In Vivo Efficacy of Bone Marrow Stromal Cells Coated with Beta-Tricalcium Phosphate for the Reconstruction of Orbital Defects in Canines

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Purpose. To repair the segmental orbital rim defects of dogs with three-dimensional (3D) tissue-engineered constructs derived from culturing autogenous bone marrow stromal cells (BMSCs) on β-tricalcium phosphate (β-TCP) scaffolds.

Methods. A 25-mm segmental defect on the canine inferior orbital rim was created. BMSCs were isolated and osteogenically induced in vitro, then were seeded onto 3D β-TCP scaffolds and implanted to repair the orbital defects after 5 days of cultivation. The group of noninduced BMSC/β-TCP, β-TCP alone, and the normal inferior orbital rim were set as controls. The orbits of all groups had spiral computed tomography (CT) scans 1, 4, 8, and 12 weeks after surgery. Gross examination, bone density, microCT, and histologic measurements were performed 12 weeks after surgery. The results were analyzed to evaluate the extent of bone repair.

Results. Twelve weeks after surgery, CT examination revealed good inferior orbital rim recovery in the induced BMSC/β-TCP group, and the bone density was 0.30 ± 0.03 g/cm² with no dominant variance, compared with the normal control (P > 0.05). MicroCT and histologic examination confirmed that the implantations led to good repair of the defects. Pore-like spongy bone surrounded the implants through the section plane, with some residue remaining in the center. In contrast, the noninduced BMSC/β-TCP implants were not fully repaired, and nonunion was evident. The bony density for this group was 0.23 ± 0.07 g/cm², which was significantly lower than that of the control group (P < 0.05). The β-TCP group was largely held by fibrous tissues.


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Orbits are two pyramid bone cavities symmetrically located between the parietal bone and the facial bones. The orbit border constitutes an irregular mouth shape in the face, functioning to shape the facial profile and to protect the eyes. Hence, much interest has been focused on the recovery of the original anatomic structure while repairing bony defects of the orbit rim. Orbital defects, caused by trauma, infection, tumor operation, and congenital malformation, often require repair and reconstruction. When the orbit is fractured, it is necessary to repair the orbit rim and facial bones. A cranio-orbital fracture requires the repair of the cranial and supraorbital margin. An orbitozygomatic fracture requires the repair of the zygoma, suborbital rim, and lateral orbital margin. A naso-orbital-ethmoid fracture requires the repair of the nasal bones and medial orbital margin. In these cases, bone or artificial materials are usually needed in the reconstruction of the orbit and the repositioning of the eyeball during orbital surgery.

Autogenous and allogeneic bone transplantation are quite effective in repairing orbital defects but bring problems such as limited bone source, time-consuming procedures, the risk of infection, and allo-immune rejection.1–3 There are several types of artificial materials that can be used in the repair, including polymeric porous polyethylene, hydroxyapatite, and titanium. However, these materials are usually nondegradable or hard to be degraded, so they may cause rejection, infection, cyst formation, or implant metastasis.4–6 Tissue engineering is a new approach for the repair of orbit defects. Due to the particular characteristics of the orbit rim, the tissue-engineering repair method differs from those used for repairing limb and mandible bones. First, the shape of the orbit margin is complicated and varies considerably according to individuals; hence, material scaffolds are premade to satisfy the shape of defect. Second, orbital margins are not weight-bearing bones, so the post-surgery stress exerted on them is low. There is a proven relationship between bony remodeling and stress, and bony tissue can be remodeled in response to stress. Therefore, in this study we investigate how bones remodel and whether a fully functional orbital rim forms in low-stress environments and with the gradual degradation of the implant.

We used autogenously induced bone marrow stromal cells (BMSCs) combined with prefabricated three-dimensional (3D) β-tricalcium phosphate (β-TCP), to construct tissue-engineered bones and repair a 25-mm-long segment defect of a canine inferior orbital rim. We discuss the feasibility of repair of irregular and non–weight-bearing orbital bones for future clinical research.

Materials and Methods

Animals and Groupings

A total of 12 adult beagle dogs, aged 1–2 years and weighing from 10.0 to 15.0 kg, were used in this study. All animals were obtained from...
Shanghai Agriculture College, tested by the Shanghai Experimental Animal Test Station, and housed in the Animal Center of Shanghai Jiaotong University affiliated with the Ninth People’s Hospital (Shanghai, China). All animals used in this study were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee of Shanghai Ninth People’s Hospital affiliated with the Shanghai Jiaotong University School of Medicine.

Twenty-four side inferior orbital rims from 12 dogs were randomly assigned to the following groups: eight side suborbital rims repaired by autogenously induced BMSC/β-TCP composites, six sides repaired by β-TCP as material controls, six sides repaired by noninduced BMSC/β-TCP composites as noninduced controls, and the other four sides as normal controls.

Experimental Process

Isolation of BMSCs and Osteogenesis Induction. Under general anesthesia with ketamine (10 mg/kg) and 5% sodium pentobarbital (0.5 mL/kg), 3–4 mL of bone marrow was aspirated from the greater trochanter of each canine femur, transferred into a heparinized centrifuge tube, and mixed gently. Then the mixture was washed with α-minimum essential medium (α-MEM; Gibco, Grand Island, NY) and centrifuged at 176.4 g for 5 minutes. The supernatant was discarded, and the pellet was diluted in equal volume of 4% acetic acid. The nucleated cells were then counted and resuspended in α-MEM medium (4 × 10^6 cells/cm^3) supplemented with 10% fetal bovine serum (FBS; HyClone, Waltham, MA). After culturing, half of the cells were induced with 50 μg/mL ascorbic acid, 10 mM β-phenylphosphoglycerol, and 10 mM dexamethasone (Sigma, St. Louis, MO) in α-MEM medium and were then cultured with noninduced cells at 37°C in an atmosphere of 5% CO₂. The second generation of cells was prepared for the cell-material complex.

Prefabricated 3D β-Tricalcium Phosphate of the Canine Orbital Rim. A beagle (aged 1–2 years, weighing from 10.0 to 15.0 kg) was randomly selected and scanned with spiral computed tomography (CT) (GE Lightspeed Ultra 16; GE HealthCare, Milwaukee, WI) at a thickness of 0.625 mm, pitch of 1.375 mm, and KV value of 200 mA. Then volume reconstruction was carried out using a thickness of 1.25 mm and an interval of 0.4 mm, and the orbit was observed in the bone tissue window. The Digital Imaging and Communications in Medicine CT data were processed to build a 3D imaging and a canine suborbital rim specimen. These specimens were sectioned in a longitudinal fashion with a diamond saw (Leica SP1600; Leica Microsystems, Wetzlar, Germany). Two holes were drilled on both ends of the infraorbital border defect, and then 1-0 silk thread (Jinhuan Medical Products Co., Shanghai, China) was used to fix the bone with scaffold holding using the premade hole (Fig. 1). The other four sides were considered as normal controls. Incisions were closed layer by layer and to prevent inflammation, and 0.8 million units of penicillin were administered intramuscularly (twice daily for three days) after surgery.

Postoperative Follow-up and Evaluation

Spiral CT Scanning. Multislice spiral CT was applied 1, 4, 8, and 12 weeks after surgery, scanning the canine head coronal view with a thickness of 0.625 mm. Three-dimensional reconstruction was performed with a thickness of 1.25 mm. The conformation of implants and synostosis were checked.

Gross Examination. Animals were euthanatized under general anesthesia 12 weeks postoperatively. A lower eyelid incision was then made, and the SC tissue was separated. The inferior orbital rim and zygoma were fully exposed, and the periosteum was dissected. Based on the 3D defect model, a medical electrical saw was used to produce a 25-mm segmental defect of the suborbital rim, and the periosteum was removed. Eight sides of suborbital rim were implanted with induced BMSC/β-TCP composites, six sides with β-TCP materials, and six sides with noninduced BMSC/β-TCP composites. Two holes were drilled on both ends of the infraorbital border defect, and then 1-0 silk thread (Jinhuan Medical Products Co., Shanghai, China) was used to fix the bone with scaffold holding using the premade hole (Fig. 1). The other four sides were considered as normal controls. Incisions were closed layer by layer and to prevent inflammation, and 0.8 million units of penicillin were administered intramuscularly (twice daily for three days) after surgery.

Bone Density Measurement. Inferior orbital rim specimens were randomly sampled 12 weeks after surgery, including four induced BMSC/β-TCP specimens, four noninduced BMSC/β-TCP specimens, four β-TCP specimens, and four normal controls. These 16 samples were then tested by fan-beam dual-energy x-ray absorptiometry (Discovery A, Hologic, Inc., Bedford, MA) to determine the bone density of specimens.

MicroCT Measurement. The 12-week postoperative specimens, including two induced BMSC/β-TCP specimens, two noninduced BMSC/β-TCP specimens, one β-TCP specimen, and one control, were subjected to the MicroCT (μ80; SCANCO Medical, Brüttisellen, Switzerland) test with a scanning thickness of 50 × 50 × 50 μm.

Histologic Measurement. Inferior orbital rim specimens sampled 12 weeks after surgery were dehydrated in a graded series of ethanol and finally in xylene, then infiltrated and embedded in methylmetacrylate. These specimens were sectioned in a longitudinal fashion to a thickness of 220 μm, using a diamond saw (Leica SP1600; Leica Instruments, Nussloch, Germany). The sections were hand-ground to 50 μm and stained with Stevenel’s blue/Van Gieson’s picro-fuchsin for histomorphometric analysis.
Statistical Analysis

All data are presented as the mean ± SD (x ± SD), and the variance was analyzed using Dunnett’s test. The statistics analysis system software (SAS, v. 6.12; SAS Institute, Cary, NC) was used with the level of significance set at $P < 0.05$.

RESULTS

Clinical Observations after Surgery

All canines displayed normal breathing and eating, and they were carefully examined for inflammation or material exposure. Slight swelling was observed but no obvious clinical signs of inflammation. Swelling disappeared in 1–2 weeks postoperatively.

Spiral CT Scanning

Since X-rays were not available for the detection of complex structures such as the orbit, CT examination and 3D structure reconstruction were used to visualize the shape of implants and the healing status of the implants in the inferior orbital rim (Fig. 2).

One week after implantation, the induced BMSC/β-TCP (Fig. 2A), noninduced BMSC/β-TCP (Fig. 2E), and TCP groups (Fig. 2I) all showed the repair and reshaping of the infraorbital rim. The implants were smooth and held their initial form. The implants also fit to the broken ends of orbital rim, and the joint line was clearly presented.

Four weeks after implantation, the surface of the implants appeared ragged. The induced BMSC/β-TCP implants held their initial shape, repaired closely, and the joint line became obscured (Fig. 2B). The noninduced BMSC/β-TCP implants repaired well, but the joint line remained obvious (Fig. 2F).

Eight weeks after surgery, the induced BMSC/β-TCP implants held their original shape, the inferior orbital rim was well formed, the implants fixed closely, and the joint lines were not visible (Fig. 2C). In contrast, the noninduced BMSC/β-TCP implants displayed shrinking (Fig. 2G). In the TCP implants declined, the gap increased, and the joint lines remained obvious, indicating nonunion of the defect (Fig. 2K).

CT examination after 12 weeks indicated that the induced BMSC/β-TCP implants maintained their original shape, and the end of the implants and the broken ends of the orbital rim had fixed with synostosis (Fig. 2D). With the noninduced BMSC/β-TCP implants, two sides presented synostosis in part, and the shape of the inferior orbital rim had not fully recovered. Ossification had occurred from the rim of the implants toward the middle, holding the smooth edge, but few ossifications were observed in the middle of the implants (Fig. 2H). Four sides formed nonunion. The TCP implants also displayed nonunion, and callus formation was visible in the broken end (Fig. 2L).

General Observations

Twelve weeks after surgery, samples were observed grossly (Fig. 3). The induced BMSC/β-TCP group showed healing of the defects, resulting in normal physiological form, and bony conjugation occurred on the broken end (Figs. 3A–D). In the TCP group, fibrous tissue links were observed on the defects, with formation of little new bone on the end (Figs. 3I, 3J). In the noninduced BMSC/β-TCP group, repair was observed in the specimens of two sides, with incomplete bony conjugation on the ends (Figs. 3E, 3F). New bone formation was observed on the other four sides; however, linking fibrous tissue was evident but with nonunion (Figs. 3G, 3H).
Bony Density Measurement

Specimens sampled 12 weeks after the operation were used for density measurements. The bony density was $0.30 \pm 0.03$ g/cm$^2$ for the induced BMSC/$\beta$-TCP group, $0.23 \pm 0.07$ g/cm$^2$ for the noninduced BMSC/$\beta$-TCP group, $0.20 \pm 0.04$ g/cm$^2$ for the material control TCP group, and $0.36 \pm 0.04$ g/cm$^2$ for the normal control group (Fig. 4). Statistical analysis of these data demonstrated that the induced group was slightly lower than the control group without statistical significance ($P > 0.05$). The noninduced and the TCP groups, however, showed dominant variance ($P < 0.05$) and were considerably lower than the control group. These results revealed that the induced BMSC/$\beta$-TCP group implants could promote orbital bone repair compared with the noninduced and TCP group implants, where regeneration via ossification was slower than material degradation.

MicroCT Observation

MicroCT images were taken at 12 weeks postoperatively (Fig. 5). Induced BMSC/$\beta$-TCP implants showed well-repaired defects and recovered their normal form for the inferior orbital rim. Pore-like spongy bone was seen to surround the implants through the section plane, without obvious material residue, but some material was left in the middle of the implant (Figs. 5A–C). In the case of the noninduced implants, some synostosis was observed but not fully repaired, and the reshaping of the orbital rim was below expectation (Figs. 5D–F). The other implants showed no bony conjugation and had a large amount of material residues (Figs. 5G–I). The lowest levels of repair were seen in the TCP group, which demonstrated nonunion.

Histologic Examination

To confirm the findings presented above, histology of hard tissue sections using Stevenel’s blue/Van Gieson’s picro-fuchsin staining at 12 weeks postoperatively was conducted (Fig. 6). Calcified bone stains bright red, with variations in intensity depending on the maturity of the bone. Noncalcified bone and osteoid stain light green, osteoblasts stain blue, and TCP stains black.

In the induced BMSC/$\beta$-TCP implants a large amount of red nonlamellated bone structure and mature lamellar bones were observed. The material and the broken ends of orbital rim were fixed with synostosis. The surrounding material was fully degraded, but some material remained in the center, which appeared irregular and was filled with red bony structures (Figs. 6A–C). High-power microscopic views showed osteoblasts and light green osteoids (Fig. 6D). In the noninduced group, red fibrous bone was observed that was irregularly distributed within the nondegraded TCP material, and there was no sign of mature lamellar bones (Figs. 6E–H). Blue and green fibrous tissues were evident, and the poorly repaired specimens displayed nonunion. In the TCP group, defects were largely held by blue and green fibrous tissues, with only a few red bone structures forming at the broken margin. Residual material was seen to be embedded in fibers (Figs. 6I, 6J). In the normal control group, specimens presented dark red mature lamellar bones (Figs. 6K, 6L).

DISCUSSION

Reconstruction of the orbital defects commenced in the 1950s, and Converse applied the bone transplantation to repair a blowout fracture of the orbit. Since then extensive research has been carried out on material and surgical procedures. Nevertheless, along with improving surgical procedures, the nature and usage of implants are also crucial. Bone transplantation, including autogenous, allogeneic, and xenogeneic transplantation, is a vital method for the repair of orbital defects and the recovery from damage.
Ilium spongy bone is usually applied in autogenous transplantation because of its good transduction, osteoinduction, and osteogenesis, and it is acknowledged to be the gold standard for the clinical bone repair treatment. Because the supply of autogenous bone is limited, it cannot satisfy large volume defects, and it may also lead to the risk of bleeding, infection, or chronic pain. Although allogeneous bones have a wide source and show good transduction, they do not allow for osteoinduction and osteogenesis and present a potential risk of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infection and immunologic rejection. Xenogeneic implants, derived from animals, provide an abundant source but must undergo various methods to eliminate foreign antigens, after which the risk of rejection may still exist. The spread of animal-derived diseases and ethical issues must also be considered with xenogeneic transplantation.

The use of artificial materials in transplantation has been a hot topic in recent decades, because of their wide availability, plasticity, and safe convenient usage. There are several kinds of materials that have been applied in orbital repair, including...
polymeric porous polyethylene, hydroxyapatite, and titanium. Polyethylene is nonvisualizable and therefore does not allow for postoperative examination. Hydroxyapatite is fragile and not able to be shaped. The mechanical properties of titanium vary remarkably from human bones, and titanium is unable to undergo vascularization. Moreover, all these materials are either nondegradable or hard to degrade. Hence, they exist as a foreign body, potentially leading to rejection, infection, cyst formation, or implant displacement. Consequently, biodegradable, osteogeneic, and osteoinductive artificial orbit material is in high demand. Researchers have tested several materials for orbital repair, including L-lactic acid, poly-L/DL-lactic acid, and polyglycolic acid. The degradation of these materials was uncontrollable, and they may release acidic substances to activate inflammation. Moreover, the interior of these materials is cell free, so osteogenesis is attributed to host bone cells, which delays healing after implantation. To date, no ideal material has been identified for clinical orbit bone repair. Since the 1990s, the rapid development of tissue-engineering techniques and the increased interdisciplinary collaboration between the fields of molecular biology, cell biology, and material science have realized bone construction via tissue engineering and now direct research on bone replacement materials.

Tissue-engineering research is currently focused on osteology and oral surgery, and animal experiments have proven the feasibility of mandible, cranium, and limb repair via tissue engineering. However, little has been reported on the application of these technologies in the repair of orbit bone. Rohner et al. investigated the repair of swine orbital walls with porous polycaprolactone scaffold/fresh autogenous bone marrow complexes. Active osteogenesis was observed in this experiment, and bone formation was higher than that in the control of material only. This study indicated that bone marrow will promote orbital repair. However, the quantity of BMSCs in fresh bone marrow is low, approximately 1/106 of the total cells. Hence isolation and purification of BMSCs, followed by in vitro cultivation, are necessary. The methods for culture and osteogenic induction of BMSCs in vitro have been improved and applied to large animal bone defect repair.

However, there are distinctions between the anatomic structure of mandibles and limbs and that of the orbital, and therefore further work is required to optimize tissue engineering for the orbital specifically.

In the present study, we created a 25-mm-long segment defect in the inferior orbital rim, with irregular arch, to allow for our investigations into the formation and rebuilding of new bone in the repair of irregular and non-weight-bearing bone. A prefabricated β-TCP scaffold was combined with autogenous BMSCs cultured in vitro. This complex was then applied to the repair of the suborbital defect. The feasibility of repair orbital rim via tissue engineering was explored, and preliminary data for clinic application have been obtained.

The construction of the complex is vital for tissue-engineering repair of the orbit, including the seed cells and the scaffold materials. In this study, BMSCs were adopted as the seed cells and β-TCP was used as the scaffold; the complex was combined and cultured in vitro. Our results indicate that BMSCs possess good regeneration and differentiation ability, in agreement with a previous report. The orbital rim is a nonburden bone, so there were no strict requirements on the intensity of the material. By increasing aperture and porosity, bone repair and vascularization could be facilitated. The average diameter of the pores in the β-TCP scaffold was 406.3 μm, and the porosity was 84.63% in this study, which satisfies the requirements of tissue engineering. The compressive strength was >2 MPa, meeting the standard for spongy bone; hence the material would fulfill the requirements for defect repair.

In this study, the induced BMSC/β-TCP complex was implanted onto the defect in the inferior orbital rim. The results demonstrated that the scaffold was degrading and new bone was being produced gradually, and the induced BMSC/β-TCP complex showed good shape and bony conjugation, with the majority of the complex being replaced by new bone tissue 3 months postoperatively. In contrast, almost no new bone formation and nonunion were observed in the TCP alone group. These results indicated that induced BMSCs promoted the regeneration and reconstruction of orbital bone in the treatment of larger bone defects. Petite et al. used noninduced BMSC complexes to repair defects in goat metatarsal bone, revealing that noninduced BMSCs possess certain osteogenic abilities. Therefore, we tested noninduced BMSC/β-TCP complexes in the treatment of defects in the orbital rim. The results showed incomplete bony repair or nonunion, and the bone density was lower than that of the normal control 3 months postoperatively. In conclusion, our findings indicate that the osteogenic ability of noninduced BMSCs is weak and that osteogenic induction is necessary for seed cells during the repair of larger bone defects.

The orbit is located in the central part of the face and is involved in the formation of the facial outline and, in particular, protection of the eyes. Hence, extreme effort should be invested in recovery of the original anatomic form when repairing bony defects of the orbit. In the case of complex orbit fracture, defects of the adjacent facial bones, such as the skull, zygoma, and nasal bone, may also need to be repaired, so a prefabricated scaffold is vital in the treatment. For this reason, we applied computer-aided design and computer-aided manufacturing (CAD/CAM) to the analysis of the canine inferior orbital and prepared a scaffold to match the defect in shape, and this resulted in good repair. One-week postoperative CT examination revealed that the inferior orbital rim of each group showed good repair, indicating that the β-TCP scaffold made via CAD/CAM fit the defects well. This method enhanced the precision and convenience of surgery and confirmed the tight joining of the implants and the broken margin of the orbital rim. The 12-week follow-up examination demonstrated that the scaffolds maintained their initial shape along with degradation of the material and new bone replacement after implantation in the induced complex group. Thus, it was shown that 3D prefabricated materials facilitated treatment and ensured proper shape recovery.

Wolff proposed that bone in a healthy person or animal will adapt to the loads it is placed under. If the loading on a particular bone increases, the bone will remodel itself over time to become stronger to resist that sort of loading. The internal architecture of the trabeculae undergoes adaptive changes, followed by secondary changes to the external cortical portion of the bone. The converse is true as well. If the loading on a bone decreases, the bone will become weaker because of turnover, and it is less metabolically costly to maintain. There is no stimulus for continued remodeling required to maintain bone mass. Therefore, when repairing the non-weight-bearing inferior orbital rim, it is necessary to determine whether the bone regenesis will match the material degradation, and whether the implants will rebuild approaching the physiological function and appearance. Our present study revealed that the induced BMSC/β-TCP group repaired the inferior orbital rim well, showing physiological appearance, building bony conjugation between the material and the broken end of orbital rim, and remodeling the fibrous bone to the lamellar bone. Taken together, these data indicate that repair and rebuilding can be achieved at the inferior orbital rim via tissue engineering. Application of CAD/CAM in the manufacture of the scaffold forms the basis of repair, and the BMSCs promote the regenesis of bone. Subsequent remodeling and shaping of
the new bone induced by the effects of the orbit microenvironment, such as muscle shrinkage or biological mechanics, lead to the recovery of physiological appearance and function. The precise mechanisms involved in this proc need to be elucidated. In addition, further follow-up studies are required to assess the long-term effects of the tissue-engineering method in orbital repair.

In summary, the tissue-engineered bones from osteogenically induced BMSCs and 3D biodegradable β-TCP can efficiently repair the orbital defects in canines. This pilot study proves the feasibility of repair of irregular and non-weight-bearing orbital bones in a tissue-engineered manner and provides experimental results for clinical research.

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