The bone collagen granules were provided by zhenghai biotechnology co. LTD Yantai, China. Cells within 5 generations were selected to be inoculated with bone collagen granules and cultured in carbon dioxide incubator for 0.5 h. The concentration of hUC-MSCs should reach  $10^7$  /ml.

### **5.3 Surgical procedure & treatment (Figure 1)**

In this study, we used 24 female JWRs (bodyweight:  $2000 \pm 300$  g). These JWRs were purchased from huafukang biotechnology co. LTD Beijing, China. All these animals were kept in the animal room of National Research Institute for Family Planning, with free access to water and food. Temperature control in the 23 to 25 °C. Humidity of 50% to 60%. Noise control under 60dB. The light cycle is 12h for day and night. Keep the room clean, dry and ventilated. The study was approved by the local research and ethics committee.

Twenty-four JWRs were randomly divided into four groups (n = 6 in each group): normal group, control group, material group and MSCs group. Rabbits were anesthetized by intravenous injection of serazine hydrochloride (concentration: 1–2mg/kg). The model of alveolar cleft was established by removing the incisors on the left side of the upper jaw (Figure 1f-h). The normal group did not receive any treatment. In the control group, collagen membrane was directly covered and skin was sutured after incisor was removed. In the material group, after the incisions were removed, the holes were filled with bone collagen granules, and then the collagen membrane was covered and the skin was sutured. In the MSCs group, after the incisions were removed, the holes were filled with bone collagen particles incubated by hUC-MSCs, and then the collagen membrane was covered and the skin was sutured. The rabbits were treated with antibiotics for 1 week to prevent infection. Blood was collected from each group at 3 and 6 months after surgery. All rabbits were euthanized and the upper jaw was examined and obtained for further evaluation.

## 5.4 Blood analysis

At 3 months after the surgery, 3 rabbits were randomly selected from each group. Blood routine, liver function, kidney function and BGP of rabbits were detected by collecting 3.5ml of venous blood from the ears. At 6 months after the surgery, the indexes of the remaining 3 rabbits were detected.

## 5.5 Micro CT analysis

3 months after the surgery, one rabbit was randomly selected from each group to make a skull model. The procedure was repeated 6 months after surgery. The skull was photographed to record its appearance. Bone regeneration in skull was evaluated by micro CT. Bone regeneration in the alveolar cleft was evaluated using a micro CT system (SIEMENS Inveon Research Workplace 4.2, Beijing). The three-dimensional repair of the injuries in each group was observed, and the trabecular bone and bone density values were recorded.

## 5.6 Histology staining

Rabbits were euthanized and histologically evaluated at 3 or 6 months postoperatively. The specimens were immersed in 4% paraformaldehyde for 24h and then decalcified in 10% EDTA. After decalcification, the tissue was first embedded with paraffin, and then paraffin sections with a thickness of 4um were prepared by the mircotome.

The morphology of the cells was revealed by hematoxylin eosin (HE) staining. The nuclei are stained blue-violet by hematoxylin and the other tissue can be stained red by eosin. The secreted products of chondrocytes are metachromatic and can eventually differentiate into osteoblasts. PeriodicAcid-Schiff stain (PAS) staining can dye chondrocytes dark purple or crimson. Collagen type 1 is found mainly in

bone and tendon fibers and can be dyed bright orange by Sirius red. We also used Image J software to calculate the relative percentage of positive staining area in each section. Osteoblasts are one of the markers of bone formation. Black cobalt sulfide deposits can be formed by alkaline phosphatase (ALP) colorimetry for the location of osteoblasts.

Bone morphogenetic protein 2 (BMP2) is one of the markers of bone formation. Primary anti-BMP2 (ab6285,1:1000 dilution; Abcam Ltd) and HRP coupled secondary antibody were used to detect the positioning in slices. Ki67 can be used to locate proliferating cells. Primary anti-Ki67 (ab15580,1:1000 dilution; Abcam Ltd) was used to detect cell proliferation by immunofluorescence. TUNEL can be used to locate apoptotic cells. Three regions were randomly selected and the percentage of Ki67 and TUNEL positive cells was quantified using Image J software.

## 5.7 Statistical analysis

All values are expressed as the mean  $\pm$  SD.  $P < 0.05$  indicates statistical significance. Data were analyzed statistically by factorial analysis of variance and the Student's t-test with GraphPad Prism software (GraphPad Prism 6).

## Declarations

### Ethics approval and consent to participate

The study was approved by the ethics committee of the National Research Institute for Family Planning.

### Consent for publication

Not applicable

### Availability of data and materials

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

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

XCS, XM and HFX designed the study. XCS, HW and JHL were responsible for the vivo surgery and performing the procedure. XCS was responsible for in vitro experiments. XCS, HW were responsible for hUC-MSCs culture. XCS, HW and HFX prepared the manuscript. XCS, HW, DZ and HFX were responsible for revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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## Abbreviations

human umbilical cord mesenchymal stem cells (hUC-MSCs)

JWRs (Japanese white rabbits)

micro CT (microfocus computerized tomography)

hematoxylin eosin (HE)

alkaline phosphatase (ALP)

PeriodicAcid-Schiff stain (PAS)

immunohistochemical(IHC)

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## Tables

Detection index	Unit	Normal group	Control group	Material group	MSCs group
RBC	10 <sup>12</sup> /L	5.727±0.460	5.757±0.802	5.660±0.067	4.803±0.759
HCT	%	36.467±2.120	37.867±2.839	37.300±1.631	31.167±4.619
RDW-CV	10 <sup>9</sup> /L	13.033±0.873	14.400±0.589	13.333±0.262	15.267±2.864
RDW-SD	%	29.400±2.192	33.767±3.420	30.867±1.793	35.367±7.014
MCV	fL	63.800±1.344	66.367±4.739	65.133±2.593	64.900±1.283
HGB	g/L	117.667±6.182	123.000±13.491	121.000±2.944	103.333±16.680
MCH	pg	20.600±0.980	21.433±0.910	21.100±0.455	21.433±0.450
MCHC	g/L	323.000±10.033	323.667±10.965	324.667±8.179	330.667±13.123
WBC	10 <sup>9</sup> /L	13.170±2.788	8.680±2.961	10.037±1.848	8.603±1.062
LYM#	10 <sup>9</sup> /L	4.685±0.672###	2.243±0.452**	2.318±0.183**	3.195±0.428*
LYM%	%	36.080±2.401	28.633±9.420	23.670±3.746	37.130±2.007
NEUT#	10 <sup>9</sup> /L	7.547±2.054	5.652±2.275	6.606±1.369	4.589±0.761
NEUT%	%	56.617±3.356	63.050±6.846	65.517±2.569	53.220±4.038
MONO#	10 <sup>9</sup> /L	0.664±0.132	0.579±0.329	0.780±0.267	0.492±0.141
MONO %	%	5.120±0.889	5.923±2.294	7.573±1.156	5.740±1.511
EO#	10 <sup>9</sup> /L	0.229±0.022	0.153±0.070	0.320±0.113	0.235±0.047
EO %	%	1.800±0.318	1.710±0.513	3.090±0.565	2.760±0.556
BASO	10 <sup>9</sup> /L	0.045±0.017	0.052±0.033	0.016±0.003	0.092±0.043
BASO %	%	0.383±0.210	0.683±0.363	0.150±0.037	1.150±0.689
PLT	10 <sup>9</sup> /L	153.333±35.188	171.667±22.867	141.000±24.536	127.667±8.498
PDW	%	15.700±0.294	15.967±0.403	15.967±0.478	15.533±0.047
MPV	fL	7.167±0.411	7.133±0.249	7.300±0.653	6.767±0.450
PLCR	%	14.267±4.606	14.833±3.167	14.300±3.511	10.567±1.761
PCT	%	0.041±0.021	0.028±0.011	0.038±0.030	0.013±0.005
CRP	mg/l	6.400±1.425	12.433±4.488	6.870±3.556	3.143±2.191#

**Table 1** Blood routine test results at 3 months postoperatively. Mean SD values were calculated for each group. \* represents the statistical difference between each group and the normal group. # represents the statistical difference between each group and the control group. \*, # P<0.05 ; \*\*, ## P<0.05 0.01. RBC:red blood cell; HCT: hematocrit; RDW-CV: red blood cell volume distribution width; RDW-SD: 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: neutrophile granulocyte; MONO: monocyte; EO: eosinophil; BASO: basophil; PLT: platelet; PDW: platelet distribution width; MPV: mean platelet volume; PLCR: platelet-large cell ratio; PCT: platelet volume; CRP: C reactive protein.

Detection index	Unit	Normal group	Control group	Material group	MSCs group
ALT	IU/L	44.533±5.424	82.167±23.572	62.333±7.903	41.400±11.051#
AST	IU/L	30.567±6.215	76.000±48.880	44.833±29.613	16.167±2.595
ALP	IU/L	47.000±17.705	59.767±10.700	67.400±13.983	23.867±5.188
TP	g/l	52.533±0.694	58.000±3.827	59.133±2.845	48.900±7.920
ALB	g/l	33.500±0.424	38.633±3.307	36.667±1.066	27.900±7.896
GLB	g/l	19.000±1.042	19.400±2.825	22.467±3.561	21.000±2.140
A/G		1.767±0.119	2.040±0.381	1.680±0.299	1.347±0.411
TBIL	Umol/L	9.247±1.036	8.080±1.408	8.917±2.806	6.953±0.345
DBIL	Umol/L	5.093±0.718	3.870±0.388	4.817±0.749	2.977±0.310*
IBIL	Umol/L	4.153±1.311	4.210±1.632	4.100±2.088	3.977±0.553

**Table 2** Liver function test results in blood at 3 months postoperatively. Mean SD values were calculated for each group. \* represents the statistical difference between each group and the normal group. # represents the statistical difference between each group and the control group. \*, # P<0.05 ; \*\*, ## P<0.05 0.01. 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.

Detection index	Unit	Normal group	Control group	Material group	MSCs group
BUN	mmol/L	7.647±1.540	9.233±3.682	7.247±2.116	11.227±4.190
CR	mmol/L	69.707±7.026	103.413±27.201	94.840±15.769	107.587±27.686
UA	mmol/L	31.467±2.155	30.733±1.126	29.600±0.141	29.700±0.000

**Table 3** Renal function results test in blood at 3 months postoperatively. Mean SD values were calculated for each group. \* represents the statistical difference between each group and the normal group. # represents the statistical difference between each group and the control group. \*, # P $\leq$ 0.05 ; \*\*, ## P $\leq$ 0.05 0.01. BUN: blood urea nitrogen CR: creatinine UA: uric acid.

Detection index	Unit	Normal group	Control group	Material group	MSCs group
RBC	10 <sup>12</sup> /L	5.467±0.364	4.927±0.190	4.250±0.964	5.433±0.233
HCT	%	36.067±1.636	36.400±4.369	26.933±5.949	33.867±2.014
RDW-CV	10 <sup>9</sup> /L	13.200±0.356	12.567±0.865	12.567±1.096	12.833±0.759
RDW-SD	%	30.967±2.253	31.433±2.782	28.600±2.128	29.300±1.203
MCV	fL	66.133±3.642	70.400±2.264	63.500±0.920	64.000±2.551
HGB	g/L	123.000±2.944	122.000±10.801	92.667±18.571	115.333±6.944
MCH	pg	22.600±0.990	23.667±0.732	21.967±0.713	21.800±0.920
MCHC	g/L	342.000±8.832	336.000±10.614	345.667±7.587	340.667±1.886
WBC	10 <sup>9</sup> /L	10.727±0.281	8.347±0.241	9.167±2.172	10.777±1.829
LYM#	10 <sup>9</sup> /L	3.700±0.009	2.299±0.544	2.908±0.603	3.947±0.737#
LYM%	%	34.517±0.926	27.457±5.857	32.310±4.066	36.503±0.922
NEUT#	10 <sup>9</sup> /L	6.165±0.287	5.265±0.285	5.518±1.541	5.913±0.946
NEUT%	%	57.443±1.217	63.157±4.320	59.440±4.470	55.020±1.007
MONO#	10 <sup>9</sup> /L	0.566±0.024	0.580±0.134	0.565±0.106	0.557±0.101
MONO %	%	5.280±0.204	6.977±1.724	6.390±1.289	5.167±0.118
EO#	10 <sup>9</sup> /L	0.264±0.088	0.169±0.012	0.158±0.110	0.330±0.045
EO %	%	2.470±0.853	2.000±0.163	1.643±0.845	3.090±0.127
BASO	10 <sup>9</sup> /L	0.032±0.013	0.017±0.004	0.019±0.002	0.023±0.009
BASO %	%	0.360±0.060	0.175±0.025	0.217±0.029	0.220±0.057
PLT	10 <sup>9</sup> /L	137.333±13.695	148.667±20.997	156.333±39.878	126.333±8.380
PDW	%	15.800±0.163	15.833±0.403	15.733±0.205	16.233±0.125
MPV	fL	7.167±0.189	6.867±0.205	6.800±0.294	6.900±0.141
PLCR	%	13.400±1.349	11.367±1.247	11.467±3.055	13.500±1.980
PCT	%	0.027±0.012	0.030±0.014	0.040±0.029	0.017±0.005
CRP	mg/l	4.373±1.426	3.990±0.670	14.663±12.212	3.957±0.651

**Table 4** Blood routine test results at 6 months postoperatively. Mean SD values were calculated for each group. \* represents the statistical difference between each group and the normal group. # represents the statistical difference between each group and the control group. \*, # P<0.05 ; \*\*, ## P<0.05 0.01. RBC:red blood cell; HCT: hematocrit; RDW-CV: red blood cell volume distribution width; RDW-SD: 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: neutrophile granulocyte; MONO: monocyte; EO: eosinophil; BASO: basophil; PLT: platelet; PDW: platelet distribution width; MPV: mean platelet volume; PLCR: platelet-large cell ratio; PCT: platelet volume; CRP: C reactive protein.

Detection index	Unit	Normal group	Control group	Material group	MSCs group
ALT	IU/L	72.733±17.093	122.400±102.499	50.700±58.432	50.800±10.968
AST	IU/L	23.567±1.126	32.167±14.290	15.400±11.052	23.400±0.294
ALP	IU/L	60.567±10.076	59.933±25.986	39.800±20.467	58.567±4.615
TP	g/l	62.000±0.648	62.433±3.058	58.633±21.150	60.167±2.829
ALB	g/l	40.600±1.283	38.400±5.233	35.367±12.761	38.800±2.765
GLB	g/l	21.367±1.698	24.033±2.323	23.300±7.763	21.333±3.583
A/G		1.917±0.222	1.633±0.349	1.540±0.554	1.893±0.438
TBIL	Umol/L	4.807±0.813	6.710±1.714	6.720±0.070	3.570±0.536
DBIL	Umol/L	2.123±0.229	4.057±1.500	3.557±1.415	2.333±0.352
IBIL	Umol/L	2.683±0.877	2.653±0.476	2.160±0.854	1.237±0.189

**Table 5** Liver function test results in blood at 6 months postoperatively. Mean SD values were calculated for each group. \* represents the statistical difference between each group and the normal group. # represents the statistical difference between each group and the control group. \*, # P<0.05 ; \*\*, ## P<0.05 0.01. 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.

Detection index	Unit	Normal group	Control group	Material group	MSCs group
BUN	mmol/L	8.763±0.403	8.570±1.241	8.263±2.780	7.987±1.025
CR	mmol/L	99.877±7.261	100.123±4.054	88.000±32.339	89.457±8.307
UA	mmol/L	33.633±3.175	30.067±1.819	30.000±10.322	29.133±0.694

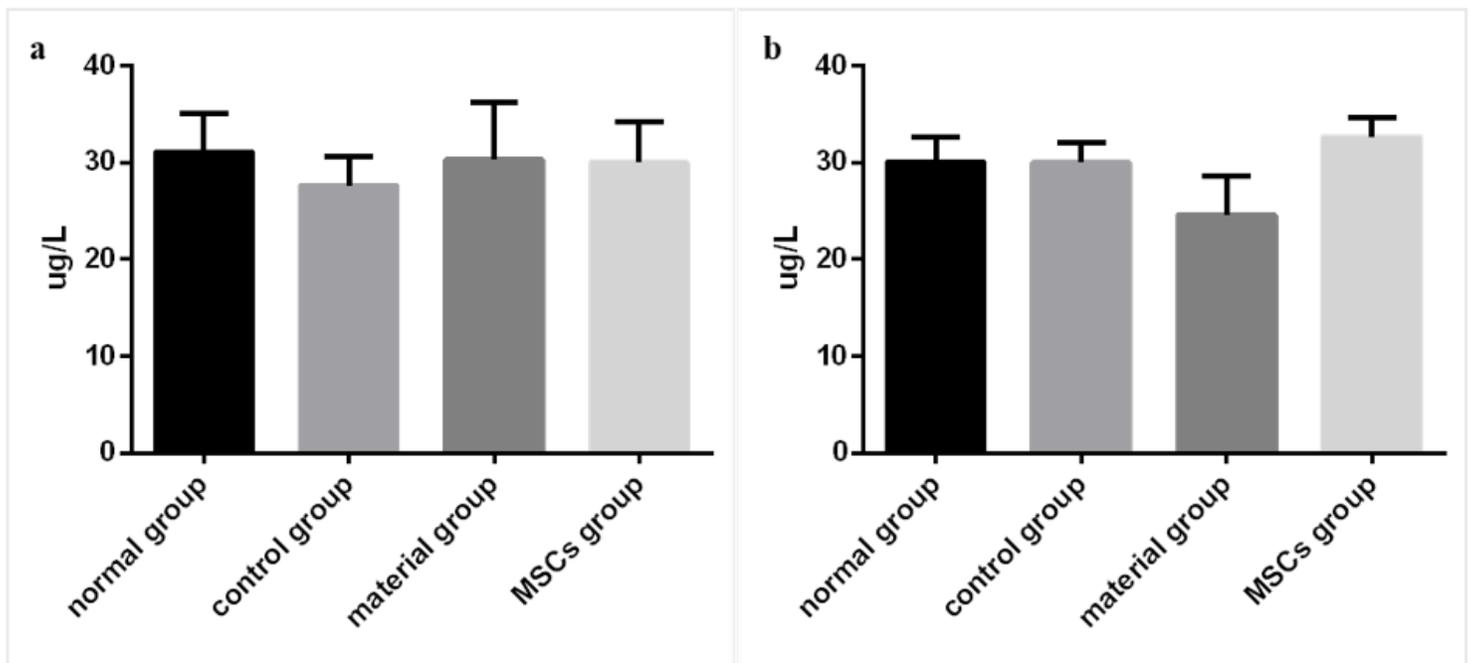
**Table 6** Renal function results test in blood at 6 months postoperatively. Mean SD values were calculated for each group. \* represents the statistical difference between each group and the normal group. # represents the statistical difference between each group and the control group. \*, #  $P \leq 0.05$ ; \*\*, ##  $P \leq 0.05$  0.01. BUN: blood urea nitrogen CR: creatinine UA: uric acid.

## Figures



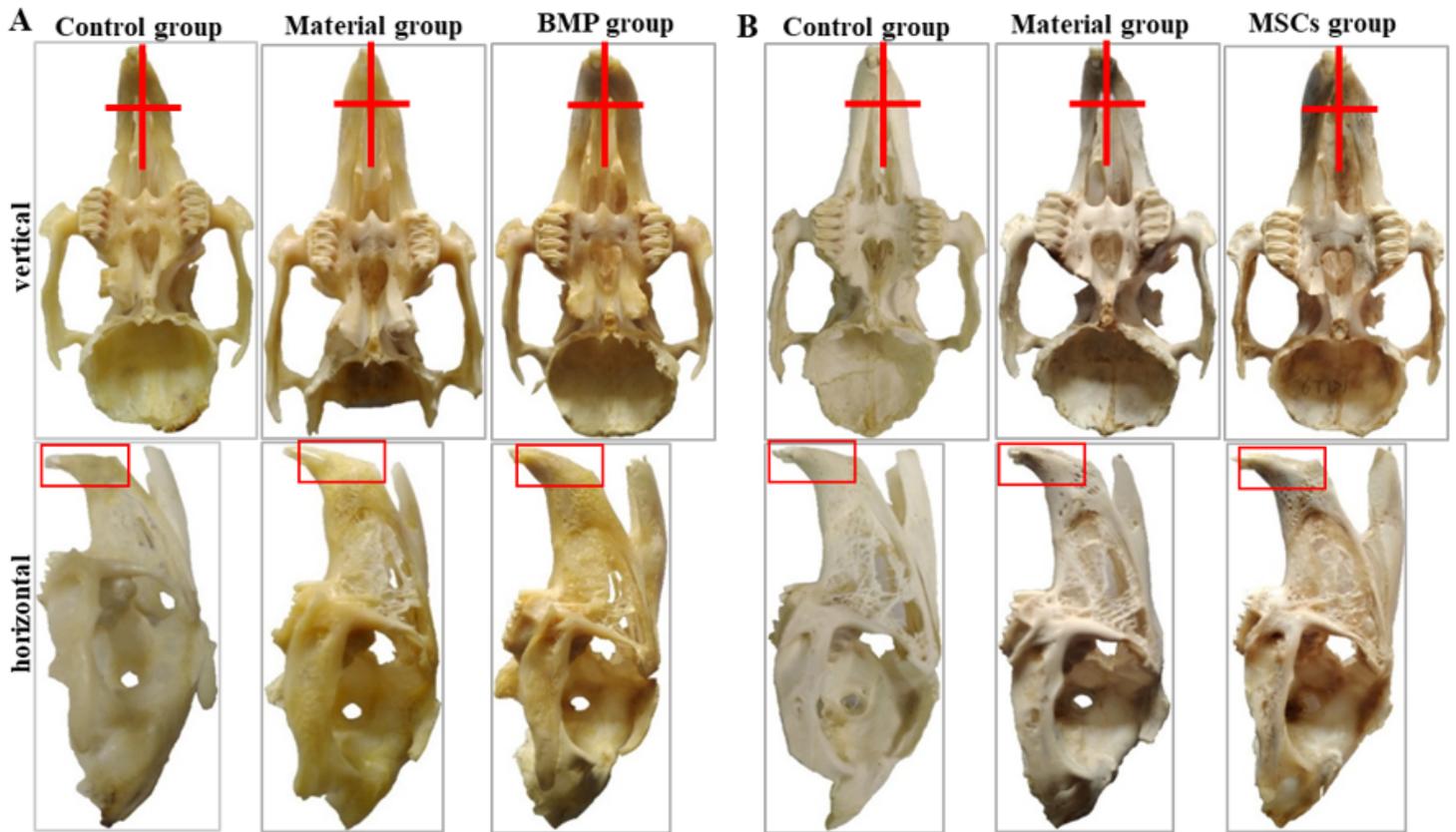
**Figure 1**

Surgical process: a collagen granules were incubated by hUC-MSCs. b fix the anaesthetized rabbits. c open the oral cavity after anesthesia. d-e incise the skin f-h remove the left incisor. i-j add collagen particles. k-i suture the skin .



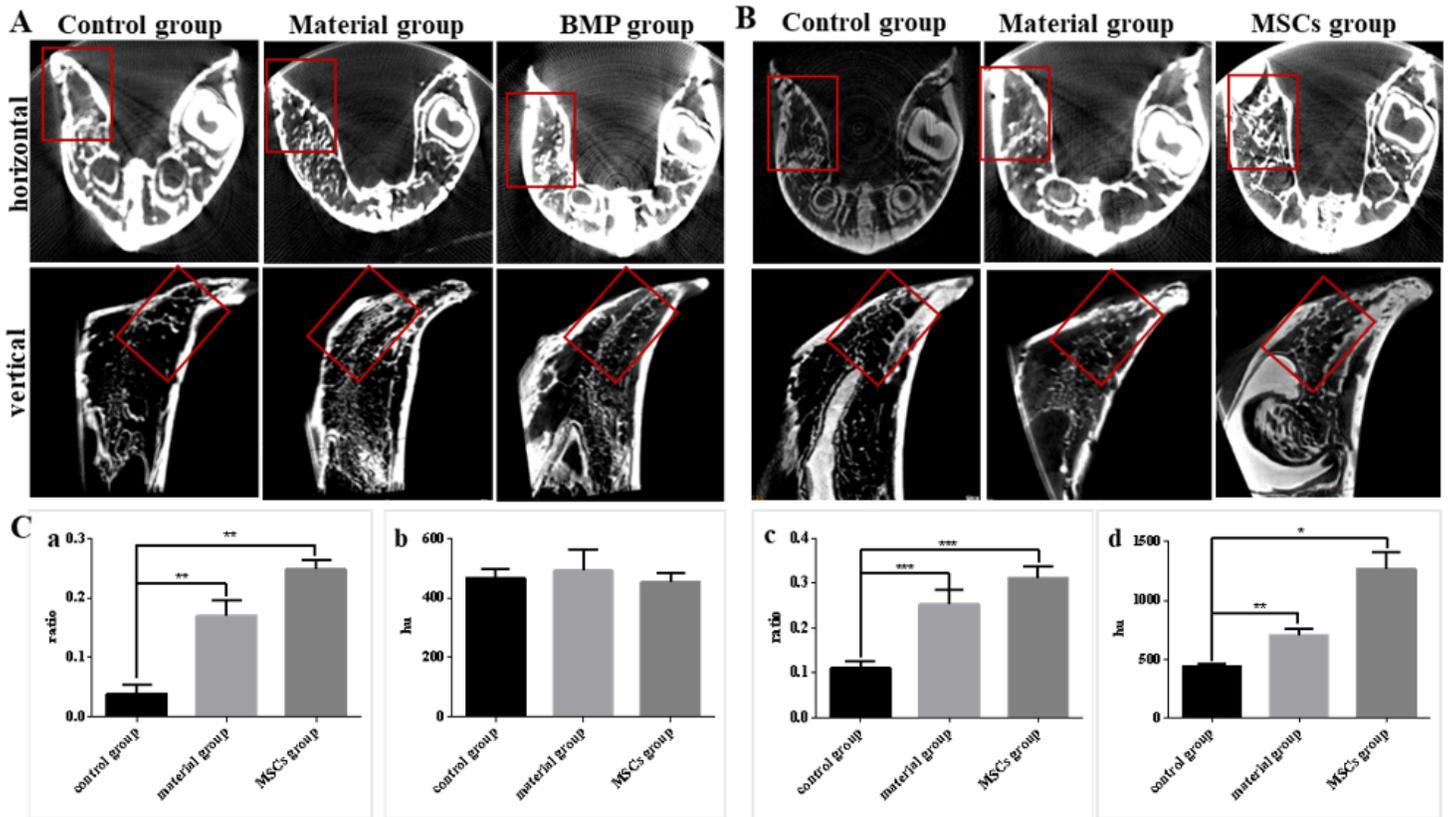
**Figure 2**

a: the content of serum BGP in each group at 3 months; b: the content of serum BGP in each group at 6 months.



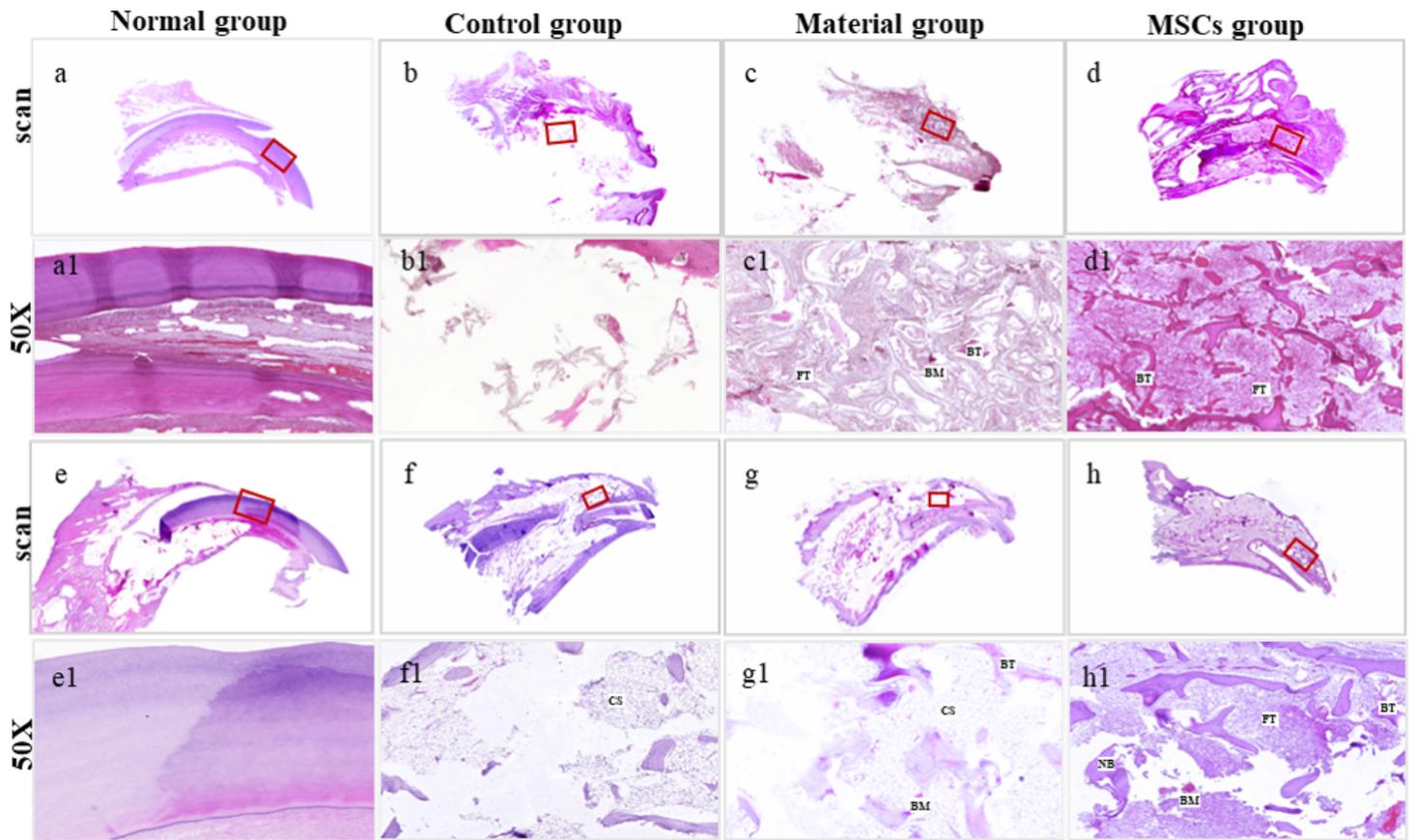
**Figure 3**

The general appearance of the skull and the red box is the surgical area. A sampling group 3 months after surgery; B sampling group 6 months after surgery. The red box and red plus sign assist in displaying the recovery of the transplanted area.



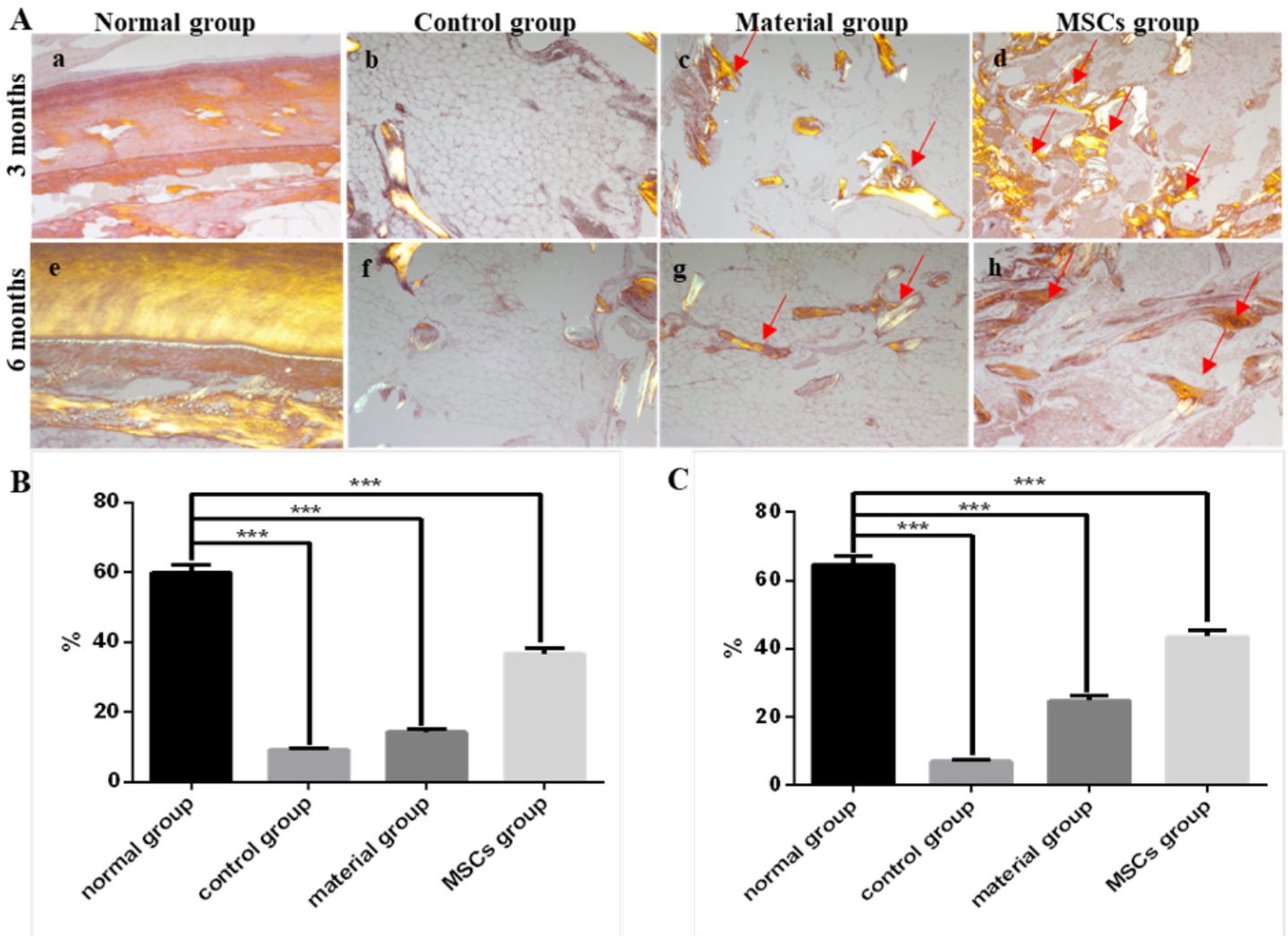
**Figure 4**

Micro CT results. A CT images from different angles at 3 months; B CT images from different angles at 6 months; C-a: the percentage of bone trabeculae at 3 months after surgery; C-b: the percentage of bone mineral density percentage at 3 months after surgery; C-c: the percentage of bone trabeculae at 6 months after surgery; C-d: the percentage of bone mineral density at 6 months after surgery. All groups were compared with the control group, and the difference was expressed as \*.The red box represents the surgical area.



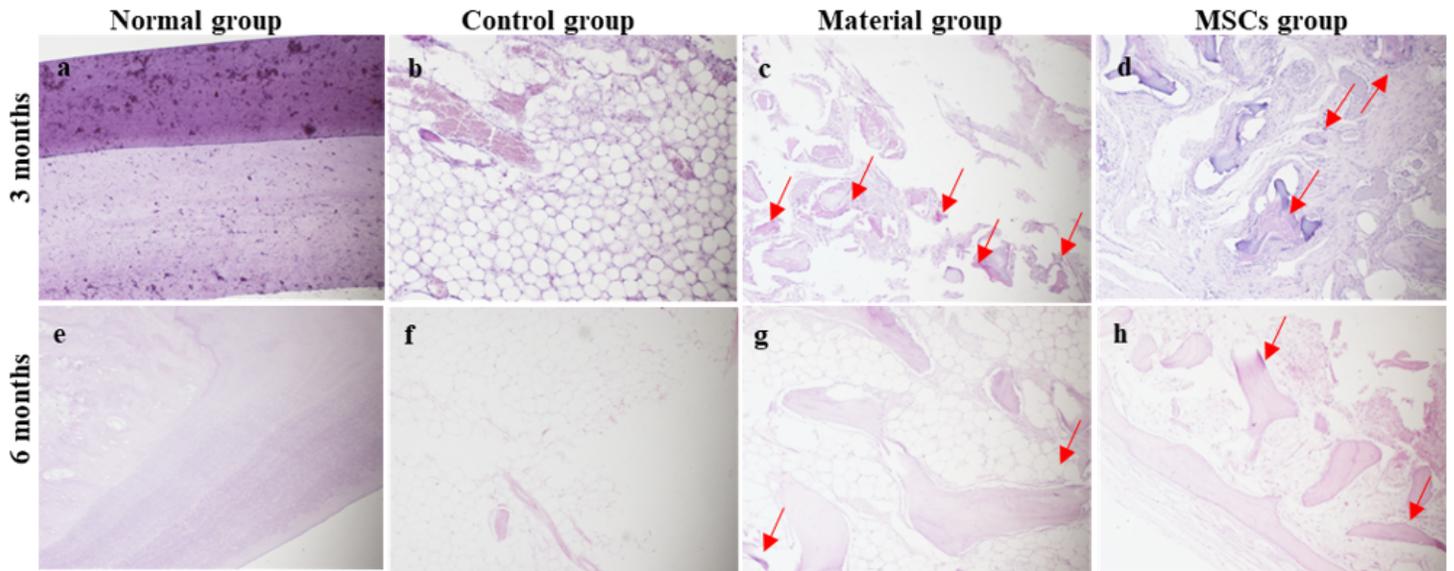
**Figure 5**

HE staining results. a-d & a1-d1 Sampling group 3 months after surgery; e-h & e1-h1 Sampling group 6 months after surgery. a,e & a1,e1 Normal group b,f & b1,f1 Control group c, g & c1, g1 Material group d, h & d1, h1 MSCs group. a-h The scan results of HE staining. a1-h1 The result of HE staining after 50 times magnification. BM: bone marrow; FT: fibrous tissue; BT: bone trabecula; NB: new bone; CS: cavitation structure.



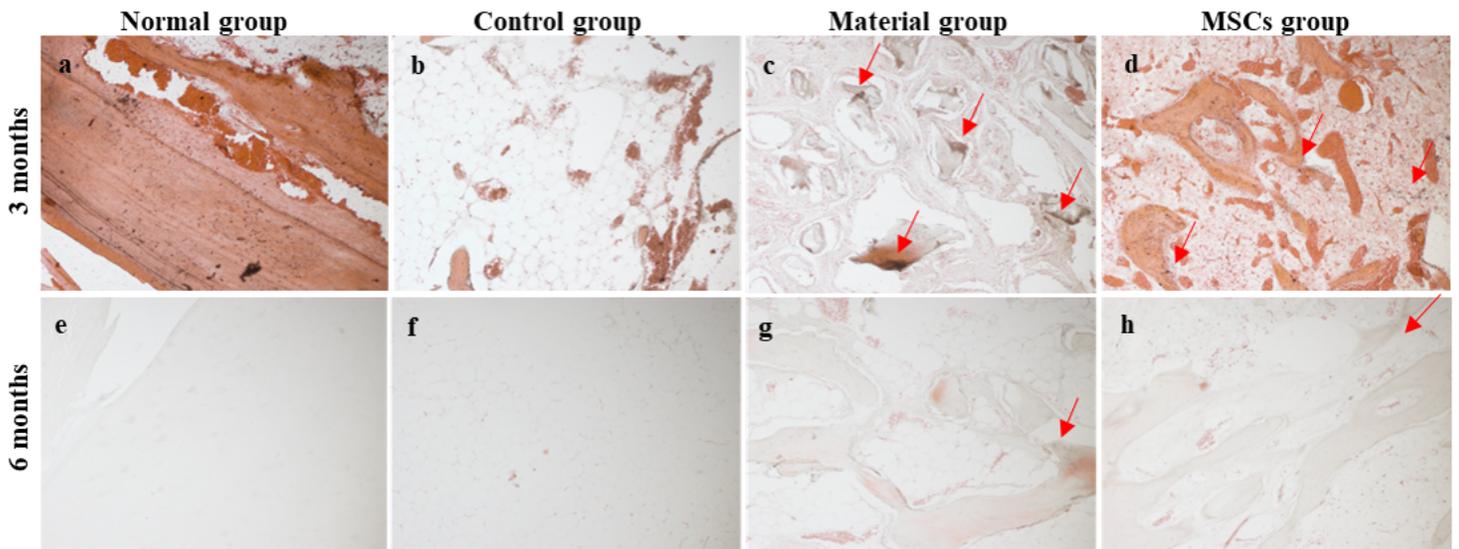
**Figure 6**

Sirius red staining results (40X). A a-d Sampling group 3 months after surgery; e-h Sampling group 6 months after surgery. a,e Normal group b,f Control group c, g Material group d, h MSCs group. Mark the area of the positive signal with a red arrow. B: The percentage of type 1 collagen in each group at 3 months after surgery. C: The percentage of type 1 collagen in each group at 6 months after surgery. All groups were compared with the Normal group, and the statistical difference was denoted by \*.



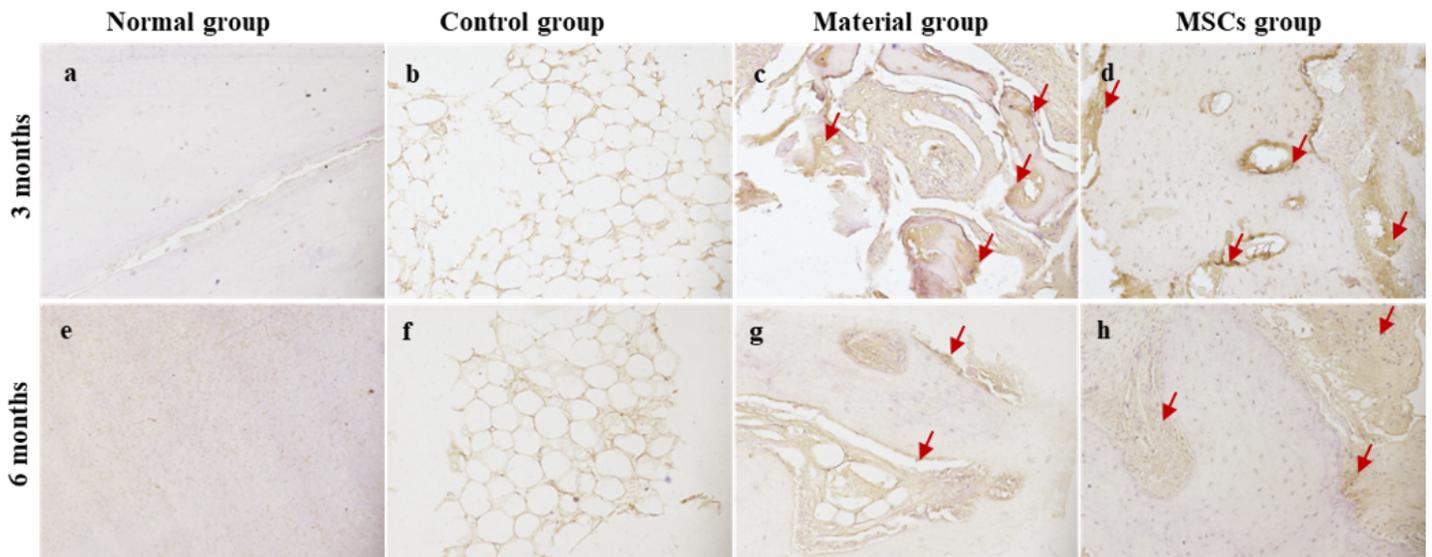
**Figure 7**

PAS staining results (100X). a-d Sampling group 3 months after surgery; e-h Sampling group 6 months after surgery. a,e Normal group b,f Control group c, g Material group d, h MSCs group. Mark the area of the positive signal with a red arrow.



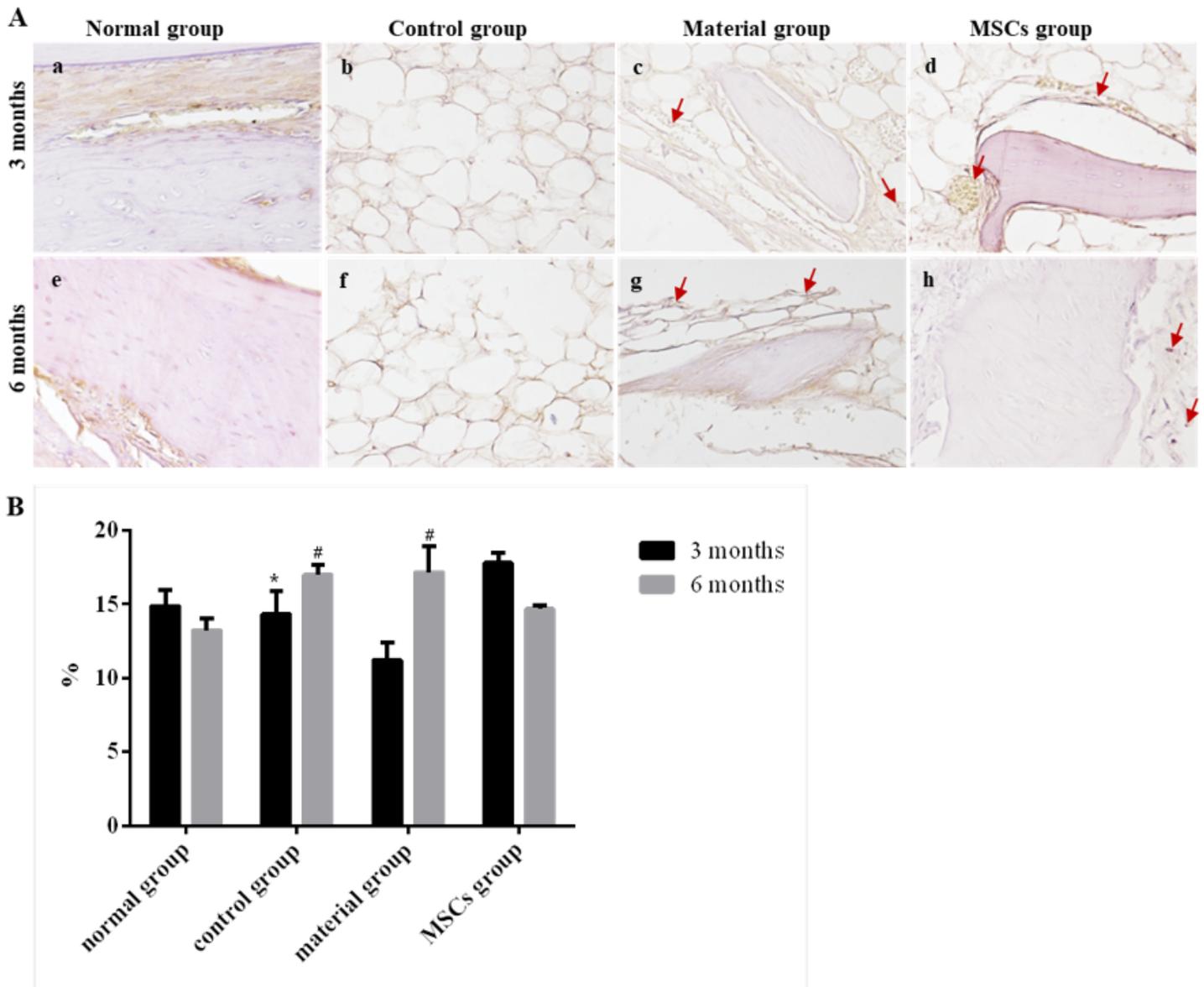
**Figure 8**

ALP staining results (100X).. a-d Sampling group 3 months after surgery; e-h Sampling group 6 months after surgery. a,e Normal group b,f Control group c, g Material group d, h MSCs group. Mark the area of the positive signal with a red arrow.



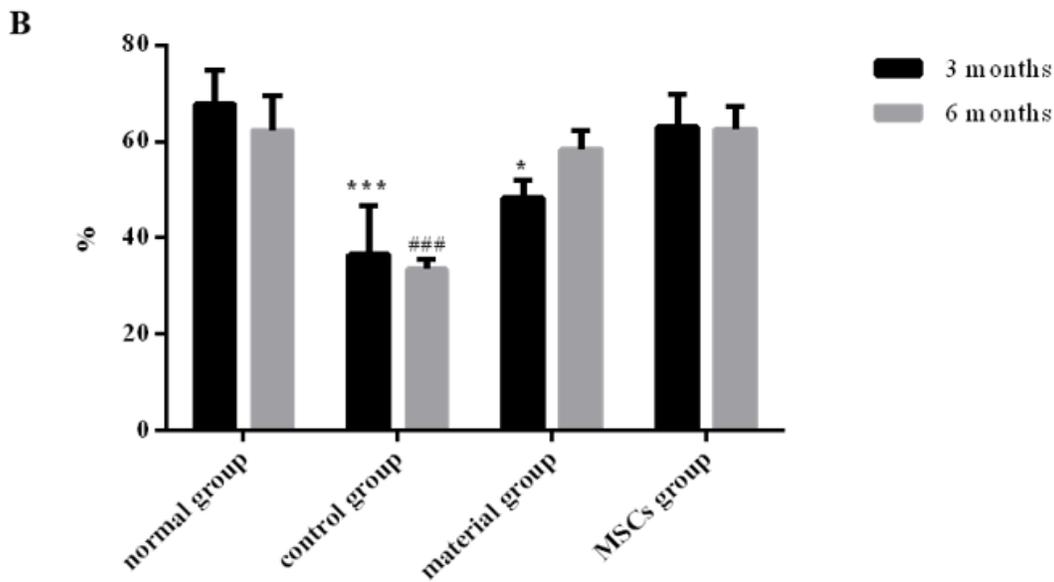
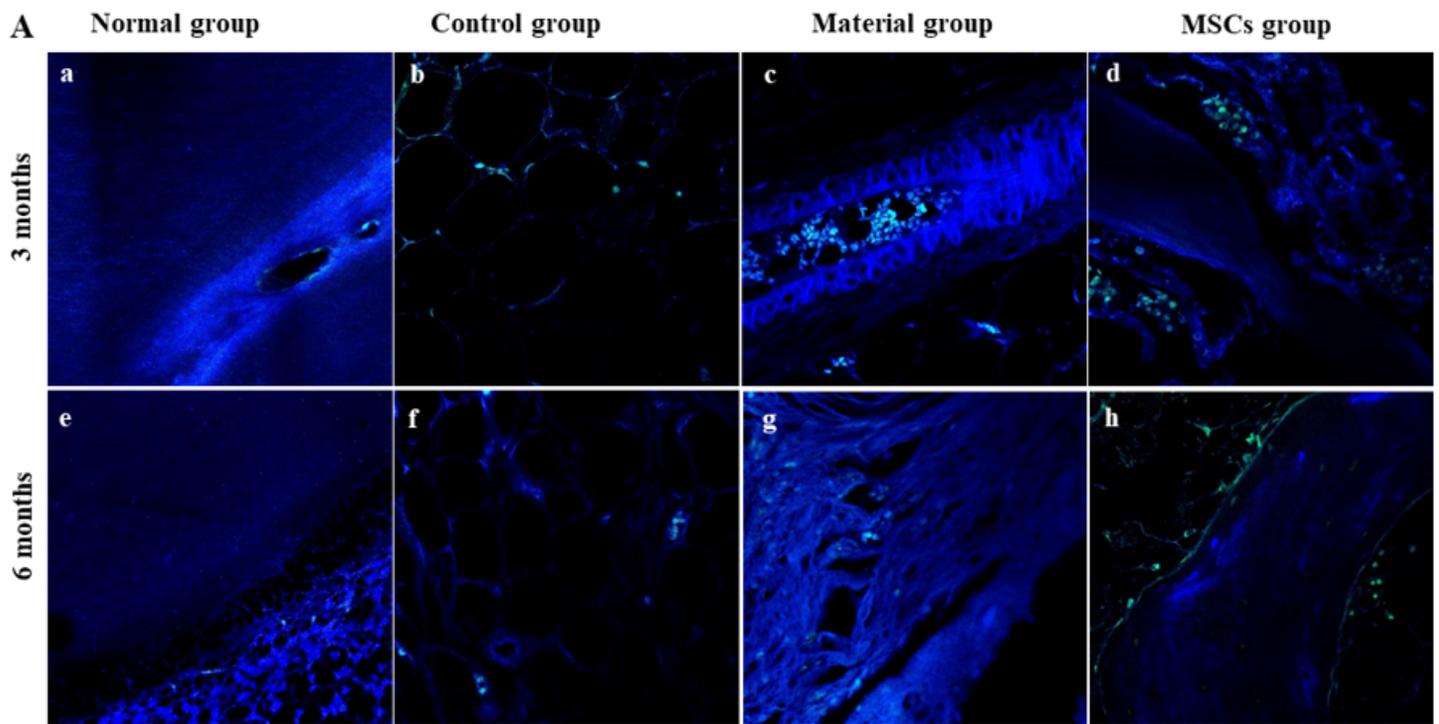
**Figure 9**

IHC results of BMP2 (200X). a-d Sampling group 3 months after surgery; e-h Sampling group 6 months after surgery. a,e Normal group b,f Control group c, g Material group d, h MSCs group. Mark the area of the positive signal with a red arrow.



**Figure 10**

TUNEL results (400X). A a-d Sampling group 3 months after surgery; e-h Sampling group 6 months after surgery. a,e Normal group b,f Control group c, g Material group d, h MSCs group. Mark the area of the positive signal with a red arrow. B Percentage of apoptotic cells in each group at 3 or 6 months. \* represents the statistical difference between each group and the normal group at 3 months. # represents the statistical difference between each group and the normal group at 3 months.



**Figure 11**

Ki67 results (400X). A a-d Sampling group 3 months after surgery; e-h Sampling group 6 months after surgery. a,e Normal group b,f Control group c, g Material group d, h MSCs group. B Percentage of apoptotic cells in each group at 3 or 6 months. \* represents the statistical difference between each group and the normal group at 3 months. # represents the statistical difference between each group and the normal group at 3 months.