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Establishment and Micro-CT Histological Evaluation of the Animal Model for Rabbit Mandibular Distraction Osteogenesis

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Abstract: Objective: To explore the application of self-made distractors to construct a unilateral DO model of rabbit mandible and evaluate the microstructure of bone specimens. Method: Designing a linear dual arm distractor and performing DO implantation surgery on 5 rabbits under local anesthesia. Stretching the mandible at rate of 1.0mm/day, 2 times/day for 10 days. After fixation for 3 months, taking mandibular specimens for gross observation, Micro-CT imaging and HE histological examination to analyze the bone density, maturity and three-dimensional structure of the new bone. Result: Except for one animal that died of unknown cause, the right mandibular body of the other animals was extended by about 10mm. The traction gap is completely filled with bright and red new bone with clear contours; Micro-CT reconstruction of three-dimensional bone images clearly shows high bone density in the gaps, with trabeculae arranging along the direction of distraction; HE staining shows that the bone trabeculae were connected into woven and layered bones, with a large number of concentric arranged bone plates surrounding the central canal to form the Haversian system, and osteoblasts proliferated actively. Conclusion: The use of Micro-CT and HE staining proves the success of the unilateral DO model of rabbit mandible using a self-made distractor, providing animal experiment for various biological factors and drug assisted methods to promote efficient DO in the later stage, and for its safe and effective clinical application.

Keywords: Distraction Ssteogenesis, Mandible, Micro-CT, Three-Dimensional Structure

1. Introduction

Distraction osteogenesis (DO) refers to the surgical technique of applying specific traction force to bone segments that retain the attachment of soft tissue and blood supply of the periosteum after osteotomy, promoting the generation of new bone in the distraction gap, and extending bone to correct bone deformities and defects. In the 1950s, Ilizarov [1-3], a scholar of former Soviet, established a series of basic theories about DO based on his previous experience, and successfully applied DO technology to treat a large number of patients with limb bone deformities. DO surgery truly entered the stage of systematic research and application.

In 1973, Snyder firstly reported the successful use of DO surgery in animal experiments for mandibular extension. Other scholars established animal models for different types of craniofacial DO surgery, and continuously improved the design of distractor. In 1992, McCarthy [4, 5] reported the successful treatment of 4 patients with hemifacial dysplasia using an external traction device. It is the first time of introducing DO technology into oral and maxillofacial surgery. In the past thirty years, with the development of a large number of basic and clinical research, DO technology has shown broad application prospects in craniofacial plastic surgery, tumor reconstruction, alveolar implantation, and other aspects. It remains a hot research field in oral and maxillofacial surgery all around the world [6-9].

In this experiment, a self-made titanium alloy distractor was used to construct a unilateral DO model of the rabbit mandible. Traditional histological HE staining and advanced Micro-CT imaging were used to evaluate the microstructure and quality of newly formed bone, to prove the success of DO animal model. It provides some animal experimental evidence for various biological factors and drug assisted methods to promote efficient DO and it's effective clinical applications in the future.

2. Materials and Methods

2.1. Experimental Animals and Instruments

Five Japanese large eared rabbits (male, 6-8 weeks old, weighing 2500±200 g) purchased from West China Animal Experimental Center, Sichuan University. Titanium alloy mandibular distractor was developed by our research group and the Key Laboratory of Oral Biomedical Engineering. It was composed of two stretch arms, one slide bar and three connecting rods. The end of the stretch arm had retentive holes, two forearms and three rear arms. There is a rotating groove at the end of slideway bar. The distance between stretch arms increases by 0.4mm for each clockwise rotation, and the maximum length of extension is actually 12mm. The length of the distractor is 36mm, and the thickness is 3mm. The diameter of rotary groove is 1.5mm, and the length is 9mm (Figure 1).

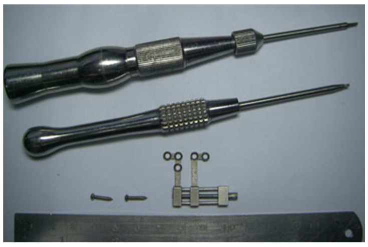

Figure 1. *Titanium Distractor, Titanium Screws and Screwdriver.*

Main instruments: Microtome (LEICA, Germany); Microscopic Image Analysis System (Olympus, Japan); µCT80 System (Scanco Medical, Switzerland) (Figure 2).

Figure 2. *µCT80 Equipment (Scanco Medical, Switzerland).*

2.2. Distraction Osteogenesis

The animals were fed with sugar water 2 days before surgery and fasted 12h before surgery. Operation procedure: According to 0.2ml/kg, the experimental rabbits were intraperitoneally injected with Su-Mian-Xin drug. After anesthesia effect, the animal was in coma, and the skin in the operative area was completely exposed. The skin, subcutaneous tissue and periosteum were cut into layers through the submaxillary incision to protect the mental neurovascular bundle. The full-layer soft tissue flap was lifted laterally to expose the lateral bone surface of the mandible. A longitudinal osteotomy line was designed in front of the mandibular first premolar. Five titanium nails were used to stabilize the distractor, avoiding root damage. Then under the washing of normal saline, the mandible was cut off along the osteotomy line in the whole laye, with grinding tablets. After rinsed and disinfected, the wound was sutured respectively with three layers of the periosteum, muscular and skin tissue (Figure 3).

Postoperative care: Intramuscular injection of penicillin 400000 u/d and 2% iodine tincture sterilized the wound for 3 days.

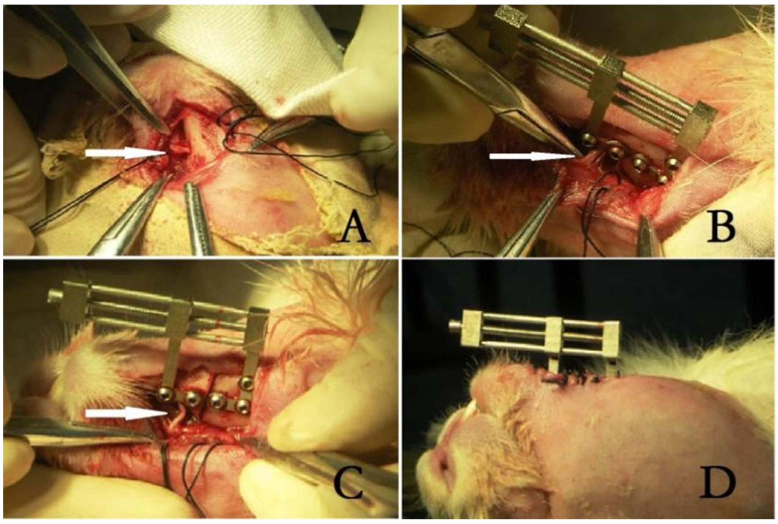

Figure 3. *The surgical process of mandibular DO. The white arrow shows the mental neurovascular bundle. A: Expose the mandible area with subjaw incision; B: The retractor fixed with 5 titanium screws; C: Full-thickness osteotomy of mandible between two distractor arms; D: Tightly suture in the different layers.*

2.3. Stretch Process and Specimen Acquisition

After 7-day delay period, stretching was performed at the rate of 1.0mm/ day, twice/ day for 10 consecutive days (stretch period: estimated width of stretch gap was 10mm). At the 12th week of fixation period, 5 animals were sacrificed. Mandibular specimens (with tensors) were dissected and fixed in 10% formalin solution at 4℃. Gross observation, Micro-CT imaging and HE histological examination were performed in sequence.

2.4. General Observation

The retention of the distractor, the combination with bone and the osteogenesis in the distraction space of the mandible were observed.

2.5. Micro-CT Imaging Evaluation

Micro-CT μCT80 system is dedicated to scanning the isolated bone specimens. The scanning parameters are set as follows: The voltage is 70kVp, the electric current is 117μA, the scanning mode is rotates 360°, the average frame is 4 frames, the exposure time is 500ms, the resolution of the planar image is 1024×1024 (medium resolution), the pixel size is 20μm×20μm, the layer spacing is 20μm.

Before scanning, the distractor was removed. The mandible specimens were vertically placed into the sample container along the long axis of the bone body. The newly-born bone in the stretch space was selected, and it's corresponding coordinate information was saved. The continuous Micro-CT images were scanned along the long axis of the mandible. After image Gaussian filtering, image information of mineralized trabeculae was extracted by computer automatic threshold. The image binarization was performed, and 3D visualization of cancellous bone in ROI region (central square region of stretch gap) was selected.

2.6. Histological Detection

The mandible specimens were removed from the fixative and trimmed. The distractor, the tooth body, the posterior edge of the ascending rami and the anterior part of the mandible were removed. And the new bone in the stretch space between the screw holes were retained. The 10%EDTA was decalcified for 4 ~ 6w, and the alcohol was dehydrated step by step. After paraffin embedding, the sections of 5μm thick were prepared on the microtome. 10 discontinuous sections of each specimen were stained by HE staining, then observed and photographed under the microscope.

3 Results

3.1. General Observation

A few days after the surgery, there was a mild infection in the operative area, which was cured after local disinfection and anti-inflammatory treatment. All the soft tissues of the wound healed totally, except one animal died of unknown cause. At the end of fixation, the animal's upper and lower incisors presented severe oblique bites (Figure 4). The animals were killed to obtain right mandibular specimens, which was prolonged by about 10mm in all cases.

The distraction space was completely filled with bright and red new bone, which was consistent with the surrounding cortex. It was difficult to distinguish the new bone and normal bone. It was also observed that small amount of new cortical bone covered the titanium screws.

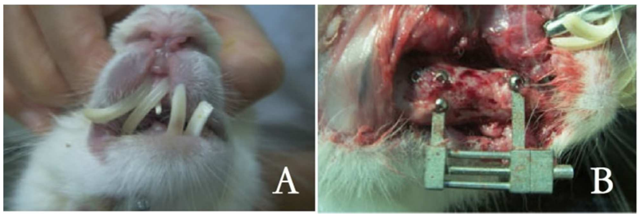

Figure 4. *General Observation of Specimens at the end of Consolidation Period. A: The Curled and Elongated Incisors, and Obvious Oblique Bite of the Mandible; B: New Bone Formed in the Distraction Space and Covered on Screws.*

3.2. Micro-CT Imaging Evaluation

At the end of the stretch period, the distraction space was completely projective. The continuity of the cortex was interrupted, and the incision lines of the bone were clearly visible at both ends of the stretch space. With the extension of fixed time, the mandibular stretch space was gradually filled with new bone. Along the stretch direction, the trabecular texture was clear and the bone density in the stretch space further increased. The medullary bone and cortical bone were completely continuous. The clear three-dimensional image of mandible was obtained through Micro-CT reconstruction (Figure 5).

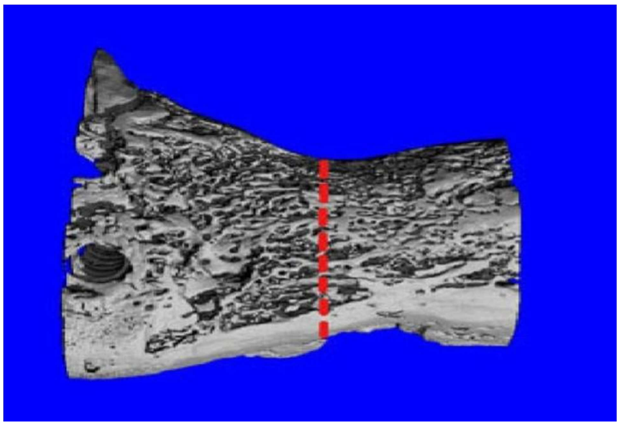

Figure 5. *Horizontal Arrangement of New Trabeculae on 3D Reconstruction of Micro-CT (red-dotted line shows the middle space).*

3.3. Histological Examination

HE staining of the specimen showed that there were large bone trabeculae connected with braided bone and lamellar bone in the distraction space. A large number of concentric circles of Havers bone plates formed the Havers system around the central canal, with obvious bone lacunae and active proliferation of osteoblasts. The new bone is of high maturity and has no obvious boundary with the surrounding normal bone. (Figure 6).

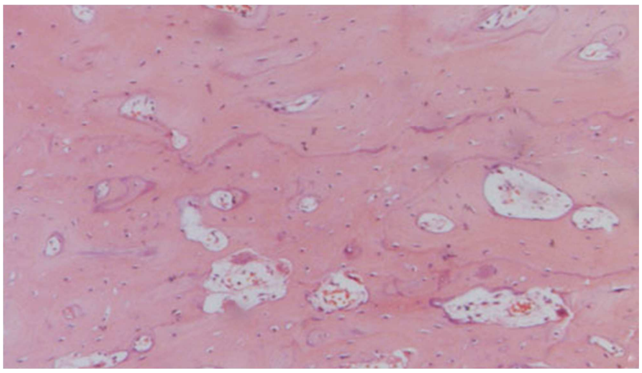

Figure 6. *Histological Examination Showing New Haversian System with Large Trabeculae Connected as Lamellar Bone (HE×50).*

4. Discussion

Successful establishment of animal model of mandibular distraction osteogenesis is the basis of DO related research. Therefore, domestic and foreign scholars used different animals such as sheep, dog, rabbit and mouse to simulate the DO process, and obtained their respective advantages and disadvantages [10, 11]. Our research group believe that the mandible shape and blood supply of rabbits are similar to human beings. It has some advantages, such as low price, docile temperament, strong tolerance to surgery, easy postoperative feeding and can also cooperate with tedious stretch operations. Therefore, in this study, rabbits were used to perform bone incision and distraction in the edentulous area between the mandibular foramenta and premolars. Then, Micro-CT, the advanced imaging technology, was used to evaluate the stretching effect.

Previous studies have proved that it is very necessary to retain adequate blood supply in the broken bone area [12]. Califano [13] believes that the DO process is a direct process of transforming mesenchymal cells into osteoblasts, and bone regeneration mainly originates from the internal and external periosteum. In order to fully reveal the osteotomy site and fixed the distractor during the operation, the periosteum of the mandible needs to be completely detached from the bone surface. If the periosteum tissue is peeled off in a large area, it is tend to osteogenesis defects in the extension area. Therefore, the surgeon should try to peel off the buccal-lingual periosteum and soft tissue in a small range. Meanwhile to avoid periosteum injury, complete suture of periosteum is required, so as to facilitate the transformation of osteoblasts into osteoblasts and the formation of new bone. The experimental results showed that our research group successfully established an animal model of the rabbit mandibular DO. The mandibular of 4 animals was lengthened by about 10mm, and new bone was formed in the interstitial area, which proved the necessity of preserving periostium during bone fracture to ensure adequate blood supply and good metabolism in the operative area.

There is some controversy about the way of new bone formation in DO [14, 15]. In the process of stretch of long bone, intramembranous osteogenesis and intrachondral osteogenesis exist simultaneously. While mandible is a flat bone. Due to the differences in anatomy and physiological functions of long bones, such as limbs, the way of new bone formation in the process of mandible DO is also different. Karp et al. [16] conducted a DO experiment on the mandible of dogs and found that the main way of new bone formation was intramembrane osteogenesis. Komuro et al. [17] found that the new bone formation of rabbit mandible was conducted simultaneously in two ways: intramembranous osteogenesis and endochondral osteogenesis. The histological results of this study showed that cartilage osteogenesis occurred in some parts of the extension area during the stretch stage and the early stage of fixation, but not in the later stage of fixation, indicating that the mandibular DO was still dominated by intramembranous osteogenesis. At the same time, the stability of the distractor has a great influence on osteogenesis. If the distractor is not stable enough, it is easy to lead to endochondral osteogenesis. The speed and quality of new bone formation in the intramembranous osteogenesis is better than that in the intramechanical osteogenesis. Therefore, it is very important to ensure the stability of the distractor during the process of mandibular DO. Our experience is that the experimental animals should be kept in an open space after surgery, instead of feeding in cage, to avoid the hanging and collision of the distractor.

Micro-CT, which has been widely used in recent years, provides an accurate imaging examination method for the biological study of bones, and can evaluate the 3D (three-dimensional) structural characteristics of bone trabeculae more objectively and scientifically [18-20]. Micro-CT is a device that adopts the X-ray imaging principle to obtain ultra-high resolution 3D imaging. That is X-rays pass through an object from all directions, then all attenuation X-rays are analyzed and measured by a computer program. The spatial resolution of 3D information can reach 100μm ~ 1μm. In Micro-CT image processing software, it is possible to observe any angle of view of the 3D image, define any area of interest (ROI), quantitatively calculate the volume, porosity, connection density, structural model index, degree of anisotropy and other parameters of the selected area inside the sample. In this study, high-resolution 3D reconstruction from Micro-CT data showed that in most animals, the new trabeculae were arranged horizontally with high bone density, the outer bone cortex was continuous, the new Havers bone system appeared, and large bone trabeculae were connected as lamellar bone. Furthermore, Micro-CT can also provide an interface of FEA (finite element analysis) to analyze biomechanical experiments such as compression, shear and torsion, which are usually required to be performed on a mechanical instrument. FEA could obtain parameters such as elastic modulus, Poisson's ratio and von Mises stress [21, 22]. This part of mechanical experiment needs further study.

In conclusion, this study explored a scientific and accurate

DO modeling and evaluation method. The bone density, maturity and 3D structure of rabbit mandibular new bone were analyzed by Micro-CT and histological examination. Good experimental results were obtained, which is worthy of experimental application.

5. Conclusion

The use of Micro-CT and HE staining proves the success of the unilateral DO model of rabbit mandible using a self-made distractor, providing animal experiment for various biological factors and drug assisted methods to promote efficient DO in the later stage, and for its safe and effective clinical application.

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Conflicts of Interest

The authors declare that they have no competing interests.

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