Free Bioverit® II Implants Coated with a Nanoporous Silica Layer in a Mouse Ear Model – A Histological Study

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ABSTRACT: The objective of this study is to evaluate the suitability of a mouse middle ear model for testing ossicular replacement materials. Twenty-four BALB/c mice are implanted with the bioglass-ceramic Bioverit® II which is coated with a silica-nanostructure or with plain Bioverit® II as a control. After 2, 6, and 12 weeks, 4 mice per group are sacrificed and both complete petrous bones are analyzed histologically. All implants revealed *in situ* an incipient growth of thin connective tissue layers over the surface, followed by a

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spreading of epithelial cells. The osseogenic response which is increasing with time is more intense in the coated Bioverit® II specimens. The absence of inflammatory cells suggests an excellent biocompatibility of the silica nano structure. As the results are comparable to a study with the same materials in rabbits, the mouse model described is highly suitable for evaluation of new ossicular replacement materials. Additionally, by gene expression analysis a more detailed insight into cellular interactions of the middle ear is offered.

KEY WORDS: biocompatibility, Bioverit® II, histology, middle ear, mouse, silica-nanocoating.

INTRODUCTION

The ossicular chain is often destroyed in patients suffering from chronic middle ear diseases like otitis media or cholesteatoma. As middle ear ossicles are the key to a functional ear, their reconstruction poses an important challenge. Implant materials which are employed in reconstructive middle ear surgery have to fulfill some fundamental criteria. They should be light weight [1], biocompatible [2], processible and long-term stable [1,2]. The risk of infection has to be excluded [1]. A rapid growth of normal middle ear mucosa around the implant is important to prevent a microbial adhesion [3].

Human autologous and homologous ossicles have been proven to be extremely suitable devices for the reconstruction of the sound conductive system historically. However, their usage is limited by restricted availability and high risk of infection, respectively.

During the last decades, alloplastic ossicular prostheses have become a popular alternative to autografts and homografts. Often used materials are e.g., ionomers, metals and ceramics [4]. Every material combines different advantages and drawbacks with regard to its biocompatibility, integration, plasticity, and physical behavior.

Bioverit® is a bioglass ceramic, which exists in four different types (I-IV) [5]. Bioverit® II, a glass-mica ceramic, is easily machined, highly biocompatible and shows no signs of biodegradation. It can be implanted even in infected locations [1] since it seems to inhibit the growth of specific bacteria [6].

Additionally, nanostructured materials possess a promising potential to promote a selective interaction between implant material and certain cell species of the surrounding tissue: the adhesion of osteoblasts is supported on nanophase surfaces, whereas the adhesion of fibroblasts and endothelial cells is decreased [7]. Observed effects are mainly influenced by the topography of the nanostructures, but depend less on

the type of material [8]. Cell-culture tests as well as *in vivo* studies using nanoporous silica coatings with pores of 3–12 nm, indicate a favorable biocompatibility [9,10]. With regard to biomedical applications, a nanostructured material has a further capability as a drug delivery system [11,12].

Turck *et al*. investigated plain Bioverit® II in comparison to Bioverit® II which was coated with a nanoporous silica layer as TORPs (total ossicular replacement prostheses) in the middle ear of rabbits [10]. The histological analysis of the samples revealed no significant findings concerning the effect of the nanostructure. As the information of histology is restricted, we have established a middle ear model in mice for ossicular replacement materials.

In the wide field of biomedical research, mice are a popular species for *in vivo* trials. Even their ears are often used in auditory research, e.g., for the examination of otitis media [13–15]. The well known genome of this species offers substantial benefits including gene arrays and systematical use of mutant and inbred strains [14].

In this study we used BALB/c mice as an immunologically well characterized strain to investigate histologically the reaction of the middle ear to the implanted materials: pure Bioverit® II and Bioverit® II which was coated by a nanostructured silica layer. To test the feasibility and the viability of this new model, we compared our results with histological findings in a rabbit middle ear study, where the same materials were used as TORPs [10].

METHODS AND MATERIALS

In this animal experiment (AZ 33.42502/07; Bezirksregierung Braunschweig, Dezernat 509, Braunschweig) we implanted bioceramics into the tympanic cavity of mice.

We obtained Bioverit® II implants from 3di GmbH, Jena, Germany. Each implant consisted of a cylinder with a length of 1 mm and a diameter of 1 mm ($\pm$ 0.1 mm). Nanostructured silica coatings were applied using a spray-coating procedure which was specifically adapted to coat this material. An acidic water–alcohol solution containing tetraethoxysilane as a silicon source and poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (average $M_n \sim 5.800$, Aldrich) as amphiphilic block copolymer was sprayed onto the implants and dried at 60°C for at least 15 min before the next spraying step. Implants were coated four to five times, resulting in a layer thickness of some hundred nanometres. After drying, the coated implants were heated to 415°C in air in order to

burn out the organic amphiphile. The nanostructure then consisted of pores opening to the surface of the coated layer. X-ray diffraction indicated pores with a diameter of ~9 nm.

We used 24 healthy female BALB/c mice (Harlan Winkelmann GmbH, Borchen, Germany) in the weight range 16–22 g. They were kept in groups of 4 animals and fed a standard laboratory diet and water ad libitum.

For sedation and anaesthesia mice received an intraperitoneal injection of 10 mg/kg xylazin (Rompun® 2%, Bayer Vital GmbH, Leverkusen, Germany) and 100 mg/kg ketamin (Ketamin Gräub®, Albrecht, Aulendorf, Germany) and a second injection after 15 min with a half dose rate. Additionally, they were given 5 mg/kg of analgesic carprofen (Rimadyl®, Pfizer Pharma GmbH, Karlsruhe, Germany) subcutaneously to reduce inflammation and pain.

The surgical site was prepared by shortening the hair of the retroauricular skin as well as possible and the area was disinfected (Figure 1(a)). Eyes were protected with dexpanthenol eye ointment (Bepanthen® Augen- und Nasensalbe, Bayer Vital GmbH, Leverkusen, Germany). Mice were positioned under a laminar flow hood. The surgical procedure was performed under 1.6- to 4-fold microscopic control (Zeiss Stereoscopic microscope, SV11 STEREOZOOM MICROSCOPE, Objektiv Plan-Apochromat S 1.0x). After the retroauricular skin incision, the auditory canal was bluntly dissected to identify the tympanic cavity (Figure 1(b) and (c)). Next to the insertion line of the tympanic membrane, a hole was made with a hypodermic needle and then drilled with a rosen burr (1.0 and 1.4 mm; Aesculap AG & CO.KG, Tuttlingen, Germany) to open the tympanic cavity for insertion of the materials (Figure 1(d) and (e)). Each left cavity was implanted with one material, which was placed in the bulla away from the ossicular chain (Figure 1(e)). The opened bulla was sealed with muscle. The tissue was sutured in two layers (musculature and skin) with a synthetic absorbable sterile surgical suture (Ethicon Vicryl® 5-0 or 6-0; Manufacturer Johnson + Johnson Intl, European logistics centre, Belgium).

Trimethoprim/sulphonamide (Cotrim-K-ratiopharm® Saft, ratiopharm GmbH Ulm, Germany) was chosen for postsurgical antibiosis and given via drinking water over a period of 7 days (0.5%).

Each of the 2 materials was implanted in 12 mice and after duration of 2, 6, and 12 weeks always 4 mice per group were sacrificed. Both petrous bones of each mouse were excised immediately after death (Figure 1(f)) and perfused in 2.5% glutardialdehyde in 0.1 M sodium cacodylate buffer pH 7.3 (Merck, Darmstadt, Germany) at +4°C overnight.

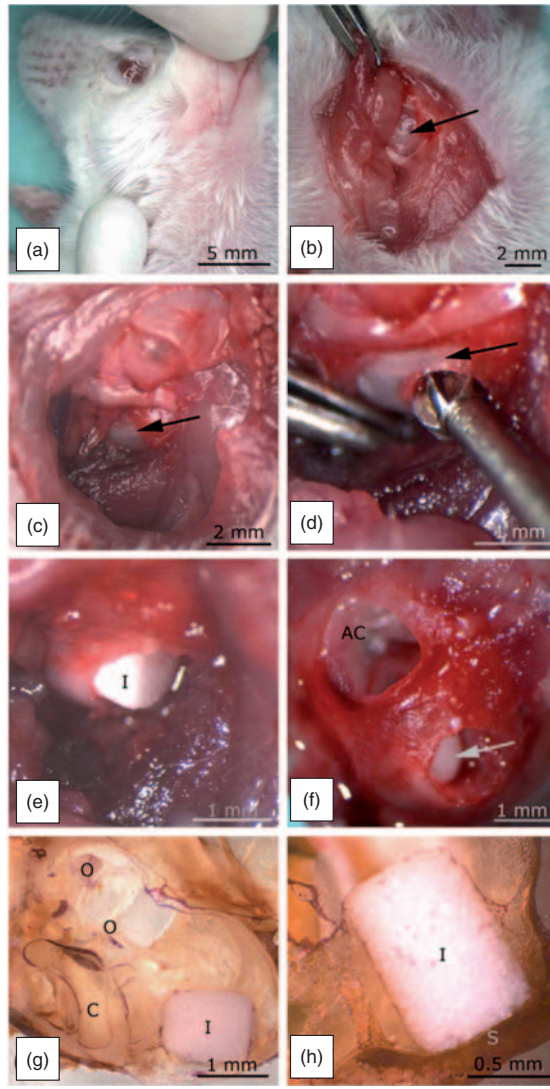

Figure 1. From surgical access to histological evaluation: Retroauricularly (a) the auditory canal is exposed (arrow in b). Next to the insertion line of the tympanic membrane (arrows in c and d) a hole is drilled into the wall of the bulla tympanica. During insertion of the implant (I) in situ (e) only an additional push is necessary to achieve the desired position (arrow in f) next to the auditory canal (AC) as seen in the already removed petrous bone (f). The grinded plane (g) illustrates the petrous bone with implant (I) and ossicles (O) inside the bulla as well as the cochlea (C). The wound healing is characterized by thin scar tissue (S) at the place of insertion after 12 weeks (h). (modified Mann–Dominici staining in g and h)

Specimens were dehydrated with graded ethanol and dried at 65°C. Embedding in epoxy resin (SpeciFix 20 Kit®, Struers A/S, Rodovre, Denmark) was accomplished under vacuum conditions.

Wet-sanded planes demonstrated the implant material *in situ* with all surrounding tissues (Figure 1(g) and (h)). Silicon carbide grinding paper (SiC Paper; Struers A/S, Rodovre, Denmark) with a grain size from 1200 to 4000 was used in a grinding and polishing machine (LaboPol-5®; Struers A/S, Rodovre, Denmark). The polished surfaces of the specimens were stained with a modified staining by Mann–Dominici. It consists of 0.5% Toluidine Blue 0 (Sigma-Aldrich Corp., St. Louis, Montana, USA), 0.1% Eosin G (Certistain®, Merck) and 0.25% Orange G (Certistain®, Merck) in 50% ethanol.

The histological documentation of four different planes of the tissue and the materials' surface was done with a stereoscopic microscope (Nikon® SMZ 1500, Tokyo, Japan) as well as with a microscope (Axioskop Zeiss, Göttingen, Germany) in 20- to 200-fold magnification illuminated from an external cold light source. The images were produced with a digital camera system (Colorview XS, Soft Imagine Systems GmbH, Münster, Germany) which was attached to the microscopes. They were analysed with Analysis 3.2 (Soft Imaging Systems GmbH) and processed with Adobe Photoshop 8.0.1.

RESULTS

All animals passed through anaesthesia and surgery without complication. Severe bleedings did occur neither during surgery nor after awakening. None of the mice showed any indisposition or reduction of alimentation as signs of an infection. Nystagmus or oblique position of the head as a result of a vestibular failure due to surgical irritation could not be observed in any case. The incision always healed by first intention (Figure 1(h)). The macroscopic examination of the outer ear at the time of euthanasia revealed a nonirritated and clean external auditory canal, and tympanic membranes were intact in all mice.

In the microscopic evaluation the inner ear (cochlea and vestibular organ) appeared always untouched and not inflamed (Figure 1(g)). The bulla tympanica remained free from any visible signs of infection or destruction. In only 4 of 24 cases a slight defect of the malleus, which did not disturb the stability of the whole ossicular chain, emerged when using the way of surgical entrance described above.

One of the implants was dislocated after 2 weeks in each group. These 2 specimens were found in the surrounding tissue of the tympanic cavity

and were not evaluated. In all other 22 cases the implants remained *in situ* lying in contact to the bulla wall close to the cochlea.

Histologically, no signs of degradation or dissolution were observed in any of the implants (Figure 1(h)). An infiltration of immune cells or an increased angiogenic activity could be found neither within the connective tissue which was directly attached to the material, nor in the surrounding tissue lying far off. The mucosa of the bulla looked completely regular in all cases (Figure 2). These findings confirm an absence of inflammatory reaction.

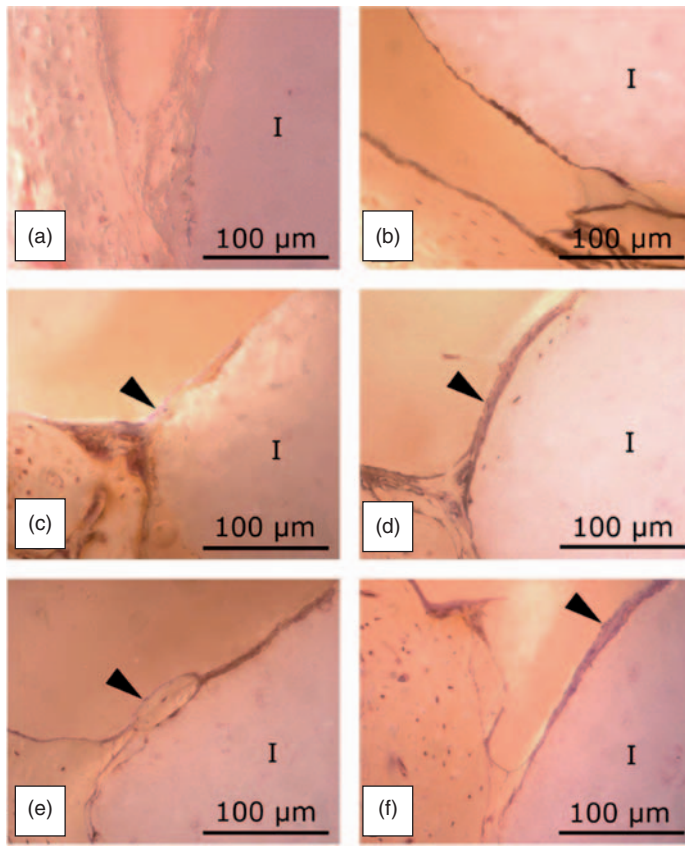

Figure 2. Continuation of the mucosal coverage of the bulla to the implants' surface: Comparison of plain Bioverit® II implants (I) at 2 (a), 6 (c) and 12 weeks (e) and coated Bioverit® II implants (I) at two (b), six (d) and twelve weeks (f). After two weeks only fibroblasts are connected; epithelial coverage is present after six and twelve weeks (arrowheads). (modified Mann–Dominici staining)

Pure Bioverit® II

At 2 weeks, all implants showed fibroblasts spreading over the surface. Areas of the material, which interacted with the wall of the bulla tympanica, possessed a continuous connective tissue layer, whereas other areas, which were projected into the free space of the tympanic cavity, were colonized by single cells only (Figures 2(a) and 3(a)).

After 6 weeks, thin and coherent connective tissue was enlarged over the surface and was sometimes covered by a single layer of epithelial cells (Figure 2(c)). The capsule was reduced in thickness on the free edge of the material (Figure 3(c)).

After 12 weeks, all implants were completely coated by a continuous connective tissue layer (Figure 3(e)). With respect to its thickness, the gradient from the contact zone to the free edge was still demonstrable. The capsule was partly covered by epithelial cells (Figure 2(e)). Some isolated connective fibres joined the bulla wall with the material in an increasing frequency over time.

Plain Bioverit® II implants initiated an osseogenic response during the whole implantation period. The frequency of ossification increased with time after implantation (Table 1). After two weeks, only in one out of 3 cases a small amount of new bone could be observed in the interspace between the material and the bulla wall. The bony tissue was in direct contact to the material.

At 6 weeks, only one out of 4 samples showed newly developed bone. The amount was slightly larger than after 2 weeks and also directly attached to the surface (Figure 4(a)). In this case, the new bone environed two edges of the implant at the junctions between material and bulla wall. A defect of the malleus in the vicinity of the material also led to growth of new bone.

After 12 weeks, 3 out of 4 specimens revealed an osseogenic response (Figure 4(c)). Partly, new bone had completely enclosed the implant, whereas it was sometimes separated by cells from the surface of the material. Other samples possessed smaller amounts of new bone in direct contact to the material.

Nanocoated Bioverit® II

After 2 weeks, a connective tissue layer had almost completely surrounded the material. The thickness was highest at the contact zone with the bulla wall (Figure 2(b)). The number of cells decreased with the distance from contact points (Figure 3(b)). On the whole, the situation was comparable to pure Bioverit® II at 6 weeks.

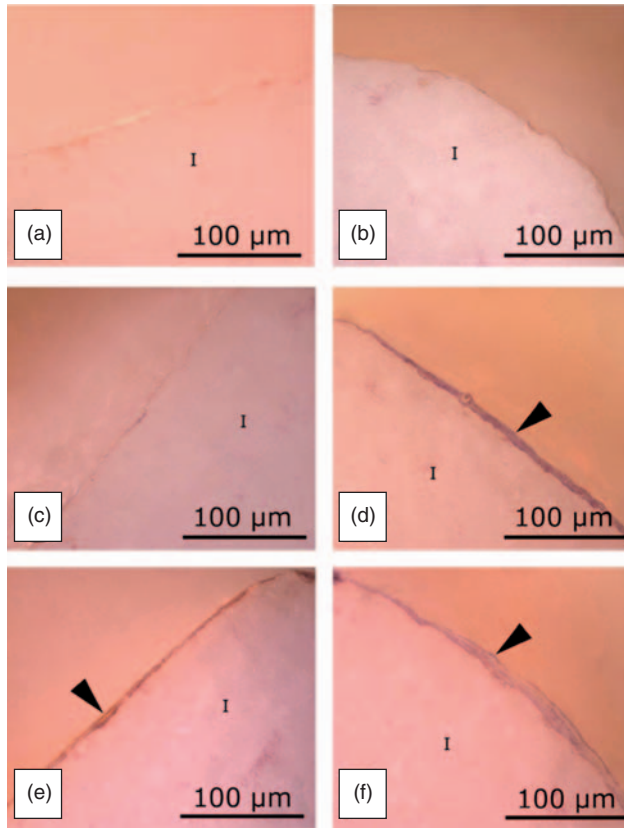

Figure 3. Capsule on the free surface of the implant: Fibrous tissue capsule and epithelial coverage (arrowheads) on the free implants' (I) surface directed to the tympanic cavity – comparison of plain Bioverit® II at 2 (a), 6 (c) and 12 weeks (e) and coated Bioverit® II at 2 (b), 6 (d), and 12 weeks (f). (modified Mann–Dominici staining).

At 6 weeks, the connective tissue layer was still thin but expanded completely over the whole surface (Figure 3(d)). The amount of cells and their distribution was similar to plain Bioverit® II at 12 weeks (Figure 2(d)).

After 12 weeks, a slight tendency to a decreased amount of cells on the whole and thus a diminution in the thickness of the connective capsule could be observed (Figures 2(f) and 3(f)). As seen in pure Bioverit® II, bridges consisting of single connective fibres sometimes filled the interspace between material and bulla wall.

The nanocoated Bioverit® II implants revealed an increasing osseogenic response during the whole implantation period which was generally more distinct than in plain Bioverit® II (Table 1).

Table 1. Extent of new bone formation in all animals of the study.

Time	Plain Bioverit® II	Coated Bioverit® II
2 weeks	–	–
	–	–
	+	+
6 weeks	–	+
	–	+
	–	+
	+	++
12 weeks	–	++
	+	++
	+	++
	++	++

Notes: – no bone formation; + little amount of new bone; ++ extensive amount of new bone

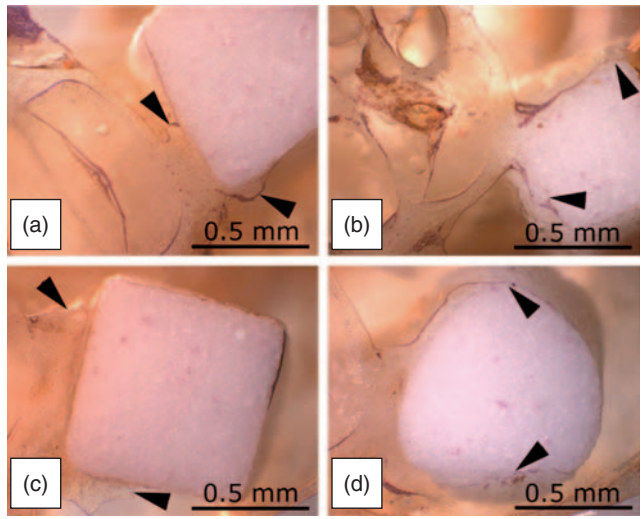

Figure 4. Development of new bone: New bone formation (between the arrowheads) is limited in plain Bioverit® II at 6 (a) and 12 weeks (c) in comparison to coated Bioverit® II at 6 (b) and 12 weeks (d). (modified Mann–Dominici staining)

After 2 weeks, the findings were similar to plain Bioverit® II. Only one out of 3 specimens showed some new bone which had developed between bulla wall and the material. Actually, a few osteoclasts were visible between implant and bone.

All 4 implants of the 6-week group exhibited new bone formation. The ossification always started from the bulla wall and surrounded adjacent

parts of the implant (Figure 4(b)). In some areas the new bone was directly attached to the material whereas other parts revealed separation by intermediate connective tissue. In comparison to plain Bioverit® II, the ossification was more expanded.

In the 12-week group again, the material of each sample showed a considerable formation of new bone which started in contact zones with the bulla wall and mantled big parts of the implant (Figure 4(d)). Usually it was attached so closely to the material that no clear borderline remained. Some smaller parts revealed intermediate connective tissue. Compared with the 6-week group of coated implants and with the 12-week group of plain Bioverit® II, the amount of new bone was obviously extended.

DISCUSSION

Animal Model

There has been extensive research investigating ossicular chain replacements in rabbits and guinea pigs [10,16–19]. Mouse middle ear models have often been used for otitis media research. However, only one research group has dealt with a middle ear model of mice for ossicular chain replacements so far [20–22].

Dost et al. found new bone formation surrounding Bioverit® I + II, hydroxyapatite and Ceravital® implants, and also in contact with the middle ear wall in guinea pigs [18,19,23]. Additionally, a significant amount of new bone around the ossicle was observed in a reconstruction with hydroxyapatite cement and dahllite cement [24]. This fact leads to the conclusion that the extensive ossification limits the use of guinea pigs for research about ossicular chain replacements [18].

Rabbits are well established in middle ear studies [10,16]. Their advantage is the size of the middle ear which makes an implantation of real ossicular replacement prostheses possible. Additionally, the rabbit model offers the chance of functional studies [17,25,26]. Unfortunately, their genome is not completely known so far which makes a genetic evaluation impossible.

Mice were often used for otitis media research [13,14,27]. Their advantages are low cost, ease of handling, and applicability to human disease [14]. Furthermore, the knowledge of their complete genome and the existence of a multitude of transgenic strains offer plenty of possibilities. Tissue and temporal specific control of gene function provides an insight into the cell biological processes [27]. The only drawback of mice is their small size which makes accurate surgery with

the aid of microscope absolutely essential and functional studies extremely difficult.

We decided to use BALB/c mice for our mouse model, which is one of the best characterized mouse strains in immunology [28]. One study has revealed that – in comparison to other mice strains – BALB/c mice were more prone for developing systemic diseases after inoculating the middle ear with a bacterial suspension. Symptoms like lethargy, ruffled fur, conjunctivitis, and balance problems occurred. These were followed by a poor prognosis [27].

In our study, mice did not encounter any infective material. However, the complete lack of any signs of restriction in their behavior, of local or systemic diseases after surgery, leads to the conclusion of a very clean procedure during implantation. In addition, the material used has a very good biocompatibility.

Biocompatibility

In another middle ear study in mice, different materials (bioglass, silastic, and plasti-pore) displayed inflammatory reactions signified by the presence of some macrophages, giant cells, and sometimes fibrous scar tissue, whereas proplast did not show any inflammation [20]. Guinea pigs did not show a foreign body reaction to Bioverit® I + II implants in the middle ear [18].

In our study, no sign of any inflammatory or foreign body reaction could be found in any of the uncoated and coated Bioverit® II implants. However, around the same material in the middle ear of rabbits, some foreign body giant cells were found [10].

Material Conditions

In rabbits, uncoated and coated Bioverit® II TORPs showed small aggregates of isolated material within the tympanic cavity after 84 days [10]. In our mouse model, where the implants did not suffer any mechanical stress we did not find any alterations even after 3 months.

Capsule Formation

The rapid growth of local middle ear mucosa around the implant material is an essential process for the successful integration of ossicular replacements.

Free titanium implants in the middle ear of rabbits showed less mucosal growth even after 504 days of implantation than implants

which were fixed as TORPs [29]. In another study, most free titanium implants were covered by epithelium after 168 days [30]. In a third study, the majority of free implants were completely covered with epithelium after 336 days, whereas some implants were not epithelialized even after 504 days [31].

In rabbits, free glass ionomer cement implants exhibited a complete mucosal coverage after 84 days in the middle ear and at 168 days some osteoid had developed [30]. If placed on unwounded mucosa, ionomer cement implants were partly covered by a mucous layer after 28 days. After 56 days, the free implants exhibited an entirely coated surface. In contrast, columellas with contact to injured mucosa were completely surrounded by middle ear mucosa already after 28 days [32].

In a rabbits' study with uncoated and coated Bioverit® II, all implants which were interposed between the footplate of the stapes and the manubrium (TORPs) showed epithelial coverage after 28 days. In comparison, the coated implants displayed a coverage which was more spread out [10].

In middle ears of mice, Merwin et al. found bioglass implants surrounded by a thin collagenous capsule. Moreover, a respiratory epithelium had grown over this surface after 1 and 2 months. In contrast, plasti-pore induced an inflammatory reaction and revealed a thick connective tissue capsule after 1 month [20]. In our study, within 2 weeks, all uncoated and coated Bioverit® II implants had a thin coverage of connective tissue which was always beginning from a contact point between prosthesis and surrounding tissue. In contrast to a cell culture study [7], the nanostructure seems to support the growth of cells on its surface.

New Bone Formation

A bony fixation between material and the footplate of the stapes is usually desired to prevent a dislocation of ossicular replacements.

An enhanced osteoblast proliferation on nanophase ceramics (alumina, titania, hydroxyapatite) was verified as compared to the conventional formulations in cell culture studies. However, this increased adhesion came along with a decreased surface occupancy [7,33]. The presence of proteins promotes the attachment of osteoblasts [7].

Furthermore, an osteoblast adhesion appears to be independent of material type and surface chemistry, but depends on the surface topography [7]. Other researchers also found a nanostructured coating having a positive effect to the osseointegration rate [34] as well as to adhesion and spreading of mesenchymal stem cells [35].

In the middle ear of mice, the replacement material proplast led to the growth of some new bone into its pores [20].

The results of the present study exhibited an augmented bone formation in case of the nanocoated Bioverit® II implants as compared to plain Bioverit® II implants after 6 and 12 weeks. The bone formation on these free implants always started from the contact zones with the bulla wall. Both materials revealed an increasing ossification over time and a higher frequency of cases with newly formed bone. These findings were in agreement with the *in vitro* data.

In contrast, in the middle ear of rabbits, a minor osseogenic response without time-related differences was observed on the surface of plain Bioverit® II and nanostructured Bioverit® II TORPs [10]. The coated implants as well as microporous TiO_2 TORPs revealed a slight tendency to a decreased bone formation in investigation periods up to 43 weeks [10,16].

It was not possible to rule out differences depending on the species. However, dimensions of contact zones with the osseogenic tissue were smaller when using TORPs in rabbits than inserting free implants in mice. Therefore, the extent of ossification seems to be regulated by the size of the contact point and also by the implantation period.

CONCLUSION

The BALB/c mouse model is highly suitable for the evaluation of new ossicular replacement materials in the middle ear. The next important step will be to analyze the specimens with gene array techniques which provide an insight into molecular responses to implant materials [36].

The silica nanocoating revealed a favorable biocompatibility as no signs of inflammation were found. Additionally, the adhesion of osteoblasts and fibroblasts has been promoted. In the ossicular replacement surgery a localized osseointegration should be supported in direction of the stapes footplate to stabilize the prosthesis *in situ*. Therefore, a local nanostructured coating may be helpful for this purpose on any favored implant material.

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