Repair of alveolar cleft bone defects by bone collagen particles combined with hUC-MSCs in rabbit

CURRENT STATUS: UNDER REVISION

BioMedical Engineering OnLine • BMC

Xue-Cheng Sun
National Research Institute for Family Planning
ORCiD: https://orcid.org/0000-0002-1689-2627

Hu Wang
Reproductive and Genetic Center of National Research Institute for Family Planning

Jian-Hui Li
Reproductive and Genetic Center of National Research Institute for Family Planning

Dan Zhang
Reproductive and Genetic Center of National Research Institute for Family Planning

Xu Ma
Reproductive and Genetic Center of National Research Institute for Family Planning

Hong-Fei Xia
Reproductive and Genetic Center of National Research Institute for Family Planning

✉ hongfeixia@126.com Corresponding Author

DOI:
10.21203/rs.2.22764/v2

SUBJECT AREAS
Biomedical Engineering

KEYWORDS
alveolar cleft, Bone collagen particle, hUC-MSCs (human umbilical cord mesenchymal stem cells), micro-focus computerized tomography (micro-CT)
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 bone defects.

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 clefts was established by removing the incisors on the left side of the upper jaw. The normal group did nothing. 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 particles 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 alvenlus 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 generation inducer combined with bone collagen materials for bone regeneration and repair.

Background
Alveolar cleft is commonly in clinics, 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 clefts that is similar to human alveolar cleft disease and can be duplicated. This method can provide a theoretical basis of the occurrence and development of the disease, and also provide a good scientific research foundation for the repair of alveolar clefts.
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 clefts.

Alveolar cleft caused only by facial muscles can be corrected or repaired by surgical suture, but there is still no effective treatment for alveolar clefts caused by bone defect. At present, the treatment methods of alveolar clefts 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 to 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 bones to treat alveolar clefts, 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. fill hydroxyapatite in the crack of the alveolar clefts 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 bones to repair alveolar fractures are 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 as 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 to 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 fissures.

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 clefts. The results indicate that hUC-MSCs combined with bone collagen particles may be a reliable alternative therapy for the repair of alveolar bone defects.

Results

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 contents in MSCs group decreased from 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 of 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 to 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 secret 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.

**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 into the two periods.

**Micro-CT imaging.** The internal images of the normal side and the operative side of each group were compared to 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 of both angle 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.

**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 was 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 stainings:** The Sirius red staining results showed that the orange color were 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 of 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 types 1 were 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 to 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 particles combined with hUC-MSCs could promote the generation of chondrocytes better than collagen particles 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 the most obvious in the normal group of 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 on 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 on 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.

**Proliferation and apoptosis analysis**

Ki67 (Figure 10B) and TUNEL (Figure 10A) methods were used to detect cell proliferation and apoptosis in each group at 3 and 6 months after surgery. TUNEL assay (Figure 10C1) showed that the apoptosis rate of the control group was similar to that of the normal group of 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 (Figure 10C2) 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 rates 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 rates remained unchanged while the apoptosis rates decreased significantly. Therefore, it can be speculated that the combination of bone collagen particles of MSCs can further to promote cell proliferation and apoptosis, thus promoting bone regeneration.

**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 [18]. This makes it an ideal candidate for the potential for stem cells in medical applications [22]. Although hUC-MSCs have good bone induction, 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 defect sites [20]. The
combination of collagen scaffolds can slow down the degradation rate of hUC-MSCs, thus prolonging the bone repair time.

Bone collagen particles used in this study are heterogeneous bone made from bovine cancellous bone after degreasing and decellularization. Bone matrix particles 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 decellularization, the allogeneic bone can effectively reduce its immunogenicity [23]. Heterogeneous 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 scans 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 into connective tissue adjacent to bone graft by certain factors of 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 conversion to hUC-MSCs to different degrees of bone defect.

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

Materials And Methods
Isolation & culture of hUC-MSCs
HUC-MSCs were isolated from Wharton’s jelly. In this study, tissue blocks 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.

Preparation of implant materials
Bone collagen particles are made of bovine cancellous bone by special decellularization and degreasing process, which can reduce its immunogenicity. It also preserves the natural structure of the bon, which is good for the growth of cells and blood vessels. The bone collagen particles were provided by zhenghai biotechnology co. LTD Yantai, China. Cells within 5 generations were selected to be inoculated with bone collagen particles and cultured in carbon dioxide incubator for 0.5 h. The concentration of hUC-MSCs should reach $10^7$ /ml.
Surgical procedure & treatment (Figure 1)

In this study, we used 24 female JWRs (bodyweight: 2000±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 controls in the 23 to 25 °C. Humidity of 50% to 60%. Noise controls under 60dB. The light cycle are 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 injections of serazine hydrochloride (concentration: 1-2mg/kg). The model of alveolar clefts 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 incisors 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.

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.

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 to each group was observed, and the trabecular bone and bone density values were recorded.

**Histology staining**

Rabbits were euthanized and histological 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 microtome.

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 secret products of chondrocytes are metachromatic and can eventually differentiate into osteoblasts. Periodic Acid-Schiff stains (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 for 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 for 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.

**Statistical analysis**

All values are expressed as the mean ± 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).

**Abbreviations**
human umbilical cord mesenchymal stem cells (hUC-MSCs)

JWRs (Japanese white rabbits)

micro CT (microfocus computerized tomography)

hematoxylin eosin (HE)

alkaline phosphatase(ALP)

Periodic Acid-Schiff stain (PAS)

immunohistochemical(IHC)

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.

Funding

This work was funded by grants from the National Key Research and Development Program of China (2016YFC1000803).

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.

Acknowledgements
The authors are very grateful to the National Key Research and Development Program of China. We are also very grateful to the animal experiment center of the National Research Institute for Family Planning for its meticulous care of animals.

Authors’ details

a Reproductive and Genetic Center of National Research Institute for Family Planning, Beijing, 10081, China

b Graduate Schools, Peking Union Medical College, Beijing, 100730, China

References

[1] Harvold, Egil. Cleft Palate, An Experiment[J]. Acta Odontologica Scandinavica, 1950, 9(1):84-87.

[2] Chierici G , Harvold E P , Dawson W J . Primate Experiments on Facial Asymmetry[J]. Journal of Dental Research, 1970, 49(4):847-851.

[3] Longaker M T , Stern M , Lorenz H P , et al. A Model for Fetal Cleft Lip Repair in Lambs[J]. Plastic and Reconstructive Surgery, 1992, 90(5):750-756.

[4] Weinberg M A , Bral M . Laboratory animal models in periodontology[J]. Journal Of Clinical Periodontology, 1999, 26(6):335-340.

[5] Liao L S , Tan Z , Zheng Q , et al. Animal experimental study on repairing alveolar clefts by using rectilinear distraction osteogenesis[J]. Journal of Plastic Reconstructive & Aesthetic Surgery, 2009, 62(12):1573-1579.

[6] Dalia E B , Smith S J , Germane N , et al. New Technique for Creating Permanent Experimental Alveolar Clefts in a Rabbit Model[J]. The Cleft Palate-Craniofacial Journal, 1993, 30(6):542-547.

[7] Bardach J , Kelly K M . Role of Animal Models in Experimental Studies of Craniofacial Growth Following Cleft Lip and Palate Repair[J]. The Cleft palate journal, 1988, 25(2):103-113.

[8] Xue-Cheng Sun,Ze-Biao Zhang et al. Comparison of three surgical models of bone tissue defects in cleft palate in rabbits[J]. International Journal of Pediatric Otorhinolaryngology.2019[124]:164-172.

[9] Binger T , Katsaros C , Martin Rücker, et al. Segment Distraction to Reduce a Wide Alveolar Cleft Before Alveolar Bone Grafting[J]. The Cleft Palate-Craniofacial Journal, 2003, 40(6):561-565.

[10] Boyne P J . Secondary bone grafting of residual alveolar and palatal clefts[ J ] J Oral Surg, 1972,
[11] Cohen M, Figueroa A A, Haviv Y, et al. Iliac Versus Cranial Bone for Secondary Grafting of Residual Alveolar Clefts[J]. Plastic and Reconstructive Surgery, 1991, 87(3):423-427.

[12] Tai C C E, Sutherland I S, Mcfadden L. Prospective analysis of secondary alveolar bone grafting using computed tomography [J]. Journal of Oral & Maxillofacial Surgery Official Journal of the American Association of Oral & Maxillofacial Surgeons, 2000, 58(11):1241-1249.

[13] Nique T, Fonseca R J, Upton L G, et al. Particulate allogeneic bone grafts into maxillary alveolar clefts in humans: A preliminary report[J]. Journal of Oral and Maxillofacial Surgery, 1987, 45(5):386-392.

[14] El Deeb M, Tompach P C, Morstad A T, et al. Long-term follow-up of the use of nonporous hydroxyapatite for augmentation of the alveolar ridge[J]. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons, 1991, 49(3):257-261.

[15] Chan B P, Hui T Y, Wong M Y, et al. Mesenchymal Stem Cell–Encapsulated Collagen Microspheres for Bone Tissue Engineering[J]. TISSUE ENGINEERING PART C-METHODS, 2010, 16(2):225-235.

[16] Johnson K D, Frierson K E, Keller T S, et al. Porous ceramics as bone graft substitutes in long bone defects: A biomechanical, histological, and radiographic analysis[J]. Journal of Orthopaedic Research, 1996, 14(3):351-369.

[17] Hibi H, Yamada Y, Ueda M, et al. Alveolar cleft osteoplasty using tissue-engineered osteogenic material[J]. International Journal of Oral & Maxillofacial Surgery, 2006, 35(6):551-555.

[18] Liu S, Hou K D, Yuan M, et al. Characteristics of mesenchymal stem cells derived from Wharton's jelly of human umbilical cord and for fabrication of non-scaffold tissue-engineered cartilage[J]. Journal of Bioscience & Bioengineering, 2014, 117(2):229-235.

[19] Jin YZ, Lee JH. Mesenchymal stem cell therapy for bone regeneration. Clin. Orthop. Surg. 10(3), 271-278 (2018).

[20] Tassi S A, Sergio N Z, Misawa M Y O, et al. Efficacy of stem cells on periodontal regeneration: Systematic review of pre-clinical studies[J]. Journal of Periodontal Research, 2017, 52(5): 793-812.
[21] Li X, Tan J, Xiao Z et al. Transplantation of hUC-MSCs seeded collagen scaffolds reduces scar formation and promotes functional recovery in canines with chronic spinal cord injury. Sci. Rep. 7, 43559 (2017).

[22] Wang Limin, Tran I, Seshareddy K, et al. A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived mesenchymal stromal cells for cartilage tissue engineering [J]. Tissue Eng A, 2009, 15(8):2259-2266.

[23] Böer U, Lohrenz A, Klingenberg M, et al. The effect of detergent-based decellularization procedures on cellular proteins and immunogenicity in equine carotid artery grafts. Biomaterials, 2011, 32(36): 9730-9737.

Table 1 Blood routine test results at 3 months postoperatively

| Detection index | Unit | Normal group | Control group | Material group | MSCs group |
|-----------------|------|--------------|---------------|---------------|------------|
| RBC             | 10^{12}/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          | 10^9/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 |
| HBG             | g/L  | 117.667±6.182 | 123.000±13.491 | 121.000±2.944 | 103.333±16.6 |
| 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±13.1 |
| WBC             | 10^9/L | 13.170±2.788 | 8.680±2.961 | 10.037±1.848 | 8.603±1.062 |
| LYM#            | 10^9/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#           | 10^9/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#           | 10^9/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#             | 10^9/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            | 10^9/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             | 10^9/L | 153.333±35.188 | 171.667±22.867 | 141.000±24.536 | 127.667±8.49 |
| 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# |

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<0.01. RBC: red blood cell; HCT: hematocrit; RDW-CV: red blood cell distribution width; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cell; LYM: lymphocyte; NEUT: neutrophil; MONO: monocyte; EO: eosinophil; BASO: basophil; PLT: platelet; PDW: platelet distribution width; MPV: mean platelet volume; PLCR: plateletcrit; PCT: prothrombin time; CRP: C-reactive protein.
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.

Table 2 Liver function test results in blood at 3 months postoperatively

| 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         |

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<0.01. 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.

Table 3 Renal function results test in blood at 3 months postoperatively

| 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        |

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<0.01. BUN: blood urea nitrogen CR: creatinine UA: uric acid.

Table 4 Blood routine test results at 6 months postoperatively
### Table 5 Liver function test results in blood at 6 months postoperatively

| Detection index | Unit | Normal group | Control group | Material group | MSCs group |
|-----------------|------|--------------|---------------|---------------|------------|
| RBC             | $10^{12}/L$ | 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          | $10^9/L$ | 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 |
| HBG             | 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             | $10^9/L$ | 10.727±0.281 | 8.347±0.241 | 9.167±2.172 | 10.777±1.829 |
| LYM#            | $10^9/L$ | 6.165±0.287 | 5.265±0.825 | 5.181±1.541 | 5.913±0.946 |
| LYM%            | %    | 3.251±0.926 | 2.745±5.857 | 3.210±4.066 | 3.650±0.922 |
| NEUT#           | $10^9/L$ | 57.443±1.217 | 63.157±4.320 | 59.440±4.470 | 55.020±1.007 |
| NEUT%           | %    | 5.566±0.204 | 0.580±0.134 | 0.565±0.106 | 0.557±0.101 |
| MONO#           | $10^9/L$ | 0.528±0.204 | 6.977±1.724 | 5.390±1.289 | 5.167±0.113 |
| MONO %          | %    | 0.264±0.088 | 0.169±0.012 | 0.158±0.110 | 0.330±0.045 |
| EO#             | $10^9/L$ | 2.470±0.853 | 2.000±0.163 | 1.643±0.845 | 3.090±0.127 |
| EO %            | %    | 0.032±0.013 | 0.017±0.004 | 0.019±0.002 | 0.023±0.009 |
| BASO#           | $10^9/L$ | 0.360±0.060 | 0.175±0.025 | 0.217±0.029 | 0.220±0.057 |
| BASO %          | %    | 137.333±13.695 | 148.667±20.997 | 156.333±39.878 | 126.333±8.380 |
| PLT             | $10^9/L$ | 15.800±0.163 | 15.833±0.403 | 15.733±0.205 | 16.233±0.125 |
| PDW             | %    | 7.167±0.189 | 6.867±0.205 | 6.800±0.294 | 6.900±0.141 |
| MPV             | fL   | 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 |

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 0.01. RBC: red 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.
### Table 6 Renal function results test in blood at 6 months postoperatively

| 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 |

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 0.01. BUN: blood urea nitrogen CR: creatinine UA: uric acid.

**Figures**
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

A TUNEL results (400X). B Ki67 results (400X). 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 . C1 Percentage of apoptotic cells in each group at 3 or 6 months. Mark the area of the positive signal with a red arrow. C2 Percentage of proliferative cells in each group at 3 or 6 months. Cells labeled in green represent cells that are proliferating. * 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.