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Osteogenic Potential of Oxycellulose – A Molecular-Biological and Histological Study in Rats

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Abstract

Oxidized cellulose is a polysaccharide substance formed by the oxidation of cellulose. Depending on the amount used, oxycellulose is completely absorbable and can be used as hemostatic agents in general and dental surgery for hemostatic purposes. Each procedure on the bone also carries the risk of bleeding, but still not much is known about the bone tissue reaction on this material. Due to this fact, the aim of this study was to evaluate the tissue reaction and osteogenic potential of the natural oxycellulose material RESORBA-CELL® standard by its implantation into rat cranial bone defects. The materials were evaluated using an established rat cranial defect model in 22 animals. One bone defect with a diameter of 5 mm was created per animal. The defects were filled with oxycellulose and left to heal for 4 weeks. Twelve samples (n=6 oxycellulose; n=6 untreated control lesions) were processed for histological evaluation. The remaining 10 samples were processed for mRNA expression analysis of genes coding for growth factors and osteogenic differentiation using quantitative RT-PCR. In oxycellulose treated bone lesions significant reduced mRNA levels of Runx2, Bglap and COL1A1 were found. The expression reached 74%, 73% and 47% of the mRNA quantity of the untreated bone, respectively. In the histological sections, the bone defects were completely filled with connective tissue / bone marrow with embedded foam cells after four weeks. In contrast to the controls, the tested material do not induced early bone healing. The residual bone seems to be rather absorbed. In conclusion, the study has shown that the RESORBA-CELL® oxidized cellulose has no osteogenic potential, but it still can be considered as a useful wound dressing in the dental surgery.

Keywords: Oxycellulose; Osteogenic potential, Rat cranial defect; Histology; RT-PCR

Introduction

Bone is the most commonly transplanted tissue after blood. Worldwide, estimated 2.2 million grafting procedures are performed annually to repair bone defects in orthopaedics, neurosurgery, and dentistry [1]. The increasing number of grafting procedures and the disadvantages of current autograft and allograft treatments (e.g. limited graft quantity, risk of disease transmission) drive the quest for alternative methods to treat bone defects. The use of synthetic bioactive bone substitute materials is of increasing importance in modern dentistry as alternatives to autogenous bone grafts. Bone graft materials are generally evaluated based on their osteogenic, osteoinductive, or osteoconductive potential.

Osteoinduction is a basic biological mechanism that occurs regularly in any type of bone healing process. Osteoinduction process starts immediately after the bone injury. This process induces a cascade of events including primitive, undifferentiated and pluripotent cells to develop into the bone-forming cells – pre-osteoblasts [2,3]. In other words the molecular mechanism of this phenomenon is to use the immature cells, and to stimulate them, so the osteogenesis process can occur. It is a dynamic process that is responsible for healing and the continuous remodeling of bone tissue. There are several cell-type specific markers that can be identified in order to characterize ongoing cellular differentiation, and bone remodeling process. Some of these, like Runx2, alkaline phosphatase (Alpl), osteocalcin (bone gamma-carboxyglutamate protein, Bglap), Acp5 (acid phosphatase type 5) and Phex, belong to the key phenotyping proteins expressed in the osteogenic cell lineage [3-6].

Runt-related transcription factor 2 (Runx2) is also called CBFA1 (core binding factor 1). It is essential for the osteoblast differentiation. This factor acts throughout the induction, proliferation, and maturation of osteoblasts and regulates expression of many osteoblast genes [7,8]. Runx2 is an essential factor in the development of the skeletal system; studies showed that lack of the Runx2 results in an immediate postnatal death of Runx2-deficient mice due to a complete lack of mineralized bone [9-11]. Mutations in the Runx2 gene result in specific diseases, haploinsufficiency causes Cleidocranial dysplasia (CCD) a condition characterized by hypoplastic clavicles and a number of other skeletal abnormalities [11]. Runx2 is also a specific regulator of osteoblast gene expression like osteocalcin, Alpl and collagen type I [12]. Osteocalcin is also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP), a non-collagenous protein found in bone and dentin. Osteocalcin is secreted solely by maturing osteoblasts and is implicated in bone mineralization and calcium ion homeostasis in human body [12]. Collagen type I is a protein which is the major component of many extracellular matrices. It is synthesized by a small number of discrete cell types, including fibroblasts, osteoblasts and odontoblasts. The COL1A1 gene encodes this protein in humans. Mutations in the COL1A1 gene are associated with severe systemic disturbances such as Ehlers-Danlos syndrome or Osteogenesis imperfecta [13].

To date, predominantly histological staining and immunohistochemical analyses were used to study the behaviour of bone substitute materials on bone graft healing. Only a few numbers of studies present the molecular mechanisms of bone formations associated with bone substitutes. Recently it was shown, that hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) up-regulate the expression of Runx2, Alpl, Acp5 and osteocalcin in biopsies from human jaws [14] and rat skulls [4,6]. When using flax composites for the treatment of bone defects significantly higher expression of IGF1 and decreased mRNA levels of Bglap and MMP8 were found compared to untreated controls [5]. Nevertheless, flax composites are able to stimulate bone regeneration. Main component of flax fibres is the natural polymer cellulose. It is well known that cellulose is hydrophilic, insoluble in water as well as most organic solvents and not biodegradable.

Oxidized cellulose is a polysaccharide substance formed by the oxidation of cellulose. It is obtained by means of oxidation of highly pure cellulose. It can be prepared in different physical forms, such as powder, textile, paper or fiber form [15]. Depending on the amount used, oxycellulose is absorbable [16,17]. Oxidized cellulose can be used as hemostatic agents in general and dental surgery for hemostatic purposes [15-18]. Each procedure on the bone also carries the risk of bleeding, but still not much is known about the bone tissue reaction on this material. Due to this fact, the aim of this study was to evaluate the tissue reaction and osteogenic potential of the natural oxycellulose material RESO-CELL® by its implantation into rat cranial bone defects.

Material and Methods

Test material

Sterile absorbable RESORBA® CELL standard (1 × 0.5 cm, Resorba Wundversorgung, Nürnberg, Germany) gauze strips are produced from natural cotton cellulose by controlled oxidation. The product is white and does not fray when cut (Resorba Medical GmbH 2013).

Experimental design and surgical procedure

Twenty-two adult Lewis 1A rats (2-month old, body weight between 250 g and 350 g and of both sexes) were used for this study. All surgical and experimental procedures were approved by the Animal Welfare Committee on the State Government (LALLF M-V/TSD/7221.3-1.1-033/11). For surgery, each rat was anesthetized with intraperitoneal injection of Ketamine (10%; CEVA Tiergesundheit, Düsseldorf, Germany) and Rompun (2%; Bayer HealthCare, Leverkusen, Germany) with a ratio of 3:2 and at an approximate dosage of 0.1 ml/100 g body weight. A midline skin incision was performed on the skull. Bone defects with a diameter of 5 mm size (using a pre-designed template) were created in each parietal region of the cranium with a trephine under constant irrigation as described previously (Figure 1A) [4-6,19]. Bone defects (n=16) were covered with oxycellulose pieces (RESORBA® Cell standard; Figure 1B and 1C) and the scalp was closed with wound clips. Six untreated animals served as controls.

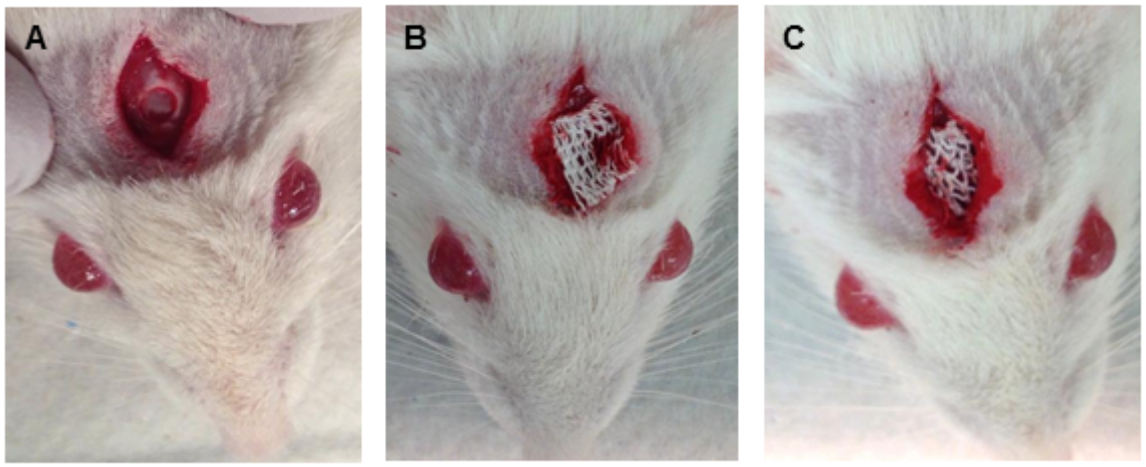

Figure 1: Rat cranium after preparation of the bone defect (A) and placement of the oxycellulose into the bone defect (B+C).

The animals were sacrificed at four weeks. The skulls were harvested and processed for gene expression analysis and histological examination. For molecular-biological examination, dissected skulls (area of the treated lesion and untreated initial bone) were shock-frozen in liquid nitrogen and stored at -80°C. For histological examination, skulls were placed in 4% PBS-buffered formalin for one week at room temperature and then decalcified for one week in ethylenediaminetetraacetic acid (EDTA). After decalcification the bone cores were rinsed with running water for 24 h; routinely dehydrated in a series of increasing concentrations of ethanol (50%, 70%, 80%, 90%, 96%, and 100%) placed in xylol for 12 h and then embedded in paraffin.

RNA isolation, reverse transcription and TaqMan RT-PCR

Homogenization of bone samples was performed in QIAzol lysis reagent (RNeasy Lipid Tissue Mini Kit, Qiagen, Hilden, Germany) using a mortar and pestle. The isolation of the total RNA was done using guanidinium-isothiocyanate (RNeasy Lipid Tissue Mini Kit, Qiagen, Hilden, Germany). An amount of 200 ng total RNA was reverse transcribed (TaqMan-reverse transcription kit, PE Applied Biosystems, Weiterstadt, Germany).

To quantify the expression of different rat genes, gene-specific TaqMan PCR primers and probes were purchased from PE Applied

Biosystems and quantitative real-time PCRs using a Toptical cycler (Biometra, Germany) were performed as described previously [4-6].

Histological processing

Serial longitudinal sections of about 5 µm were stained with hematoxylin/eosin (H.E.) for recognizing various tissue types and Masson Goldner trichrome for differentiation between collagen, muscle and bone tissue.

A blind histological evaluation of all sections stained was undertaken by two independent investigators. The slices were observed and photographed under a Nikon light microscope (Eclipse E600) equipped with a calibrated digital camera (DXM1200, Nikon, Tokyo, Japan). Histomorphometric measurements were performed on images at a magnification of 10× using image analysis software (Image J, NIH, v. 1.61).

Statistical analysis

All statistical analyses were performed using the SigmaStat 3.5 Software (Systat Software, Inc.,1735, Technology Drive, San Jose, CA 95110, USA). Statistical analyses were made using the Mann-Whitney rank sum test. Data are given as means ± S.D. $P<0.05$ was considered statistically significant.

Results

Real-time RT-PCR

All the animals have recovered without problems from the surgery. No complications such as allergic reactions, abscesses or infections were observed throughout the study periods.

For weeks after oxycellulose insertion the animals were sacrificed, rat skulls prepared and dissected in two parts: 1) treated lesion and 2) untreated initial bone tissue. Both bone samples of each rat were used for molecular-biological analyses and the intra-individual gene expression of bone specific genes in both skull parts was compared to each other (Table 1). In oxycellulose treated bone lesions significant reduced mRNA levels of Runx2, Bglap and COL1A1 were found. The expression reached 74%, 73% and 47% of the mRNA quantity of the untreated bone, respectively. All other tested genes do not show any difference compared to controls (Table 1).

Gene	Bone tissue of		P value
	Treated lesion	Untreated area	
Acp5	0.12 ± 0.024	0.116 ± 0.015	n.s.
Alpl	0.096 ± 0.017	0.084 ± 0.009	n.s.
Bglap	0.87 ± 0.13	1.85 ± 0.25	P=0.002
Col1a1	3.04 ± 0.56	4.15 ± 0.42	P=0.041
IGF1	0.46 ± 0.09	0.39 ± 0.05	n.s.
IGF2	1.17 ± 0.18	1.04 ± 0.17	n.s.
Phex	0.073 ± 0.022	0.031 ± 0.002	n.s.
Runx2	0.002 ± 0.0002	0.0027 ± 0.0003	P=0.015
VEGFA	0.093 ± 0.015	0.095 ± 0.015	n.s.

Table 1: Gene specific transcription levels in rat bone tissue samples. The mRNA levels are given in relation to those of 18S rRNA. Means ± S.E.M. are given in all cases for n=10 samples. P values indicate statistical significant differences between bone tissue of treated lesions and untreated initial bone tissue, Mann Whitney U rank sum test. n.s.=not significant.

Histology

In the histological sections, there were no signs of a cellular inflammatory infiltrate and reaction against foreign bodies. The untreated defects were completely filled with connective tissue after four weeks. Furthermore, the onset of bone regeneration was found. The amount of regenerated bone was approximately 47.8 ± 1.3%. Likewise untreated defects, in oxycellulose treated defects cell-rich areas of connective tissue/bone marrow were observed. Embedded in the connective tissue areas with foam cells (transformed macrophages) were found (Figure 2). In contrast to the controls, the tested material do not induced early bone healing. The residual bone seems to be rather absorbed (Figure 2).

Discussion

Oxidized cellulose materials are usually placed in the surgical site to achieve hemostasis [16-18,20]. They can be used in surgical and minimally invasive procedures to support hemostasis in capillary, venous, and minor diffuse arterial bleeding, in which conventional hemostatic measures such as sutures or ligatures are ineffective or unfeasible [16-18,20]. In addition to topical hemostasis, the bacteriostatic and antiseptic properties of oxycellulose products can prevents the secondary infection by inhibiting the growth and spread of gram-positive and gram-negative microorganisms and aerobic and anaerobic bacteria [17,21,22]. Manipulations and intervention in the bone can also bleed profusely. Normally such bleeding bone was treated with bone wax [17]. In our study surgically prepared bleeding bone defects were covered with oxycellulose pieces and the tissue

reaction to the material was molecular-biological and histological examined.

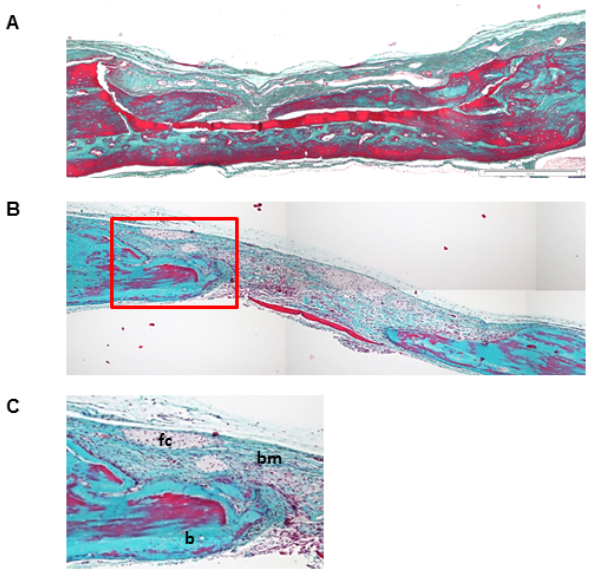

Figure 2: Masson Goldner trichrome staining of a calvarial cross-section embedded in paraffin after 4 weeks of healing. (A) untreated control lesion, (B) oxycellulose treated bone lesion, magnification x20; (C) higher magnification of the highlighted area (red frame), magnification x100. b=bone; bm=bone marrow / connective tissue; fc=foam cells.

Recent studies indicate quick resolution and complete resorption of the oxidized cellulose material during the second week after implantation [23]. Otherwise some reports suggest that oxycellulose dressings used in general surgery can swell and increase its volume leading to mechanical compression on vital anatomical structures and radiographic artifacts mimicking abscesses and tumors in CT and ultrasonography [24,25]. In our study, four weeks after implantation of the oxycellulose, no intact material could be detected. The oxidized cellulose was completely resorbed from macrophages. Transformed macrophages (foam cells) were found embedded in the connective tissue, which fill the bone defect. Furthermore, the tested material in our study did not show any osteogenic potential. Moreover it partially inhibited early bone healing as compared to control. The results indicate that the mRNA amount of Runx2 was statistical significantly lower after treatment with oxycellulose. The mRNA levels of osteocalcin (Bglap) and Col1a1 were also statistically significant decreased in treated bone lesions compared to untreated initial bone tissue. The decrease of the Runx2 shows that tested material inhibits the maturation of the osteoblasts. Runx2 as a specific regulator of osteoblast gene expression may have also influenced the expression level of osteocalcin and collagen type I. The results obtained by molecular biology were confirmed by the histological preparations. Oxycellulose treated bone defects showed no bone regeneration, and resorption of remaining bone. Our findings are consistent with a number of previous studies [17,26-29]. Armstrong et al. examined bone defect treated with the oxidized regenerated cellulose. Healing of the bone defects was evaluated by micro-computed tomography (micro-CT) and histological research. His study showed that tested material inhibited early bone healing [26]. Ibarrola et al. studied bone healing in a rat tibial bone defects using light microscopy. In this study the oxidized cellulose caused an intense inflammatory response and bone repair was reduced [27]. A subperiosteal implantation of oxidized cellulose in five patients demonstrated no radiographic evidence of bone formation after 12 months [28]. In contrast, bone regeneration in surgical defects in the iliac crest of dogs was found after application of oxidized cellulose [30].

In conclusion, the study has shown that the RESORBA-CELL® oxidized cellulose has no osteogenic potential; moreover it partially inhibits an early healing of the osseous defects in rats. Although the substance does slightly negatively affect the healing of the bone defect it still can be considered as a useful wound dressing in the dental surgery because of its hemostatic, bacteriostatic and antiseptic properties.

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