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Efficacy of nanoporous silica coatings on middle ear prostheses as a delivery system for antibiotics: An animal study in rabbits

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Abstract

Nanoporous silica layers are able to host molecules and release them over a certain period of time. These local drug delivery systems for antibiotics could be a new approach in treatment of chronic otitis media. The aim of this study was to examine the efficacy of nanoporous silica coatings on middle ear prostheses as a delivery system for antibiotics in vivo.

Pseudomonas aeruginosa was inoculated into the middle ear of rabbits to induce an otitis media. The control group received coated Bioverit® II implants without antibiotics. Coated prostheses with loaded ciprofloxacin were implanted into the middle ears of the study group. After one week, the rabbits were sacrificed.

The clinical examination as well as the microbiological and histological examinations of organs and middle ear irrigation revealed clear differences between the two groups. *P. aeruginosa* was detected in every middle ear of the control group and was almost completely eliminated in the study group. Organ examinations revealed the presence of *P. aeruginosa* in the control group and a prevention of a bacterial spread in the study group.

The nanoporous silica layer as antibiotic delivery system showed convincing efficacy in induced pseudomonal otitis media in the rabbit.

Keywords

Animal model, drug release, infection, middle ear, nanoporous

1. Introduction

Treatment of chronic otitis media (COM) is often complicated by recurring bacterial infections, which disturb the healing process, lead to persistent otorrhea, and might even induce meningitis, brain or mastoid abscess [1-3]. Even after surgery aiming at eradication of the infection and despite major progress in chemotherapy, microorganisms can persist and reproduce. Besides, the connection between the oral cavity and the middle ear through the Eustachian tube enables bacterial colonization which can hardly be avoided.

In many cases of COM the ossicular chain is destroyed and reconstruction is necessary. Microbial biofilms can form on freshly implanted material surfaces and impair the performance of the prostheses [4]. If the implants are infected or complications like extrusion occur, they have to be removed. An important factor for a successful implantation is a fast integration of the prostheses. Therefore, tissue cells of the organism have to cover the implant before bacteria adhere to the surface and may lead to implant rejection [5, 6]. There are several approaches to avoid bacterial adhesion and reproduction on medical devices. One possibility is the application of a material with mild antibacterial activity, such as ionomer cement, bioactive glass ceramic or hydroxyapatite. For these materials, the antibacterial efficacy differs depending on the bacterium species [7]. Also bioceramics like Bioverit[®] or Al₂O₃ ceramic show a slight antibacterial effect against gram-negative bacteria, but complete elimination is not achieved. In contrast, glassy carbon serves as a source of nutrition for bacteria so that this material is unsuitable for ossicular reconstruction [8]. Another possibility is the surface modification of implants, for instance using special coatings with antibacterial activity. Berry et al. investigated phosphorylcholine-coated fluoroplastic tympanostomy tubes in comparison to silver-oxide impregnated and plain fluoroplastic tubes incubated with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The coated tubes showed resistance to biofilm formation by both pathogens, whereas in plain tubes, growth of *P. aeruginosa*, and in silver-oxide impregnated tubes, biofilms consisting of both microorganisms were detected [9].

An antibacterial effect can also be provided by a local drug delivery system releasing a drug at the critical site to kill bacteria in the surrounding area. Often, biodegradable polymers are used as a drug reservoir and release system [10]. Nanoporous silica materials have been proposed as potential drug release systems in 2001 [11]. In the beginning, investigations have largely focused on in vitro applications and simple model drugs like ibuprofen, but more recent investigations have been conducted with a wide variety of drugs and biologically active molecules [12-15]. Nanoporous silica materials possess regular pores on the lower nanoscale (3-10 nm) and are also designated as mesoporous materials, according to the definition of the International Union of Applied Chemistry [16]. Their pore diameters are sufficiently large for the adsorption also of larger drug molecules. Nanoporous silica materials possess large pore volumes and high surface areas, allowing the absorption of large amounts of drugs, thus providing sufficient concentrations for local treatment. The surface of silica materials is reactive due to the presence of silanol groups. This allows for facile modification by silanization reactions and thus opens possibilities for enhancing the drug loading and for controlling the drug release [17, 18]. The research in the biomedical application of this material has so far focused on nanoporous silica nanoparticles, whereas the device presented here is based on a coating, i.e. a continuous thin film, on a prosthesis. Using this implant-supported coating as a basis for drug delivery constitutes a direct pharmacological application form.

The nanoporous silica coatings we use have been evaluated in preceding studies [13, 19-21] and have demonstrated excellent tolerance and tissue compatibility in cell culture and animal experiments, with one study in the middle ear of rabbits extending over a period of up to 360 days [19]. Many studies on nanoporous silica nanoparticles have proven their general biocompatibility [17,18, 22-24] which has also been proven in some animal studies [23, 25-27]. However, some studies have exhibited potentially harmful properties. In one investigation,

nanoporous silica particles injected intraperitoneally or intravenously in a mouse model lead to death or euthanasia [25]. A further investigation in a mouse model showed that subcutaneously placed nanoporous silica nanoparticles could increase tumor growth [28]. In contrary, the FDA has recently approved the first in human trial of nanoporous silica nanoparticles applied in the bloodstream [29].

The object of this study was to examine the application of a drug delivery system in reconstructive middle ear surgery. For this purpose, Bioverit®II middle ear prostheses were equipped with a nanoporous silica coating which was chemically modified to incorporate large amounts of ciprofloxacin [13]. These prostheses were implanted in the middle ear of rabbits, a useful animal model for middle ear surgery [30]. To demonstrate the antibacterial efficacy, otitis media was induced by *P. aeruginosa* application into the middle ear.

2. Materials and methods

2.1 Animals

For this study, 24 female New Zealand White rabbits were purchased from Charles River, Sulzfeld, Germany. The rabbits were reared up to a weight ranging from 3.2 to 4.2 kg in the Institute for Laboratory Animal Science and Central Animal Facility of Hannover Medical School, Germany. This study was conducted in accordance with the German law for animal protection and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes. All experiments were approved by the Local Institutional Animal Care and Research Advisory committee and permitted by the local government (Ref.: 33.9-42502-04-09/1734).

2.2 Implants

In this study Bioverit®II prostheses from 3di GmbH (Jena, Germany) were used as basic material for ossicular reconstruction. The implants consisted of a cylinder with a length of 2.5 mm and a diameter of 1 mm and at a right angle a plate with a diameter of about 3 mm (Figure 1).

Figure 1: Coated Bioverit® II implant.

The implants were coated using a dip-coating procedure to establish the nanoporous silica layer. In a fashion similar to the production of flat samples and as described in more detail elsewhere [13], coatings were produced by dip-coating the implants from a solution containing ethanol, water, hydrochloric acid, tetraethoxysilane as a silica source and a poly(ethylene glycol)-poly(propylene glycol)-block-co-polymer (from Sigma-Aldrich, similar to Pluronic P-123 from BASF) as the structure-directing agent. The implants were dipped employing a DC Small Dip-Coater (NIMA, Coventry, England) operated in a climate box at a constant humidity adjusted by 50% w/w glucose solution. The coated implants were then left at constant humidity for five minutes. The samples were then dried at 60 °C for 30 min. This procedure was repeated twice. Finally, the samples were dried overnight and afterwards calcined at 415 °C for four hours (rate of heating/cooling 1 °C min⁻¹).

In order to improve the degree of loading with ciprofloxacin, the nanoporous coating was chemically modified so that its surface carries sulfonic acid groups. The surface silanol groups of the silica were first reacted with a mercaptosilane and then oxidized with hydrogen peroxide [13, 31]. For this purpose, the implants were first cooled to 0 °C in 89 ml of dichloromethane before 12.76 ml of 3-mercaptoptrimethoxysilane were added. The solution was gently stirred for 22 h without renewing the ice bath. The implants were then washed with dichloromethane and ethanol and dried at 100 °C for five hours. Afterwards, they were placed in 100 ml of an aqueous hydrogen peroxide solution (30% w/w) for 48 h at room temperature, followed by washing with water and absolute ethanol. Finally, the implants were dried at 60 °C for two hours and cooled to room temperature.

The insertion of ciprofloxacin into the sulfonate-modified nanoporous coating was carried out in a 60 mM solution of the drug at pH 4 and at 37 °C for three days. At pH 4, the sulfonic acid groups are deprotonated and thus negatively charged whereas the molecules of the ciprofloxacin are protonated and thus positively charged, leading to a strong electrostatic attraction [13]. After the insertion, the implants were rinsed shortly with water to wash off remaining solution at their outer surface. Afterwards the samples were dried for two hours at room temperature at constant air humidity adjusted with 50% w/w glucose. From the preceding work on ciprofloxacin release from glass slides coated once with modified nanoporous silica layers, it can be estimated that per 1 cm² of the macroscopic surface of the implant, ca. 2 µg of ciprofloxacin can be released, corresponding to ca. 0.32 µg of ciprofloxacin per implant (with a calculated implant surface of 0.16 cm²).

We implanted two types of prostheses, which were allocated to two groups. In the control group ($n=7$), Bioverit® II implants with a sulfonate-modified silica coating without antibiotic were applied. The study group ($n=7$) consisted of implants with a ciprofloxacin-loaded modified nanoporous silica layer.

2.3 Bacteria

For testing the efficacy of local antibiotic drug delivery in the middle ear, a complex procedure was developed.

In an initial experiment, a clinical *P. aeruginosa* isolate obtained from an abscess as well as the strain PAO1 were used for experimental infection to test their suitability for this study. As the clinical isolate showed increased pathogenicity resulting in premature euthanasia of an animal, the biofilm forming strain PAO1 was used for all further experiments and the concentration of the bacterial suspension adapted. Sensitivity of the *P. aeruginosa* strain PAO1 to ciprofloxacin was assured.

The bacteria were cultured on blood agar and incubated at 37°C for 24 hours. Subsequently, they were suspended in sterile saline and set to an optical density of 1 (OD=1) measured at 578 nm, corresponding to a concentration of 1x10⁶ colony-forming units (CFU)/10 µl. This suspension was diluted 1:1000 in sterile phosphate-buffered saline solution (pH 7.40) to obtain a concentration of 1x10³ CFU/10 µl which was confirmed by plating the suspension. This dilution was used for experimental infection of the middle ear during surgery.

2.4 Middle ear surgery and post operative care

Narcosis, preparation for surgery and access to the middle ear were performed according to previous studies [19, 32].

After opening the tympanic cavity, the ossicular chain was removed and the prostheses were implanted. Before replacing the tympanomeatal flap, 0.2 ml of the bacterial suspension (1x10³/10 µl) was injected into the bulla. Gelita (B. Braun, Melsungen, Germany) was used as a tamponade to close the external auditory canal. The wound rims were closed with non-absorbable surgical suture material (Supolene®, RESORBA Wundversorgung, Nürnberg, Germany).

The rabbits were examined twice a day. We recorded their general and neurological conditions and examined the wound areas. Besides, the animals received carprofen (Rimadyl[®], 4 mg/kg s.c., Pfizer, Berlin, Germany) as an anti-inflammatory and analgetic drug for two days. If the rabbits showed remaining fever after two days, carprofen treatment was continued. In case an animal displayed neurological disorders, we administered vitamin B1 (Betabion[®] 100mg, 10 mg/kg p.o., Merck Serono, Darmstadt, Germany) and vitamin B6 (Vitamin B6-Hevert[®] Tabletten, 5 mg/kg p.o., Hevert-Arzneimittel, Nussbaum, Germany) to support neurological functions. Six rabbits had to be fed with blended special powder for herbivore (Critical Care[®], Albrecht, Aulendorf, Germany) because of insufficient autonomous feed intake. Additionally, these rabbits received subcutaneous injection of electrolyte solution (Ringer-Acetat-Lösung, B. Braun, Melsungen, Germany) for fluid substitution. To support the intestinal flora we administered Bird Bene Bac[®] Gel (Albrecht, Aulendorf, Germany) if the rabbits suffered from diarrhea.

Blood samples were taken daily from the auricular artery in an ethylenediaminetetraacetic acid (EDTA) tube and examined using Vet ABC[®] Animal Blood Counter (Scil animal care company GmbH, Viernheim, Germany), especially to control the number of leucocytes.

2.5 Evaluation of the clinical condition

To evaluate the general clinical condition, we assessed behaviour and posture, feed intake, defecation, body weight and rectal temperature. We created a scoring system for the first four parameters mentioned. The daily scores of each animal were added to a total clinical score and then calculated as the means of the two groups (Table 1). Daily measured body weights were compared with the initial weights. Rectal temperature was calculated as the mean of two measurements per day.

Table 1: Scoring system for clinical general examination.

Clinical parameters		Score
Posture and behaviour	lively/cheerful, attentive, curious, eating, typical movement and posture	0
	calm, attentive, eating, reduced movement, normal posture	1
	very calm, incurious, weak, sternal- abdominal position	2
	apathetic, lateral position, rolling	3
Feed intake	normal	0
	slightly decreased	1
	missing	2
Defecation/urination	normal	0
	modified	1
Total clinical score		0-6

Concerning the neurological status, the symptoms head tilt, nystagmus, mechanical head motion, imbalance, circling in cage and rotation around the longitudinal axis were evaluated. The number of animals of each group showing the different neurological symptoms was exposed. Additionally, the neurological status was evaluated with the aid of a scoring system (Table 2).

Table 2: Scoring system for neurological examination.

Neurological symptoms	Combinations	Score
None	None	0
Head tilt Nystagmus Mechanical head motion	1-2 of these symptoms or symptoms only in excitement	1
Imbalance Circling in cage	3-5 of these symptoms	2
Head tilt Nystagmus Mechanical head motion Imbalance Circling in cage Rotation around the longitudinal axis	3-5 of these symptoms, always incl. rotation around the longitudinal axis	3

2.6 Euthanasia and necropsy

Animals of both groups were euthanized one week after infection at the latest. Animals received an intramuscular injection of ketamin (Ketamin Gräub[®], 25 mg/kg i.m., Albrecht, Aulendorf, Germany) and midazolam (Midazolam-ratiopharm[®], 1.25 mg/kg i.m., ratiopharm, Ulm, Germany). Additionally, propofol (Propofol-Lipuro 1%[®], 2 mg/kg i.v., B. Braun, Melsungen, Germany) was administered through a venous access in the auricular vein to deepen the narcosis. Subsequently, the rabbits were sacrificed using pentobarbital (Release[®], 600 mg/kg i.v., WDT, Garbsen, Germany). The animals were dissected and organ alterations were documented. Tissue samples of lung, liver, spleen, and kidney were taken for microbiological examination. Additional samples of other organs, when appearing affected, were also taken. The external acoustic meatus was dissected and a middle ear irrigation with 1ml saline solution was administered through the tympanic membrane. The appearance of this solution was evaluated (0: turbid or bloody; 1: mildly purulent; 2: highly purulent) and then stored for further microbiological evaluation. Afterwards we opened the bulla from the ventral side and took a swab sample of the middle ear mucosa as a second sample for further microbiological evaluation. The implant was retrieved and rinsed with 100 µl saline solution to determine possible *P. aeruginosa* adhesion and growth on the prostheses. Additionally, we evaluated the macroscopic findings in the middle ear based on a scoring system (0 = no abscess, 1 = abscess). Finally, the skullcap was opened and a swab sample of the brain was taken for possible detection of *P. aeruginosa*. The brain was removed and examined histopathologically to detect inflammatory lesions, especially around the auditory nerve.

2.7 Microbiological examination

Tissue samples of organs, rinsing solutions of implants, and swab samples of the brain were cultured in thioglycolate (TG) medium (Thioglycolat Medium U.S.P., Oxoid Deutschland GmbH, Wesel, Germany). The lavages as well as the swabs of the middle ears were cultured on TG medium, standard blood agar (Blood Agar Base No.2, Oxoid Deutschland GmbH, Wesel,

Germany) and Gassner agar (Oxoid Deutschland GmbH, Wesel, Germany). Bacterial growth in the TG medium-based samples was evaluated for one week; for the blood- and Gassner agar-based samples, observation was restricted to a maximum of 48 h.

The longitudinal axis presence of *P. aeruginosa* was confirmed by an oxidase test. To definitely identify *P. aeruginosa* we used the API system (API®20NE, bioMérieux Deutschland GmbH, Nürtingen, Germany) on a random basis. Quantification of *P. aeruginosa* was achieved by culturing dilution series of middle ear irrigations and rinsing solutions of implants and is given as CFU/10 µl.

2.8 Statistical methods

GraphPad Prism 5.0 was used for statistical analysis. The parameters of the general clinical examination were statistically analyzed using the Mann-Whitney U- test. The chi-square test and Fisher's test were applied to evaluate the neurological findings and the results of the necropsy and qualitative microbiological examination. Quantitative microbiological results were statistically analyzed by an unpaired T-test.

3. Results

Based on the initial study, the *P. aeruginosa* laboratory strain PAO1 was selected for all further analyses as it induced typical general clinical conditions and also local clinical pictures consistent with an otitis media. In the control group receiving no local antibiotic delivery, one rabbit had to be euthanized after four days and one rabbit after six days owing to poor health conditions.

3.1 General clinical examination

The two groups showed obvious differences in their general condition (Figure 2). While the control group was severely impaired, the animals of the study group barely showed any disorders of their general condition. Clinical scores, consisting of the parameters posture and behaviour, feed intake, as well as defecation (normal or disturbed, i.e. diarrhea or constipation) and urination, were significantly different between control and study group at day one ($p = 0.015$) and day three ($p = 0.041$) (Fig. 2a). Two rabbits had to be euthanized preterm in the control group.

In the control group, posture/behaviour score 1 was achieved by four animals (57.1%) in the first five days. The two animals, which had to be euthanized preterm, achieved scores 2 and 3 throughout the entire period. One animal of the control group displayed normal behaviour and was allocated score 0. In general, an improvement in clinical parameters was noted after day four of the experiments. On the last two days, all remaining rabbits were no longer affected in their behaviour and posture.

In the study group, five of seven animals were lively, attentive, curious and moved typically and were therefore assigned score 0, except for day 2, where all animals were slightly affected. Only two rabbits of this group were calmer and moved less than the others at additional time points (score 1). The posture/behaviour scores 3 and 4 were not allocated in this group.

The feed intake was reduced or non-existent in 6 of 7 rabbits of the control group on the first day. On the second day, four animals still showed no or hardly any feed intake. On the next days, the animals showed normal feed intake except for the two rabbits that had to be sacrificed preterm.

In the study group we recognized normal feed intake, with the exception of one rabbit which showed decreased intake for three days in total.

All rabbits of the control group suffered from various modifications of defecation, e.g. diarrhea or constipation on the first four days. On the fifth day two rabbits and on the sixth day one rabbit were still allocated to score 1. Only on the last day we noticed normal defecation and urination in all remaining rabbits.

In the study group, four of seven animals suffered from different consistency of their faeces on the first four days. On the fifth and sixth day, only one rabbit showed modified faeces, and on the last day defecation and urination was normal.

A reduction in the body weight was observed in six animals of the control group and three of these rabbits lost more than 17 % of their initial body weight during the whole study period (Figure 2b). On the last two days of the study period the body weight was constant or slightly increased although one animal still lost weight. In contrast, only one rabbit of the study group lost 15.9 % of its initial body weight and two animals lost 6.3 % and 5.1 %. The others lost less than 5 % or gained weight over the whole study period. Statistically significant differences between the two groups were not observed.

Six of the seven rabbits in the control group developed fever on the first two days (Figure 2c). On the third day four animals still showed fever which remained until day 5 in two animals in spite of carprofen treatment. On the last two days the remaining rabbits showed rectal temperature in the reference range.

In the study group, only one rabbit developed fever on two days. The remaining animals had rectal temperatures in the reference range during the whole study period, whereas the temperature increased a little from the third day onwards. The Mann-Whitney U-test revealed a significant difference of body temperature between the groups on the first day ($p = 0.001$).

Figure 2: Course of total clinical score, body weight change, rectal body temperature and white blood count in rabbits of the control and study group (# = premature death of two animals of the control group). Mean + SD.

- a) Total clinical score from day 1 to day 7 post infection, * $P = 0.015$ (day 1), $P = 0.041$ (day 3) Mann-Whitney *U*-test.
- b) Body weight change of both groups in percent related to their initial weight (day 0).
- c) Rectal body temperature of both groups from day 1 to day 7; untreated animals developed fever in the first three days despite of carprofen, * $P = 0.001$ (Mann-Whitney *U*-test).
- d) Leukocytes of both groups from day 0 (preoperative leukocytes count) to day 7.

3.2 Blood examination

Leukocytosis appeared in every animal of the control group (Figure 2d, Control group). Four rabbits developed leukocytosis for only one day, and one animal for two days. Two rabbits showed a high leukocyte count every day. Only in the control group, we recorded a temporary drop in the number of leukocytes at day 3 post infection.

In the study group, leukocytosis was detected in two rabbits, in the first for one, in the second for four days. In this group we recognized an increase in leukocyte numbers on the first day post infection, but on the following days they showed a slight fluctuation in the leukocyte count. There were no statistically significant differences between the two groups.

3.3 Clinical neurological examination

Vestibular signs were recorded in the control group as well as in the study group and did not differ significantly. A graphic illustration of the occurrence of the neurological symptoms is shown in Figure 3. In the control group five of seven rabbits suffered from different vestibular signs on certain days. Extreme head tilting combined with rotation around the longitudinal axis was found in two animals, while the others displayed mild head tilt on some days. Nystagmus and imbalance was seen in four animals, three rabbits circled in their cages and one rabbit showed mechanical head motion. Additionally, one animal presented bilateral strabismus. When evaluating the symptoms during the whole study period with reference to the scoring system, 63 % of the animals achieved score 0, 23 % score 1, 4 % score 2 and 10% score 3. It has to be taken into account that the two animals which died preterm showed the most severe neurological symptoms and that these animals are not included in the calculation above after their death.

In the study group the same number of rabbits (71.4 %) showed vestibular symptoms, but to a lower degree. Mild head tilt was observed in only three rabbits for two days. Nystagmus was noticed in four animals, but only in a state of excitement after disturbance on two days. Three rabbits showed signs of mechanical head motion and imbalance, while two rabbits were found circling in their cage. Rotation was seen in only one animal on the first day. Based on the scoring system, 69 % of the rabbits on average could be assigned to neurological score 0 and 23 % to score 1. Neurological scores 2 was observed on two days in 6 % of the rabbits, while score 3 was observed in only one rabbit on the first day.

a) Circling in cage

b) Mechanical head motion

c) Head tilt

d) Imbalance

e) Nystagmus

f) Rolling

Figure 3: Clinical neurological examination. Occurrence of circling in cage (a), mechanical head motion (b), head tilt (c), imbalance (d), nystagmus (e) and rolling (f).

3.4 Necropsy

In the control group, we discovered several organ alterations in three rabbits. One rabbit showed discoloration and an irregular surface of the lungs, ascites and an enlarged spleen. The rabbit, necropsied on day four, developed a slightly yellow liver, bloody bile and a reddish discoloured urinary bladder. In another rabbit we observed ascites. In contrast, we found no conspicuous organ alterations in the study group. The Fisher's test revealed no significant difference ($p = 0,192$).

The middle ear irrigation exhibited different degrees of turbidity. In the control group most of the irrigations (71.4 %) were highly purulent (irrigation score 2) while the rest was mildly purulent (14.3 %, score 1) or only turbid (14.3 %, score 0). These results differed significantly from those of the study group ($p = 0.013$), where 57.1 % of the animals presented a mildly purulent irrigation (score 1) and 42.9 % a turbid irrigation (score 0). Score 2 was not assigned in the study group.

We also calculated a significant difference ($p = 0.005$) between the groups concerning the macroscopic findings in the middle ear (Figure 4). In the control group, all rabbits had developed a middle ear abscess (score 1), whereas in the study group every rabbit with the exception of one presented a normal middle ear (score 0).

Figure 4: Macroscopic findings in the middle ear. Abscess in the right middle ear of an animal of the control group (a); Insight into an unaffected left middle ear (b).

3.5 Microbiological examination

The microbiological examination of organ samples of control rabbits revealed the presence of *Escherichia coli* and *Klebsiella pneumoniae pneumoniae*. In three animals these bacteria were found in lungs, liver and spleen, in one rabbit additionally in the kidneys, urine and bile. In comparison, the animals of the study group showed no microorganisms in their organ samples.

P. aeruginosa was cultured from the treated ears of all rabbits of the control group. All middle ear irrigations cultured on blood and Gassner agar as well as in thioglycolat medium were positive for *P. aeruginosa*. With one exception, we also detected *P. aeruginosa* on the implant, in the swab sample of the middle ear mucosa and in the swab sample of the brain (in two rabbits swab samples of the middle ear and in one rabbit a swab sample of the brain were not taken).

In the study group, *P. aeruginosa* was not detectable in two animals. In one rabbit, *P. aeruginosa* was detected only in the swab sample of the middle ear after enrichment in TG medium for five days. In another animal, the test pathogen occurred only in the irrigation of the middle ear. In the remaining rabbits, *P. aeruginosa* was cultured from the swab sample and the irrigation of the middle ear. On the implant, *P. aeruginosa* was detected in two animals, whereas in one of these

specimens the bacterium grew only after enrichment in thioglycollate. All swab samples of the brain were negative in the study group. The results of the qualitative microbiological examination are shown in Figure 5.

Figure 5: Microbiological examination (qualitativ). Presence of *P. aeruginosa* in middle ear irrigation, implant irrigation, swab sample of the middle ear mucosa and swab sample of the brain (* $P = 0.021$, fisher's test) of control and study group in percent.

Significant differences between the groups were observed in the swab sample of the brain ($p = 0.021$) whereas the other findings did not differ significantly. The bacterial counts for the middle ear and the implant irrigations are presented in Figure 6 for both groups. Considerable lower amounts of *P. aeruginosa* were detected in the study group, however, without reaching statistical significance.

Figure 6: Microbiological examination (quantitativ) of middle ear irrigation (a) and implant irrigation (b) of control and study group.

3.6 Histopathological examination

In the histological examination of the brains, a severe purulent meningoencephalitis was detected in two rabbits of the control group (Fig. 7a, b), while one of these rabbits revealed also a

perivascular lymphoid infiltration and lymphoid meningoencephalitis (Fig. 7c). A focal accumulation of lymphoid cells in meninges and cerebellum was discovered in a third rabbit. The brain of the animal which had to be euthanized ahead of schedule on day 6 was not examined. In contrast, only one rabbit of the study group suffered from mild lymphoid meningoencephalitis, whereas all other samples exposed normal findings.

The examination of liver and lung samples of one animal of the control group with alterations in these organs revealed a pneumonia and fatty degeneration of the liver.

a)

b)

c)

Figure 7: Histological examination. Brain tissue sections of two animals of the control group (H&E-stained).

- a) Preparation of the animal which died on day four, severe neutrophile infiltration in the parenchyma.
- b) Higher magnification of the marked area in a).
- c) Moderate exudation of lymphocytes, lymphoid cuffs around the vessels.

4. Discussion

Nanoporous silica materials represent a promising approach as drug delivery systems for a variety of medical applications [17, 18]. For example, these systems could be used in orthopaedic surgery supporting the bone repair process [10] or as a vehicle to administer a monoclonal antibody with antitumor activity to inhibit tumor growth [33].

We hypothesized that nanoporous silica materials could serve as a drug delivery system in middle ear surgery. After developing an experimental delivery system which was tested in vitro [13] we intended to examine whether this system works on middle ear implants as well. For this purpose, we have produced nanoporous silica coatings on the implants, functionalized them and loaded them with ciprofloxacin. We furthermore have developed an animal model in rabbits comprising an infection placed with intention. The middle ear of rabbits was chosen because of favourable surgical approaches, as had been confirmed in previous studies [32].

The pseudomonas laboratory strain “PAO 1”, which had also been used in cell culture studies, seems to be less pathogenic than the field strain isolated from an abscess of a rabbit. This strain, which was inoculated in the first animal of our preliminary study caused severe symptoms. With the “PAO 1” strain, obvious clinical symptoms were present in the rabbits, but the strain is less aggressive so that the infections could be persecuted according to the clinical conditions of the animals for a longer period of time. Therefore, “PAO 1” was chosen as the appropriate model agent for induced otitis media in rabbits.

The clinical general examination revealed clear differences in the general condition of the two groups, especially during the first three days. Rabbits of the study group were more lively, curious and mobile than the control group, which showed also reduced feed intake accompanied with weight loss. The rectal temperature in the control group increased over the reference range despite of the administration of carprofen, which demonstrates the severity of infection. Additionally, pyrexia is relatively uncommon in rabbits and occurs for example only in heat stroke and severe systemic infection [34]. Defecation was not significantly different between the two groups which could be explained by the general susceptibility of rabbits' gastro-intestinal tract in connection with disorders of feed intake and many other factors like stress or antibiotic treatment over a long time [35]. In general, a slight aggravation of clinical signs was seen on the second day in the study group which could be a result of increased demand of the immune system, for example for removing dead bacteria.

The seeming improvement of the general condition of the control group after day four is in part due to the premature death of two animals. However, the clinical condition of the other control animals improved at day five or six, likely reflecting an enhanced control of the bacteria by the immune defence. Nevertheless, it can be concluded that the rabbits of the study group were less affected in their general condition especially in the first days, as confirmed by the total clinical score, the rectal body temperature (being in the reference range) and only slight reduction of body weight.

The findings in the neurological examination also provided evidence that the infection in the control group was more pronounced than in the study group, where the animals showed only mild vestibular signs. Symptoms like head tilt, spontaneous nystagmus, imbalance and circling indicate peripheral lesions [34, 36], which could be provoked not only by otitis media or interna, but also by irritation of the auditory nerve during surgery. This indicates that the described symptoms in the study group could be consequences of surgery and are not necessarily associated with infection due to *Pseudomonas*. The results of the necropsy confirm this assumption, because only one rabbit of the study group developed pus in the middle ear, so that the neurological findings in the other rabbits have to be attributed to mechanical irritation during surgery. In contrast, every rabbit of the control group without antibiotic showed an abscess in the middle ear which suggests the spread of the infection to the inner ear and the appearance of more severe symptoms like head tilt, nystagmus and imbalance may be expected. The appearance of rotation seems to correlate with the grade of head tilt, because animals with most severe head tilt also showed rotation about their longitudinal axis. This was also observed by Künzel et al. in a study with rabbits infected with *Encephalitozoon cuniculi* [37]. Besides, rolling could also point to central disorders [38] that may be due to an ascending infection with *P. aeruginosa*.

Concerning the blood examination, it has to be noted that carprofen was administered to inhibit pain and severe inflammatory reactions and likely influenced leukocyte counts. Nevertheless, leukocyte counts differed between the two groups. In the control group, an enhanced migration of the white blood cells to the site of infection likely resulted in an abrupt decrease in cell numbers in the blood on day four, followed by a subsequent increase. In the study group no considerable changes in leukocyte numbers were noted, indicating that the infection has been under control from day 1 on. The increase in both groups on the first day likely reflects the mobilization of leukocytes from their reservoirs subsequent to surgery and the inoculation of bacteria into the middle ear.

Organ alterations, which were observed in the necropsy of the controls, illustrated the high impairment of the animals caused by the induced infection. The invasion of bacteria from the gastrointestinal tract can be regarded as a cause for these alterations, which was proven by the microbiological examination of tissue samples revealing the presence of Enterobacteriaceae. This bacterial translocation from the gastrointestinal tract to extra intestinal organs was likely induced by bacterial overgrowth as a result of anorexia and the failure of immune cells to kill the microorganisms immediately after breaching the mucosal barrier. This could be due to a lack of

immune cells in the gastrointestinal tract because of their enhanced requirement in combating the induced infection in the middle ear [39]. The third cause for supported bacterial translocation could be an endotoxic shock due to death and lysis of gram negative bacteria, resulting in reduced perfusion of organs like the gastrointestinal tract and therefore an injured intestinal mucosa [40, 41]. The impairment of organs was also proven by histological examination of tissue samples. Thus, pneumonia and fatty liver were detected in an animal with proven coliform bacteria in these organs, although degeneration of the liver could also have been caused by anorexia.

Clear evidence for the efficacy of the nanoporous coating loaded with antibiotics is also given by the results of the middle ear irrigation in combination with the macroscopic findings of the middle ear. In more than half of the control animals highly purulent middle ear irrigations were found, whereas the study group revealed only mildly purulent irrigations in 57 % and normal findings in the rest of the samples. The opening of the middle ear also points at the reduction of bacteria in the study group: only one rabbit presented a mild abscess, in contrast to the occurrence of abscesses in all rabbits of the control group.

Finally, the microbiological examination confirmed the almost complete elimination of bacteria in rabbits of the study group. Due to a limited number of animals and relatively high variation in bacterial counts, that is often observed in quantitative cultures, bacterial counts of both groups were not significantly different. However, qualitative detection of *P. aeruginosa* in middle ear and implant irrigations as well as in the swab samples of the middle ears indicated an obvious effect of the released ciprofloxacin on the microorganisms. In two rabbits of the study group, *P. aeruginosa* was completely eliminated as shown by negative cultures of all samples. The examination of the other rabbits revealed positive findings either in the middle ear irrigation, in the swab sample or in the implant irrigation, whereas in the control group, except for one animal, all samples were positive. In addition, samples of the study group displayed growth of *P. aeruginosa* often only after enrichment, indicating a small number of viable bacteria in the middle ear.

The drug delivery system investigated here may not have eliminated all bacteria, but it in any case was able to prevent ascending infection which was demonstrated by microbiological examination of brain swab samples. It was ascertained that no brain sample of the study group was positive for *P. aeruginosa*. In the control group, 57 % of the samples revealed the presence of *P. aeruginosa*, although it has to be considered that from one animal no sample was taken.

These findings correlate with the histological examination of the brains, presenting various alterations in three rabbits of the control group and only a mild meningitis in one animal of the study group. In contrast, there was no correlation between the histology and the severity of neurological symptoms, except for the rabbit with the most severe meningitis which showed massive neurological disorders and died four days after treatment. Such observations were also made in rabbits with encephalitozoonosis showing no clinical symptoms, but severe histological brain alterations [42]. Unfortunately, the brain of the rabbit that had to be sacrificed ahead of time was not examined and cannot serve as a further example for central disorders. Nonetheless, the histological examination of the brain demonstrated the ability of the drug delivery system to prevent a wide bacterial expansion.

Compared to the systemic application of drugs, local delivery systems can provide higher drug concentrations at the decisive site by avoiding the transport and wide distribution via the blood system to the tissue. This appears especially important when the blood circulation is insufficient [43]. As our previous in vitro experiments had shown [13], the amount of ciprofloxacin released from nanoporous silica coatings is able to avoid bacterial proliferation. In the present study, the drug delivery system also prevented bacterial growth, although it did not eliminate all bacteria in every rabbit of the study group. A risk associated with the incomplete elimination of bacteria is the development of bacterial resistance, so that a total eradication of bacteria is aimed at. A possible cause of the incomplete elimination could be the high bacterial concentration inoculated

into the middle ear which was chosen to present significant differences between the groups, including clinical symptoms. Therefore it should be considered to inoculate a lower bacterial concentration, which would probably more closely correspond to the actual situation in otitis media. Additionally, the pharmacologically effective doses of antibiotics for such local applications are not known. The amount of drug delivered will have to be adapted to the specific cases, for example for bacterial infections in the middle ear of rabbits or humans.

To show first effects of the released ciprofloxacin, the study period was set to one week. Further studies are intended, which will be performed over a longer period of time in order to document the development of the clinical situation and finally the ability of the drug delivery system to combat – in cooperation with the host immune defence – all bacteria.

An important feature of the drug delivery system described here is the fact that it covers the whole surface of the implant. Even if a complete elimination of bacteria cannot be achieved, this fact helps to prevent bacterial colonization of the surface of the implant. This preventive measure is of course achieved more effectively by an antibiotic delivery device which itself is located on the surface as compared to a systemic application. As bacteria trying to adhere to the implant surface experience a very high local concentration of the drug, the formation of a bacterial biofilm can be prevented. A biofilm consists of a community of surface-associated bacterial cells in an extracellular polymeric substance (EPS) matrix. The formation of biofilms on implants can be a reason for remaining infections and, finally, extrusion of prostheses [44]. Such communities are marked by a strong resistance to antibiotic treatment, because of their reduced metabolic activity [44-46]. Antibiotics often achieve to kill planktonic bacteria, but the biofilm remains unaffected and serves as a continuous bacterial reservoir. The formation of a biofilm takes place in several successive steps and begins with bacterial adhesion on the surface so that killing these bacteria is an important factor in avoiding biofilm formation. Besides material properties, like roughness or hydrophobicity [47-49], surface treatment of medical devices such as antibacterial coatings plays an important role in the inhibition of bacterial biofilms [9]. The results of our study suggest a further approach in handling this problem.

Nanoporous silica has again been shown to be a highly biocompatible material. This animal study thus lends further credit to its application in biomedical devices. As presented here, nanoporous silica coatings on prosthetic devices can be especially useful as they can serve as local release systems for drugs or other bioactive molecules which deliver these agents directly at the place of interest. For example, a delivery system for bone morphogenetic protein 2 (BMP2) based on a nanoporous silica coating has been developed and has given favourable results in elaborate in vitro tests [50].

5. Conclusion

The present study demonstrates convincingly the efficacy of nanoporous silica layers as a drug delivery system for antibiotics on middle ear implants. The locally released antibiotic almost completely eliminated the bacteria and prevented further bacterial proliferation and invasion of other organs. We have furthermore demonstrated the ability of the drug delivery system to reduce clinical symptoms, resulting in obviously less affected animals concerning their clinical condition. Therefore, nanoporous silica materials as local drug delivery systems on middle ear implants could serve as a promising support in the treatment of otitis media and prevent implant-associated infections. The fact that a first in-human trial of nanoporous silica has recently been approved [20] should allow for a fast translation of such a drug delivery system into the clinic. Furthermore, the concept of using nanoporous silica coatings on implants as local drug delivery vehicles should be easily transferable to other drugs, including larger biomolecules, and to other types of prostheses.

The development of the animal model described in this study, consisting of an intentionally placed bacterial infection in the middle ear of rabbits, has been a cornerstone in the confirmation

of the efficacy of the device described here. With the aid of this model, further measures against implant-associated infections, i.e. also antibacterial coatings instead of drug release coatings, can be investigated.

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