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Efficacy of tissue engineered bone grafts containing mesenchymal stromal cells for cleft alveolar osteoplasty in a rat model

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ABSTRACT

The development of sufficient tissue engineered bone grafts for alveolar cleft osteoplasty could reduce the necessity of autogenous bone grafts and its donor site morbidity. The aim of the study was to evaluate tissue engineered bone grafts in an artificially created bone defect.

Bone grafts were created *in vitro* colonizing a synthetic hydroxyapatite–tricalciumphosphate scaffold (BONITmatrix®) with either undifferentiated mesenchymal stromal cells (group 1) or osteogenic differentiated mesenchymal stromal cells (group 2). Cells were multiplied from bone marrow of donor rats. Unmodified scaffolds (group 3) and the tissue engineered bone grafts were inserted into artificial maxillary defects of 54 Lewis rats. In 18 animals the defects remained unfilled (control). After one, three and six weeks the rats were sacrificed. The defect was evaluated radiologically and histologically with regard to the remaining defect volume and diameter. Statistical analysis followed.

The bone grafts led to a specific bone formation at the defect margin. No complete reunion of any defect was observed within the healing time. After six weeks, the remaining defect volume was 6.86 ± 3.21 mm^3 (control), 4.08 ± 1.36 mm^3 (group 1), 5.00 ± 0.84 mm^3 (group 2) 5.50 ± 1.05 mm^3 (group 3). The remaining defect diameter measured 2.63 ± 0.52 mm (control), 2.39 ± 0.23 mm (group 1), 2.53 ± 0.22 mm (group 2) and 2.70 ± 0.66 mm (group 3). In all experimental groups the defect volume and diameter decreased over time, which was significant for group 1 ($p = 0.014$), group 2 ($p = 0.025$) and group 3 ($p = 0.048$). The defect volume and width was significantly reduced for bone grafts containing undifferentiated cells compared to control ($p = 0.035$) or scaffolds only ($p = 0.05$).

Conclusion: Tissue engineered bone grafts induce a pronounced bone formation in artificial bone defects compared to unfilled controls or scaffolds only.

1. Introduction

The prevalence of cleft lip with or without cleft palate is about 1 per 700 live births in humans and thereby the most common congenital craniofacial anomaly (Moreau et al., 2007). Currently, an essential part of the therapy concept of alveolar clefts is bone grafting before eruption of the permanent canine in the stage of mixed dentition (Horswell and Henderson, 2003). This secondary osteoplasty stabilizes the dental arch, creates a sufficient bone site for the erupting permanent teeth, closes oronasal fistulae and supports the surrounding soft tissue and the nasal alar base (Witsenburg, 1985).

The currently established material for alveolar osteoplasty is autogenous iliac bone graft because it contains living immune-compatible bone cells and has strong osteogenic properties (Bajaj et al., 2003). Nevertheless, bone harvesting from the iliac crest is associated with disadvantages such as donor site morbidity, limited availability and postoperative temporary mobility impairment (Younger and Chapman, 1989; Moreau et al., 2007; Nguyen et al., 2009b). These drawbacks led to the search for alternatives, e.g. alternative donor sites for autogenous bone grafts, allogenic biomaterials or tissue engineered bone grafts.

An alternative for autogenous and allogenic grafts could be the creation of artificial tissue constructs by tissue engineering. These bone grafts are composed of osteogenic cells, an osteoconductive three-dimensional scaffold and osteoinductive growth factors (Lane et al., 1999; Hutmacher, 2000). Tissue engineered bone grafts

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are clinically applied, e.g. to augment the alveolar ridge or to fill bone defects after cyst enucleation (Choi et al., 2004; Ueda et al., 2005; Pradel et al., 2006).

However, these bone grafts are rarely studied in alveolar cleft models. Before application in humans a characterization of potential tissue engineered bone grafts is recommendable. The following studies analyzed bone grafts of different compositions in cleft animal models with a surgically created alveolar defect: Mayer et al. examined a polylactide-co-polyglycolide – scaffold with and without recombinant bone morphogenetic protein-2 (BMP-2) in a dog model (Mayer et al., 1996). An experimental study in a rat model for gingivoperiosteoplasty with collagen and hydroxyapatite scaffolds with and without recombinant BMP-2 was realized by Nguyen et al. (Nguyen et al., 2009a). Yoshioka et al. analyzed the bone regeneration of carbonated hydroxyapatite particles colonized with mesenchymal stem cells in artificial jaw clefts in a dog model (Yoshioka et al., 2012). The mentioned studies concluded that the application of tissue engineered bone grafts could have the potential to become an alternative to autogenous bone grafts. However, further studies are necessary to identify the best suitable bone graft. The idea of applying tissue engineered bone grafts for alveolar bone grafting has been studied in humans sporadically.

A pilot study for tissue engineered bone grafts consisting of osteoblasts and demineralized bone matrix for secondary alveolar cleft osteoplasty in humans was realized by Pradel and Lauer (Pradel and Lauer, 2012). In this study, no significant difference could be observed between autogenous and a tissue engineered bone graft regarding the defect ossification after six months.

The present study analyzed bone grafts of silicon oxide-hydroxyapatite–tricalciumphosphate and mesenchymal stromal cells in a rat model of cleft alveolar osteoplasty. The cells were either osteogenic differentiated or undifferentiated and cryopreserved before colonization of the scaffold. The efficacy of the bone grafts with regard to the remaining defect size was evaluated by cone-beam computed tomography, histomorphology and histomorphometry after healing times of one, three and six weeks, respectively. We hypothesized an accelerated bone formation in the alveolar defect by the application of tissue engineered bone grafts.

2. Materials and methods

2.1. Animals

The study was performed using 72 female Lewis rats with a body weight of 180–200 g at the beginning of the experiment. The animals were housed in a light and temperature controlled environment and nourished with pellets and water *ad libitum*. The rats were subdivided randomly into the experimental groups. All interventions were performed in accordance with the Commission for Animal Studies at the District Government Dresden, Germany.

2.2. Tissue engineered bone grafts

2.2.1. Isolation and cell culture

The mesenchymal stromal cells (MSCs) for colonization of the scaffolds were isolated from the bone marrow of donor rats (female Lewis rats, body weight 200 g). First, anesthesia was induced via intraperitoneal injection of 100 mg/kg body weight ketamine (Riemser Arzneimittel AG, Greifswald, Germany) and 10 mg/kg body weight xylazine (Pharma-Partner Vertriebs-GmbH, Hamburg, Germany). The femur was removed and the donor animals were subsequently killed in a CO_2 bath. Under sterile conditions the proximal and distal epiphysis were separated from diaphysis and the bone marrow of the epiphysis punctured. The aspirate was centrifuged at 1200 rpm for 5 min. The supernatant was aspirated and resuspended with 5.5 ml of minimum essential medium (MEM Alpha Modification, PAA Laboratories, Traun, Austria), phosphate buffered saline buffer without Ca^{2+} and Mg^{2+} (PBS, Life Technologies Corporation, Darmstadt, Germany), 10% fetal calf serum, 2% (4 – (2-hydroxyethyl)-1-piperazineethanesulfonic acid – buffer 1M), (HEPES, PAA Laboratories) and 1% penicillin/streptomycin (PAA Laboratories).

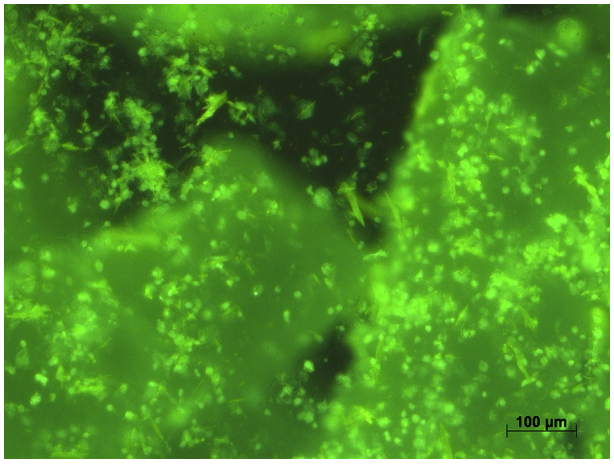

Fig. 1. Synthetic bone substitution material BONITmatrix® colonized with mesenchymal stromal cells fluorescence stained by spDiOC18 dye.

The transfer of the cells into culture flasks (T75 cell culture flask, Greiner Bio-One, Frickenhausen, Germany) and the microscopic examination of cell adherence followed. The cells were cultured *in vitro* for a period of 14 days and the culture medium was changed every three days. Next, the cells were split into passage 1 and cultured in the culture flasks until a confluence of 95% was achieved (Fig. 2A).

2.2.2. Cryopreservation

For cryopreservation cells of the first passage were grown to 95% confluence. Then, they were detached using trypsin/EDTA 0.25%/0.02% (Life Technologies Corporation, Darmstadt, Germany) and separated into single cell suspensions. In a cryo-vial the cells were cryopreserved in a solution of culture medium containing 10% fetal calf serum and 5% cell preservation fluid dimethyl sulfoxide using a freezing device (Mr. Frosty freezing container, Thermo Scientific, Waltham, USA). The cell solution was frozen at 1 K/min to −80 °C and subsequently transferred into liquid nitrogen. Before colonization of the bone grafts the cryopreserved cells were thawed in a water bath at 37 °C and fresh culture medium was added at 37 °C.

2.2.3. Colonization of the scaffolds

The commercially available granular (size 0.6 × 4.0 mm) bone substitute BONITmatrix® (DOT GmbH, Rostock, Germany) was used in the form of granules as scaffold for the tissue engineering grafts. The synthetic material consists of 87% nanocrystalline calcium phosphate (60% hydroxylapatite and 40% β-tricalcium phosphate) and 13% of a silica matrix. According to the manufacturer the porosity of BONITmatrix® is about 80%, whereas the single granules have a porosity of about 60%. The bone substitute material is produced in a patented sol–gel technique without sintering (DE 100 03 824).

Before colonization the sterile scaffold materials were portioned into a 24-well plate and incubated with 2 ml culture medium for 24 h at 37 °C. Subsequently, the material was washed with PBS in preparation for the cell colonization.

The cells of the second passage were washed with 2 × 6 ml PBS and then detached using 2 ml of trypsin/EDTA. The mobilized cells