ORIGINAL ARTICLE

Matrix change of bone grafting substitute after implantation into guinea pig bulla

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Background: Many different surgical techniques have been developed to remove open mastoid cavities. In addition to autologous materials, alloplastic substances have been used. A very slow absorption of these materials and extrusion reactions have been reported. We investigated a newly developed, highly porous bone grafting material to eliminate open mastoid cavities, in an animal model. To characterise the transformation process, the early tissue reactions were studied in relation to the matrix transformation of the bone material.
Material and methods: NanoBone® (NB), a highly porous bone grafting material based on calcium phosphate and silica, was filled into the open bullae from 20 guinea pigs. The bullae were examined histologically. Energy dispersive X-ray spectroscopy (EDX) was used to investigate the change in the elemental composition at different sampling times. The surface topography of the sections was examined by electron microscopy.
Results: After 1 week, periodic acid-Schiffs (PAS) staining demonstrated accumulation of glycogen and proteins, particularly in the border area of the NB particles. After 2 weeks, the particles were evenly coloured after PAS staining. EDX analysis showed a rapid absorption of the silica in the bone grafting material.
Conclusions: NanoBone® showed a rapid matrix change after implantation in the bullae of guinea pigs. The absorption of the silica matrix and replacement by PAS--positive substances like glycoproteins and mucopolysaccharides seems to play a decisive role in the degradation processes of NB. This is associated with the good osteoinductive properties of the material. (Folia Morphol 2012; 71, 2: 109–114)

Key words: obliterating open mastoid cavities, open mastoid cavity, highly porous bone augmentation materials, NanoBone®, silica

INTRODUCTION

For rehabilitative therapy of chronic middle ear infections, the mastoid must be drilled, often in combination with removal of the posterior wall of the ear canal. This can result in inflammation of the open mastoid cavity, which can lead to various problems in patients. These include chronic inflammation of the open mastoid cavity with secretions, changes in acoustic behaviour [7], and in certain situations, dizziness and an impaired self-cleaning function with build-up of ear wax. In these cases, surgical (partial) obliteration of the open mastoid cavity is often performed.

A variety of methods have been developed for cavity obliteration using different materials. Autologous materials such as bone meal or cartilage from the septum or the concha are often applied in combination [3]. These have a high biological potency

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but are only available to a limited extent [11]. Usually, an additional surgical removal is required, which might increase donor morbidity and the likelihood of surgical complications [6].

Alloplastic materials have also been used to eliminate open mastoid cavities [4]. The applied substances, such as hydroxyapatite or tricalcium phosphate ceramic, which are similar in composition to that of natural bone, should stimulate the formation of new bone during resorption of the material [16]. Alloplastic substances have the advantage of simplicity and 'unlimited' availability. However, rejection of these substances has been described not only during the healing phase after implantation, but also after several months, usually associated with acute inflammation and granulation. These observations have led to the increasing use of autologous materials for obliterating open mastoid cavities [6]. In recent years, new, highly porous bone augmentation materials have been developed and have been applied successfully in oral and maxillofacial surgery.

NanoBone® (NB) (ARTOSS GmbH, Rostock, Germany), based on calcium phosphate and silica, is sintered at low temperatures (700°C) and in a sol-gel process. The very high osteoconductivity and improved resorption of NB after implantation have been described [8]. It seems that the silica plays an important role in the bone remodelling process [5].

This new bone grafting material was tested for its ability to obliterate open mastoid cavities, in an animal model. When used for obliteration of pneumatised bullae from guinea pigs, NB showed a rapid osteoinduction and absorption of the silica matrix [13]. In the early stages after implantation, the bone material could play a decisive factor in the remodelling process of the newly formed bone [15]. To characterise the transformation and provide information on the complex metabolic processes in relation to osseointegration and osteoinduction, the early tissue reactions were examined in relation to the matrix transformation of the bone material, and its interaction with the surrounding tissue.

MATERIAL AND METHODS

The investigations were performed in accordance with paragraph 8, section 1 of the German Animal Protection Law and have been approved by the local animal care committee.

The bone grafting material used (NB) is produced by a sol-gel technique. Nano-crystalline hydroxy-

apatite (HA) is embedded and homogeneously distributed in a silica gel (SiO_2). The nanocrystalline HA corresponds approximately to the structure of the autologous bone. Silica gel should promote collagen and bone formation. The granules used had a diameter of about 0.6 mm.

Twenty guinea pigs (male, Hartley Donkin, average weight 585 g) were anesthetised with a combination of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (4 mg). After retroauricular incision, the skull bone of the bulla was exposed. The bulla was opened with a drill. NanoBone® was soaked with the blood that was released from the incision of the skin. The middle ear space of the guinea pigs was filled with NB. The mucosa layer was left in the bulla. After periods of 1, 2, 3, and 4 weeks, groups of five animals were euthanised under general anaesthesia with an intracardiac injection of pentobarbital (100 mg/kg). For histological examination, the entire bulla was removed along with the adjacent structures. The removed specimens were fixed for 24 h in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2) then decalcified at room temperature in 10% ethylenediaminetetraacetic acid (EDTA) for 3 weeks and embedded in paraffin. For microscopic examination, 3 μm thick sections of the samples were stained with haematoxylin and eosin (HE), periodic acid-Schiffs reagent (PAS), and Alcian blue. For investigations with the electron microscope and energy dispersive X-ray spectroscopy (EDX), the sections were deparaffinised on a plastic slide. The electron microscopic studies of the surface were carried out with a DMS960 scanning electron microscope (Zeiss, Jena, Germany). EDX analysis (remX GmbH, Bruchsal, Germany) was carried out on 3 μm thick gold-palladium coated sections.

RESULTS

All guinea pigs survived the surgery with no apparent health problems. Interventions as a result of wound infections or other complications were not required.

Histology of the slice preparations

One week after implantation, there was a local inflammatory reaction with accumulation of neutrophils and plasma cells surrounding the NB bone grafting material (Fig. 1A). After the second week, the bone grafting material was embedded in a loose connective tissue. During the fourth week, we identified trabecular bone between the NB granules (Fig. 1B). For the detection of neutral and acidic mucosubstances, staining of the sections with PAS and
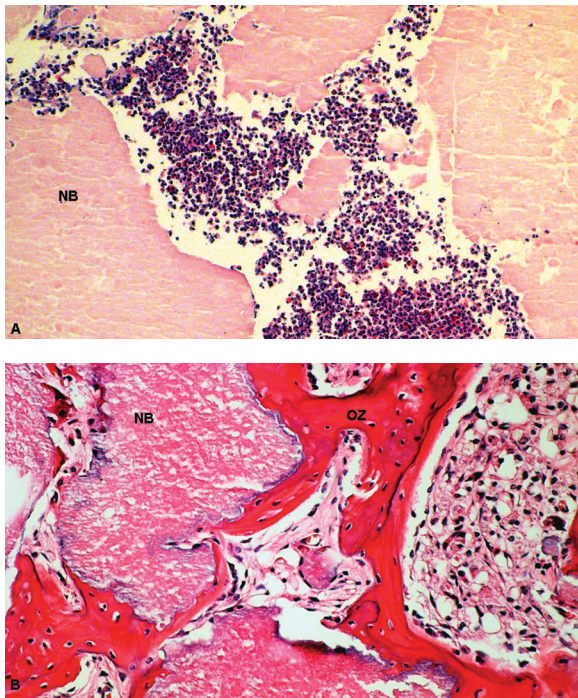
Figure 1. Histological examinations of implanted NanoBone®
(NB) after 1 (**A**) and 4 (**B**) weeks with ossification zones (OZ)
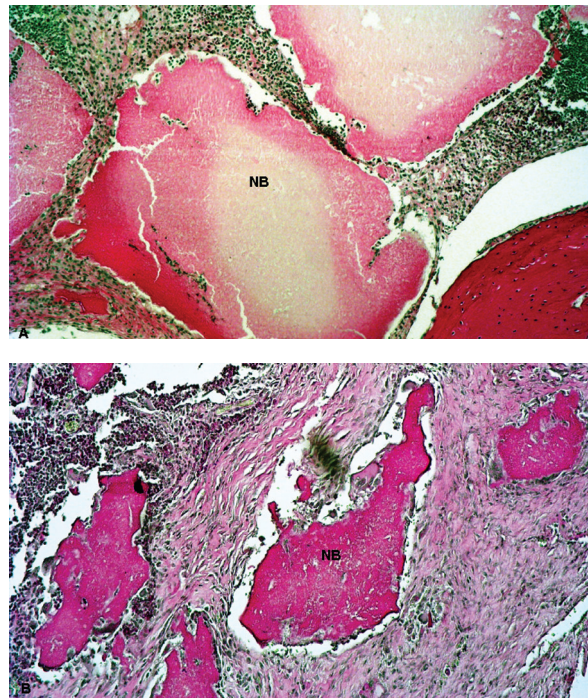(haematoxylin and eosin staining, ×100).

Figure 2. Histological examinations of implanted NanoBone®
(NB) after 1 (**A**) and 2 (**B**) weeks (PAS staining, ×100).

Alcian blue was performed. In the PAS stained sections, one week after implantation, we found enrichment of neutral mucosubstances such as glycogen, mucopolysaccharides, and muco- and glycoproteins on all of the edges of the NB particles (Fig. 2A). After another week, the particles were stained evenly (Fig. 2B). In the third week after implantation, the colour of the particles decreased while the ossification zones in the border area around the bone graft material were significantly stained.

In the Alcian blue stained sections, 1 week after implantation, we found that the internal structures of the NB particles stained intensely, indicating acid mucosubstances (Fig. 3A). After another week, the bone grafting material was uniformly weakly coloured, while the fringing ossification zones had a narrow strip of intense coloration (Fig. 3B). Fibroblastic tissue reactions such as encapsulation or foreign body reactions around the particles were not found.

**Electron microscopy/energy dispersive
X-ray spectroscopy**

Electron microscopy of decalcified NB particles before implantation showed the highly porous silica structure (Fig. 4A). One week after implantation of NB in the bullae of guinea pigs, the porous structures had
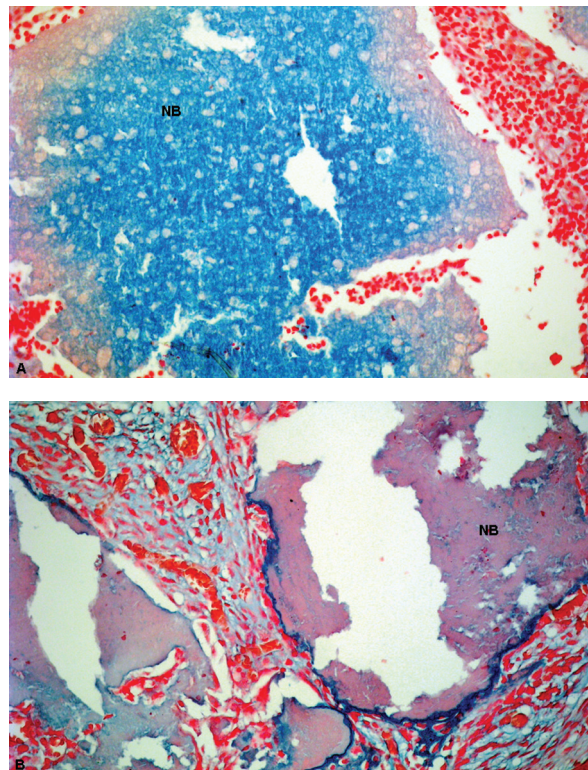

Figure 3. Histological examinations of implanted NanoBone®
(NB) after 1 (**A**) and 2 (**B**) weeks (Alcian blue staining, ×150).
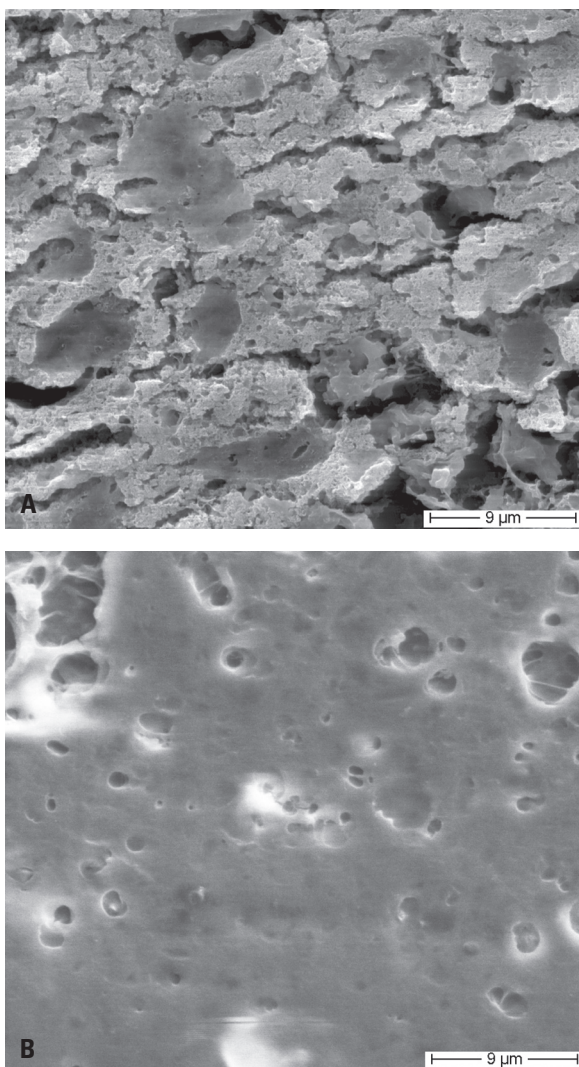
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Figure 4. Electron microscopy of decalcified NanoBone® particles before (**A**) and 1 week after implantation (**B**).
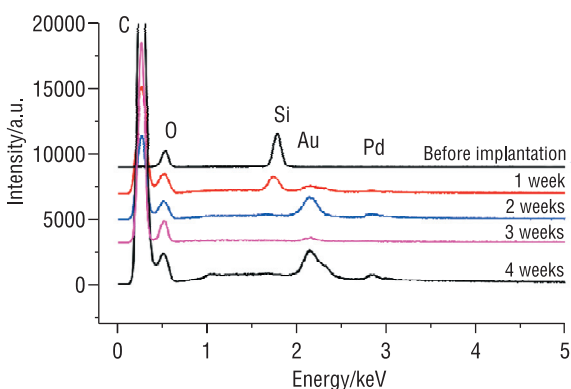

Figure 5. Energy dispersive X-ray spectroscopy analysis: change over time of concentration of the elements Carbon (C), Oxygen (O) and Silicon (Si) 1 week until 4 weeks after implantation; Au — — Aurum; Pd — Palladium; technically related measurement.
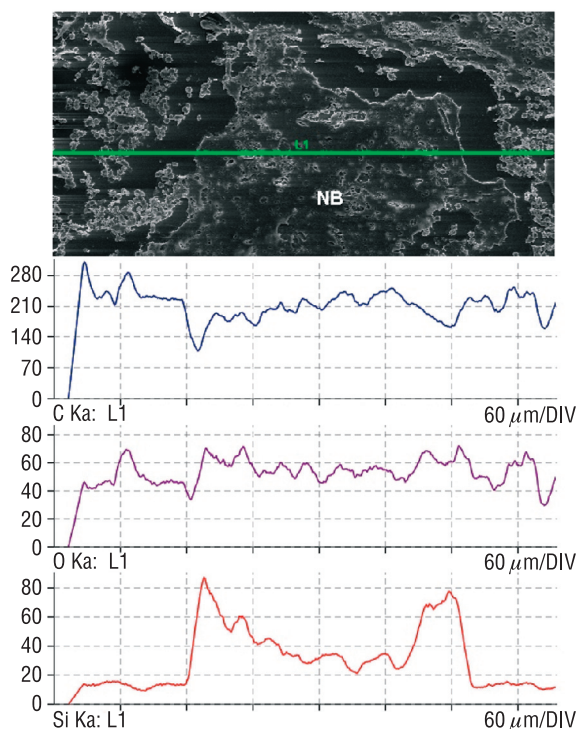

Figure 6. Energy dispersive X-ray spectroscopy analysis of the elements (line scan): Carbon (C), Oxygen (O) and Silicon (Si) 1 week after NanoBone® (NB) implantation.

vanished. A homogeneous matrix had appeared (Fig. 4B). In order to determine the composition of the homogenous matrix, we carried out EDX of the samples. After implantation, the NB particles were semi-quantitatively analysed for carbon, oxygen, and silicon. For progress of resorption of the decalcified bone material, we estimated the degree of silica. While a significant proportion of oxygen and silicon could be detected in the decalcified material before implantation, the silicon content had decreased significantly in the NB particles one week after implantation. Silicon was virtually undetectable in the second week after implantation. In the first two weeks after implantation, an increased carbon and oxygen content was measured in the NB particles (Fig. 5). EDX elemental analysis was carried out at various locations on the particles and the surrounding tissue. A week after implantation, there were clear differences in signal intensity for silicon all over the particles. We performed EDX line analysis of the sections and this was compared to the surrounding tissue. The NB particles showed a higher concentration of silicon. It was noticeable that the silicon intensity was stronger in the marginal area of the particles than in the centre (Fig. 6).

DISCUSSION

The bone grafting material NB was implanted into a preformed cavity, the guinea pig bulla, without abrasion of the mucous membrane lining the cavity or the bone. There was rapid bone formation and osseointegration of the material.

Bone substitutes have been investigated after implantation in artificial bone defects in animal models [12] but have mainly been used to fill bone defects such as those used in sinus augmentation [2]. With the obliteration of a pneumatised and preformed cavity, the increased air might play a special role. The used granules might be too big to fulfil a small cavity like the guinea pigs bulla constantly. Despite such adverse conditions, we found a rapid osteoinduction in the guinea pig bulla in our investigation. Basically, in rodents such as guinea pigs, it is very easy to induce bone growth [14]. However, in other studies, NB also showed very good resorption compared to the osteoconductivity of biphasic bone substitute material of 60% hydroxyapatite and 40% tricalcium phosphate [9]. Acidic and neutral mucosubstances such as glycoproteins, mucopolysaccharides, and phospholipids are important factors for osteoneogenesis and osseointegration. These accumulate initially in the border area and later throughout the particles of the bone grafting material.

Knowledge of the composition of the stained mucosubstances would be interesting and informative. Specific immunohistochemical studies should be carried out on specific glycoproteins such as osteogenin and bone morphogenetic protein, which are closely related to osteoneogenesis. The pore heterogeneity of macro- and micropores and the high porosity of the material, in excess of 80%, could be important factors for the enrichment. The silica portion of the investigated NB bone grafting material was 24%. Silicon plays a sheathing role in bone metabolism and can stimulate osteoblast proliferation [5, 10]. In our study there was a rapid reduction of silicon in the NB particles until, at 2 weeks after implantation, no silicon was detected. Even after the first week, a significant decrease in silicon concentration was seen in the bone grafting material although the breakdown of silicon has not been resolved sufficiently. The solubility of silica at physiological pH is extremely low [1]. Even a hypothetical degradation by cells could not explain the reduction because the macropores in the bone grafting material were too small for macrophages. A possible pathway for silica could be through an enzymatic reaction.

The carbon and oxygen content in the examined sections was adopted as parameters for the organic substances. The EDX analysis showed an increase in organic compounds while silica decreased. The silica was replaced by an organic matrix — a matrix transformation that took place within the NB particles. In addition, even after 1 week, electron microscopy showed the otherwise rough and porous surface in sections through the NB particles but now wrapped in a homogeneous matrix.

In the EDX analysis, a special feature was found on the fringes of the particles: here the silicon content had increased compared to the centre of the particles. This result was confirmed by measurements on a number of particles. The reason for this effect has not yet been determined. The early and rich vascularisation of the connective tissue surrounding the particle, and the porosity of the bone material with accumulation of extracellular matrix proteins in the internal structures of the bone grafting material, are certainly some of the reasons for the transformation matrix described.

CONCLUSIONS

In the bone grafting material examined after implantation, NanoBone® showed a rapid matrix change in the bullae of guinea pigs. This could be associated with the good osteoinductive properties of the material. Further investigations may provide an insight into the mechanisms of the matrix change.

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