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PŮVODNÍ PRÁCE

Antimicrobial Activity of the Most Common Antibiotic-Releasing Systems Employed in Current Orthopedic Surgery: *in vitro* Study

Antimikrobiální účinnost nejběžnějších systémů uvolňujících antibiotika, které se používají v současné ortopedické chirurgii: *in vitro* studie

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ABSTRACT

PURPOSE OF THE STUDY

Infections of joint replacements represent one of the most serious problems in contemporary orthopedics. The joint infections treatment is usually multimodal and involves various combinations of drug delivery and surgical procedures. The aim of this study was to evaluate and compare the bacteriostatic and bactericidal properties of the most common antibiotic carriers used in orthopedic surgery: bone cements mixed with antibiotic and porous calcium sulfate mixed with antibiotic.

MATERIAL AND METHODS

Three commercial bone cements (Palacos®, Palacos® R+G, Vancogenx®) and commercial porous sulfate (Stimulan®) were prepared with a known concentration of vancomycin (a glycopeptide antibiotic). Specifically, for the purpose of our study, the testing specimens were prepared to release 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 mg of vancomycin into 1 liter of solution. The specimens with increasing amount of antibiotic were placed in a separate tubes containing 5 mL of Mueller-Hinton broth inoculated with a suspension (0.1 m, McFarland 1) of the reference strain CCM 4223 *Staphylococcus aureus* to evaluate their bacteriostatic properties (broth dilution method). After this initial incubation and evaluation of the broth dilution method, an inoculum from each tube was transferred onto blood agar plates. After another 24-hour incubation under the same conditions, we evaluated the bactericidal properties (agar plate method). As many as 132 of independent experiments were performed (4 specimens × 11 concentrations × 3 repetitions = 132).

RESULTS

The bacteriostatic properties of all investigated samples were excellent, perhaps with the exception of the first bone cement (Palacos®). The sample Palacos® started to exhibit bacteriostatic properties at concentrations ≥ 8 mg/mL, while all other samples (Palacos R+G®, Vancogenx®, and Stimulan®) were bacteriostatic in the whole concentration range starting from 1 mg/mL. The *bacteriocidic properties* did not show such clear trends, but correlated quite well with different properties of the investigated samples during mixing – the most homogeneous samples seemed to exhibit the best and the most reproducible results.

DISCUSSION

The reliable and reproducible comparison of ATB carriers is a difficult task. The situation is complicated by high numbers of local antibiotic carriers on the market, numerous antibiotics used, and differences in clinical trials at different laboratories. Simple *in vitro* testing of bacteriostatic and bacteriocidic properties represents a simple and efficient approach to the problem.

CONCLUSIONS

The study confirmed that the two most common commercial systems used in the orthopedic surgery (bone cements and porous calcium sulfate) prevent bacterial growth (bacteriostatic effect), but they may not be 100% efficient in complete elimination of bacteria (bacteriocidic effect). The scattered results in the case of bacteriocidic tests seemed to be connected with the homogeneity of ATB dispersion in the systems and with the lower reproducibility of the employed agar plate method.

Key words: local release of antibiotics; bone cements; calcium sulfate; antimicrobial susceptibility.

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INTRODUCTION

Septic complications are one of the most serious problems in contemporary orthopedic surgery. Infections of joint replacements are always a serious complication and occur in 1–2% of primary implantations (1). For example, osteomyelitis is often difficult to treat due to differences of bone tissues, requiring a rapid and multimodal approach including antibiotic (ATB) administration and, especially in the case of chronic osteomyelitis, surgical treatment. In patients with impaired blood supply to the limbs or diabetes mellitus, osteomyelitis may be an indication for amputation (20). Other examples of urgent diagnoses requiring early multimodal treatment including ATB administration are septic arthritis or joint infections accompanied by septic shock (21). Local surgical treatment of the infected site with thorough debridement is key to controlling the infection. A feared complication is recurrent infection, which increases mortality and the risk of reinfection (19). The most common etiological agents of infections of bones, joints and joint replacements are *Staphylococcus aureus* (which is the most common agent in general), *Staphylococcus epidermidis*, and also *streptococci*, *enterococci*, *enterobacteria*, *pseudomonads* and anaerobic bacteria (9).

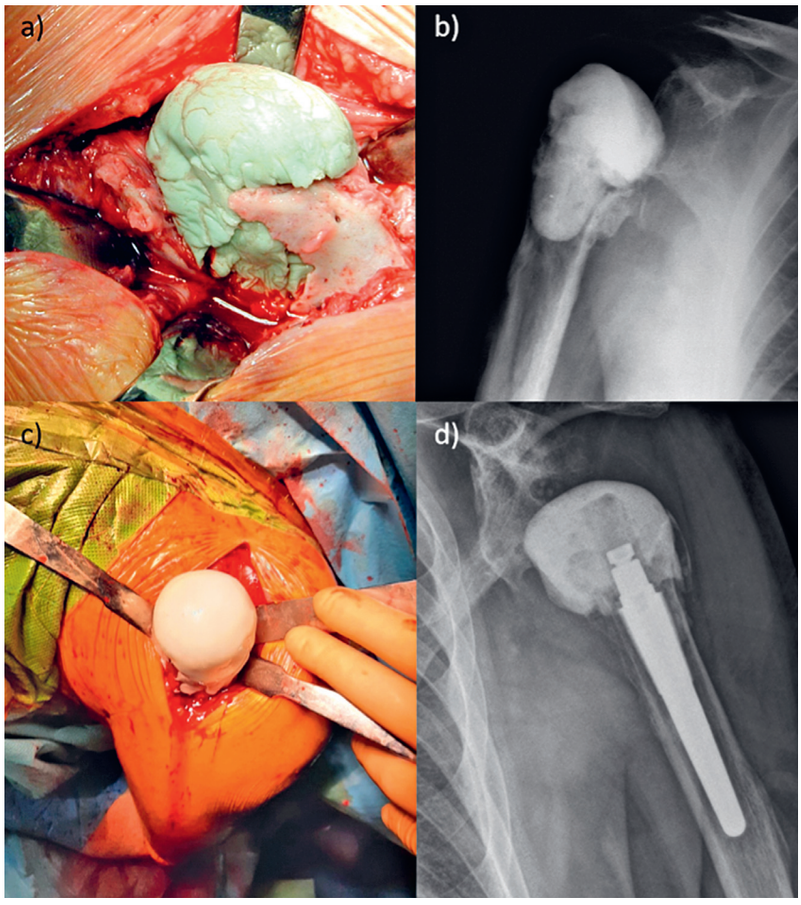

Fig. 1. Examples of the use of Palacos R+G® bone cement (a, b) and Vancogenx® (c, d) to produce a shoulder replacement spacer. Perioperative images (a, c), X-ray after spacer implantation (b, d).

Therapies of bone and joint infections usually involve both **total and local use of ATB**. Total (oral or parenteral) ATB administration may have toxic effects on the kidneys (which were observed for gentamicin and vancomycin) or cause other adverse effects at higher concentrations. Also, the penetration of ATB from the bloodstream to the site of infection may be limited (e.g. to poorly vascularized or infection-modified tissues, necrotic areas, bone sequesters, etc.). On the other hand, locally-released ATB reach high concentrations directly at the site of infection. Moreover, suitable systems containing antibiotics (ATB carriers) can release ATB in a controlled and adjustable way. The ATB carriers can be divided into several groups according to their nature or origin: synthetic polymers, natural polymers (biopolymers), inorganic materials, composite materials or special materials such as bone grafts (23). An overview of the ATB carriers that enable local release of antibiotics is given in Table 1. The basic classification of the different carrier types is based on previous studies from our department (23), but the table has been updated based on the most recent literature (see references directly within Table 1). The key property of an ATB carrier (12) is **biodegradability**, as it impacts on the following surgical treatment of the patient. The non-biodegradable ATB carriers require subsequent removal and therefore further surgery, whereas the biodegradable carriers degrade in the body on their own. The development and use of ATB carriers, especially biodegradable ones, is proving to be effective for the eradication of chronic osteomyelitis (12). The main representatives of **non-biodegradable ATB carriers**, which are employed in current orthopedic surgery, include various forms of the synthetic polymer polymethylmethacrylate (PMMA; so-called "bone cement", e.g. Palacos®, Vacogenx ®, etc.). PMMA is still the most commonly used antibiotic carrier used for the prevention or treatment of bone and joint infections and joint replacements (especially in the form of spacers), as evidenced by a number of studies (18) and the statistics from the clinical practice in the University Hospital in Motol (as illustrated also in Figure 1). Other representatives of non-biodegradable carriers are apatite-wollastonite ceramic glass, hydroxyapatite, calcium phosphate (tricalcium phosphate) and composite materials (e.g. calcium sulfate with calcium carbonate, hydrogenated triglyceride and gentamicin sulfate). The most widely used **biodegradable ATB carriers** include calcium sulfate, polylactide and col-

Table 1. Overview of ATB carriers. Explanation of the key terms used in the table: bioactivity = bioactive materials can form direct contact with living tissue without the formation of a connective tissue interlayer; osteoconduction = bone formation by migrating osteogenic cells; osteoinduction = bone formation at a site where osteogenic cells are not primarily present, influenced by growth factors that affect cell differentiation. We note that the exact properties of all listed materials in vivo are influenced not only by their chemical composition (which is described in the table), but also by their internal structure and surface morphology, e.g. resorbability decreases with increasing crystallinity and increases with porosity of the material and also depends on the blood supply to the surrounding tissue (28, 29, 35).

Group of carriers	Name of carrier, examples	Properties, interaction with body tissues
Synthetic polymers	polymethyl methacrylate (PMMA, bone cement, e.g. Palacos®, Vancogenx ®) (34)	non-biodegradable
	polyanhydrid (4)	biodegradable (4)
	polyhydroxyalkanoate (23)	biodegradable (23)
	polyhydroxybutyrate-co-hydroxyvalerate (23)	biodegradable, osteoinductive (23)
	polykaprolactone (PCL) (28, 30)	biodegradable (7, 30)
	polylactide	biodegradable
Natural polymers	fibrin (23)	biodegradable (23)
	collagen, gelatin sponge (e.g. Garamycin®, Septocoll®) (4, 24, 28)	biodegradable (4, 28)
	demineralized bone matrix (DBM): human (Accell Connexus®) or bovine (Colloss®E) (23)	biodegradable, osteoconductive, osteoinductive
	blood coagulum	biodegradable
Inorganic and ceramic materials	bioactive glass (Na_2O-CaO-$SiO_2P_2O_5$) (28)	non-biodegradable, osteoconductive (7)
	apatite-wollastonite ceramic glass (A-W GC, bioactive ceramics)	non-biodegradable, osteoconductive
	hydroxyapatite (HA, e.g. Endobon®) (28)	biodegradable (in the form of small particles), osteoconductive (28)
	tricalcium phosphate (TCP, e.g. Poresorb®) (10, 23, 28)	biodegradable (in the form of small particles), osteoconductive and osteoinductive (4, 28)
	calcium sulfate (e.g. Stimulan®, Osteoset T®) (4)	biodegradable (1, 4)
Composite materials	hydroxyapatite with collagen (e.g. Collapat® II, Healos®) (3)	biodegradable, osteoconductive (3)
	hydroxyapatite with calcium phosphate and fibrillar collagen (28)	biodegradable, osteoinductive (28)
	hydroxyapatite with calcium phosphate and poly(DL-lactide)	biodegradable, osteoinductive (28)
	nano-HA with poly(3-hydroxybutyrate-hydroxyvalerate)-polyethylene glycol (PHBV-PEG) (23)	biodegradable
	bioactive glass with polymethyl methacrylate (23)	non-biodegradable
	mesoporous bioglass with poly(D,L-lactide-co-glycolide) (PLGA)	biodegradable
	bioactive bone cement (BABC; apatite-bisphenol-alpha-glycidyl methacrylate filled with wolastonite glass particles)	non-biodegradable, partially osteoinductive
	calcium phosphate (e.g. Poresorb®) with autologous blood or bone marrow aspirate	biodegradable, osteoconductive, osteoinductive (28)
	calcium phosphate lysine	biodegradable, osteoinductive (28)
	calcium phosphate with poly(lactide-co-glycolide) (PLGA)	biodegradable (28)
	calcium phosphate with bovine collagen	biodegradable (28)
	calcium sulfate with bone cement (23)	non-biodegradable
	calcium sulfate with autologous bone graft (23)	biodegradable
	calcium sulfate with demineralized bone matrix (23)	biodegradable
	calcium sulfate and hydroxyapatite	biodegradable (in the form of small particles), osteoconductive, osteoinductive
	calcium sulfate with calcium carbonate and hydrogenated triglyceride (e.g. Herafill®beads G)	biodegradable
Bone grafts	autologous or allogeneic (23)	osteoconductive (23)

Fig. 2. Example of prepared specimens of Stimulan®/vancomycin (a), Palacos®/vancomycin and Vancogenx® (b), tube with Stimulan®/vancomycin in culture broth with inoculated Staphylococcus aureus strain (c), row of tubes with Palacos® R+G/vancomycin cement in broth (d).

lagen (12). In practice, calcium sulfate pellets (abbreviation CS; chemical formula $CaSO_4$) known under the commercial name Stimulan® are often used, to which thermostable and thermolabile antibiotics can be added during preparation (4, 23).

This study is a part of a joint project of University Hospital in Motol and the Institute of Macromolecular Chemistry, Czech Academy of Sciences, in which we are developing a completely new type of biodegradable and shapeable polymeric antibiotic carrier with the composition TPS/PCL/ATB, where TPS = highly homogeneous thermoplasticized starch (25, 32) and PCL = poly(ε-caprolactone). Our system allows controlling the rate of ATB release (30) and shows very promising bacteriostatic effects (7). Therefore, the aim of this paper was to (i) validate the reliability and reproducibility of standard microbiological methods in evaluating the antimicrobial effects of commercially available antibiotic carriers and, at the same time, (ii) quantify the antimicrobial efficacy of the two main types of antibiotic carriers most commonly used in orthopedic practice: bone cements (various types of PMMA polymer with added ATB) and porous calcium sulfate ($CaSO_4$ with added ATB). In the next study, we intend to compare the properties of commercial samples with the properties of our TPS/PCL/ATB systems from our ongoing research.

MATERIAL AND METHODS

Material

We tested representative samples from the two most common groups of commercially available systems with

local release of antibiotics (ATB carriers) that are used in orthopedic surgery:

- The first group of ATB carriers used in orthopedics are bone cements (i.e. poly(methyl methacrylates), PMMA's). In this work we used three widely used commercial bone cements (Palacos®, Palacos® R+G, and Vancogenx®.
- The second group of common ATB carriers used in orthopedics are porous calcium sulfates ($CaSO_4$). In this work, we employed $CaSO_4$ with trade name Stimulan®.

All tested ATB carriers were bought from their manufacturers and mixed with defined values of vancomycin (a glycopeptide antibiotic) as described below. It is worth noting that two of the studied ATB carriers came with pre-mixed antibiotics, which were incorporated into the systems by their manufacturers: (i) Palacos® R+G bone cement was supplied with 0.5% gentamicin and (ii) Vancogenx® bone cement was supplied with 4.2% of gentamicin and 2.5% of vancomycin. In this study, the additional antibiotic in Palacos® R+G and Vancogenx® (i.e. gentamicin) was taken as an integral part of the system. The investigated antibiotic (i.e. vancomycin) was either already present (in Vancogenx®) or added during specimen preparation in suitable concentration (in Palacos®, Palacos® R+G, and Stimulan®) as described in the following subsection. In other words, we added vancomycin to all systems except for Vancogenx®, which had already contained 2.5% of vancomycin from the manufacturer.

Preparation of ATB carriers and individual specimens for microbiological tests

The systems for local release of ATB were supplied as two-component mixtures (liquid component and solid/powder component). As noted above, some of the systems could have already contained a pre-mixed ATB, added by the manufacturer). The preparation of the basic systems with local release of ATB was carried out exactly according to the manufacturers' instructions, which were part of the commercial packages. The general procedure was the same for all systems: the solid (powder) component was mixed with the liquid component and ATB in a clean, stainless-steel bowl and then the mixture was thoroughly mixed until a homogeneous mass was formed. Each ATB carriers (Palacos®, Palacos® R+G, Vancogenx® and Stimulan®) was prepared each with a known final concentration of vancomycin. The prepared samples were cut precisely into testing specimens so that a defined amount of antibiotic was always released into 1 liter of broth. Specifically, for the purpose of our study, the testing specimens were prepared to release 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 mg of vancomycin into 1 liter of solution. The final concentration of the released ATB was controlled by the testing specimen size. As many as 132 of independent experiments were performed (4 specimens × 11 concentrations × 3 repetitions = 132). The experiments with the two different samples (Vancogenx® and Stimulan®) were repeated 4 times, while the experiments with the two similar samples (Palacos® and Palacos® R+G) were repeated 2 times, which gives the average number of repetitions = 3, as mentioned above. Figure 2 illustrates the prepared specimens and their usage in microbiological experiments.

Testing of an antimicrobial activity

For each sample, both bacteriostatic and bacteriocidic properties tested. Each specimen was placed in a separate tube containing 5 mL of Mueller-Hinton broth inoculated with a suspension (0.1 m, McFarland 1) of the reference strain CCM 4223 Staphylococcus aureus (as illustrated in Figure 2). This was followed by incubation for 24 hours under controlled conditions in a biological incubator at 37 °C. After 24 hours, the result was evaluated. The turbid broth indicated bacterial growth, while clear broth documented sufficient inhibition of bacterial growth. In this way, we could characterize bacteriostatic properties and estimate minimal inhibitory concentration (MIC). After this initial incubation and evaluation of the broth dilution method, we inoculated inoculum from these tubes onto blood agar plates and after another 24-hour incubation at 37 °C, we evaluated the possible presence of the bacterial colonies. The presence the colonies on the agar surface indicated that some bacteria survived, while their absence proved complete elimination of bacteria. In this way, we could characterize bacteriocidic properties and estimate minimal bactericidal concentration (MBC).

Details concerning microbiological experiments used in this study

In clinical microbiological practice, the terms **bacteriostatic properties** and **minimum inhibitory concentration (MIC)** are connected with quantitative sensitivity of a microorganism to an antibiotic. The MIC (in mg/L) is the lowest concentration of the antibiotic that will prevent bacterial growth in the sample. For MIC testing, we used the **broth dilution method**, which is the most common microbiological method in research (16), and which is also recommended by EUCAST (31). Its essence is the inoculation of a bacterial culture into a series of tubes or wells of a microtiter plate, which are filled with culture medium (broth) and increasing concentrations of antibiotic. The ability of an antibiotic of a given concentration to inhibit bacterial growth is assessed from the broth of turbidity. The turbidity of the broth indicates bacterial growth and ineffectiveness of the antibiotic (9). The reference strain CCM 4223 *Staphylococcus aureus* was used in our study.

The terms **bacteriocidic properties** and **minimal bactericidal concentration (MBC)** are connected with total elimination of bacteria in given environment. The MIC is the lowest concentration of an antibiotic that is able to reduce 99.9% of the microorganisms in the sample and thus prevent their multiplication. The bacteriostatic and bacteriocidic properties are usually tested together: an inoculum from the tube with a broth containing ATB carrier (i.e. the inoculum from broth dilution method) is transferred onto blood agar plate (separately for each tube with a given antibiotic concentra-

Table 2. Processing-related properties of the investigated samples, which are connected with easiness of the preparation, homogeneity of ATB in the specimens, and final quality of the sample.

Material	Maximum time for mixing (before solidification)	Pre-mixed ATB in the system	Viscosity of material and easiness of the mixing
Palacos®	2–3 min	no	high viscosity, bad mixing
Palacos R+G®	2–3 min	yes	high viscosity, bad mixing
Vancogenx®	3–5 min	yes	lowest viscosity, the best mixing
Stimulan	> 5 min	no	medium viscosity, average mixing

tion) – that is why the procedure is called the **agar plate method**. The plates with agar where no bacterial colonies grow after 24 hours of culture are negative, i.e. the ATB was able to kill the bacteria, exhibiting bactericidal effect (9). It should be noted that MBC testing is not commonly encountered in clinical practice due to the greater time required and certain difficulties with reproducibility (although standardized procedures have been described). In clinical practice, an antibiotic concentration four times greater than the bacteriostatic concentration (MIC) is considered bactericidal (6).

RESULTS

Results of all microbiological experiments performed in this study are summarized in Figs. 3 and 4. Both figures show results for the same series of samples, i.e. the three types of bone cements and the porous calcium sulfate. Figure 3 displays the broth dilution test results, while Figure 4 displays agar plate test results. As explained in the Experimental section above, broth dilution tests deal with bacteriostatic properties (inhibition of bacterial growth and MIC), while agar tests deal with bacteriocidic properties (complete elimination of bacteria and MBC). The difference between the bacteriostatic and bacteriocidic experiments and results illustrated in Figure 5: in broth dilution tests (Fig. 5a) we observe turbidity if the bacterial growth was not inhibited, while in the agar tests (Fig. 5b) we observe bacterial colonies unless all bacteria were eliminated.

The *bacteriostatic properties* (broth dilution method, Figure 3) of all investigated samples were excellent, perhaps with the exception of the first bone cement (Palacos®). The sample Palacos®

Dilution test	Concentration of ATB in the system [mg/L]										
	0	1	2	4	8	16	32	64	128	256	512
Palacos	+	+	+	+	-	-	-	±	-	-	-
Palacos R+G	+	-	-	-	-	-	-	-	-	-	-
Vacogenx #1	+	-	-	-	-	-	-	-	-	-	-
Vacogenx #2	+	-	-	-	-	-	-	-	-	-	-
Stimulan #1	+	-	-	-	-	-	-	-	-	-	-
Stimulan #2	+	-	-	-	-	-	-	-	-	-	-

Fig. 3. Results of the broth dilution tests for all investigated samples. The four investigated samples (Palacos®, Palacos R+G®, Vancogenx® , and Stimulan®) were used for preparation of specimens, which released 0, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 mg of ATB per 1 liter of solution. Two specimens were prepared for each combination of sample and concentration. For the first type of bone cement, we performed one series of experiments for each of the two subtypes (Palacos® and Palacos R+G®), i.e. each specimen was measured 2×. For the second type of bone cement (Vancogenx®) and for the porous calcium sulfate (Stimulan®) we carried out two series of experiments, i.e. each specimen was measured 4×. The plus (+) sign denotes positive result (non-inhibited bacterial growth in both cases), the minus (-) sign denotes negative result (complete inhibition of bacterial growth in both cases), and plus-minus sign (±) denotes ambiguous result (non-inhibited growth in the first sample and complete inhibition in the second sample or vice versa).

Agar test	Concentration of ATB in the system [mg/L]										
	0	1	2	4	8	16	32	64	128	256	512
Palacos	+	+	+	±	±	±	±	±	±	±	±
Palacos R+G	+	±	-	+	-	-	-	+	-	-	-
Vacogenx #1	+	-	-	-	+	-	-	-	-	+	-
Vacogenx #2	+	-	±	±	-	±	-	-	±	-	-
Stimulan #1	+	+	-	-	-	-	-	-	-	-	-
Stimulan #2	+	+	+	+	+	+	+	+	+	+	+

Fig. 4. Results of the agar plate tests for all investigated samples. The four investigated samples (Palacos®, Palacos R+G®, Vancogenx® , and Stimulan®) were used for preparation of specimens, which released 0, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 mg of ATB per 1 liter of solution. Two specimens were prepared for each combination of sample and concentration. For the first type of bone cement, we performed one series of experiments for each of the two subtypes (Palacos® and Palacos R+G®), i.e. each specimen was measured 2×. For the second type of bone cement (Vancogenx®) and for the porous calcium sulfate (Stimulan®) we carried out two series of experiments, i.e. each specimen was measured 4×. The plus (+) sign denotes positive result (bacterial growth observed in both cases), the minus (-) sign denotes negative result (no bacterial growth in both cases), and plus-minus sign (±) denotes ambiguous result (bacterial growth observed in the first sample and not observed in the second sample or vice versa).

started to exhibit bacteriostatic properties at concentrations ≥ 8 mg/mL, while all other samples (Palacos R+G®, Vancogenx®, and Stimulan®) were bacteriostatic in the whole concentration range starting from 1 mg/mL. The fact that the sample Palacos® showed ambiguous result at concentration of 64 mg/mL could be either experimental error or it might be connected with inhomogeneous mixing of ATB into PMMA during sample preparation, as discussed in the next paragraph.

The *bacteriocidic properties* (agar plate method, Figure 4) did not seem to show as clear trends as the bacteriostatic properties evaluated from solution broth method. However, the bacteriocidic properties were in remarkable agreement with experimental parameters connected with sample preparation, which are summarized in Table 2. The first sample (Palacos®) exhibited the worst results (no bacteriocidity up to concentrations 2 mg/mL and ambiguous results for all concentrations ≥4 mg/mL of the concentration range. This corresponded to the fact that the sample could be mixed for just a short time before solidification, ATB had to be added during the mixing process manually, and viscosity of the mixture during preparation was rather high (Table 2, the upper row). The second sample (Palacos R+G®) was slightly better than Palacos®, which could be attributed to the fact that ATB was pre-mixed in the system (the material contained 4.5 wt.% of gentamicin and 2.5 wt.% of vancomycin). We note that each specimen was prepared in such a way that it released the defined amount of vancomycin. Consequently, the release of gentamicin from Palacos R+G® was an additional contribution to bacteriocidity of the sample. Moreover, the pre-mixed ATB in the sample should lead to the more homogeneous distribution of ATB each specimen, which should eliminate the cases when a small testing specimen contains lower or even zero ATB concentration. The other processing-related parameters of Palacos® and Palacos R+G® were the same (Table 2, the upper two rows); this is quite logical as the two samples are based on the same prepolymer matrix. The best bacteriocidic properties were obtained for Vancogenx samples: The samples were bacteriocidic in the whole concentration range, even if there were some fluctuations that could be attributed to experimental errors and/or inhomogeneous distribution of ATB in the system. This corresponded to the fact that Vancogenx samples could be mixed for longer time before solidification, the ATB was premixed in the sample, and the lowest viscosity of the system enabled efficient mixing (Table 2, the third row from the top). The last sample (Stimulan®) showed rather ambiguous results (compare the last two rows in Figure 4), which seemed to result from inhomogeneous distribution of ATB in the specimens – the mixing time was the highest, but ATB had to be mixed in manually and viscosity of the material during mixing was just average.

DISCUSSION

The ATB carriers employed in orthopedics can be divided into three groups:

(i) the most common ATB carriers, which are polymethylmethacrylates and porous calcium sulfate,

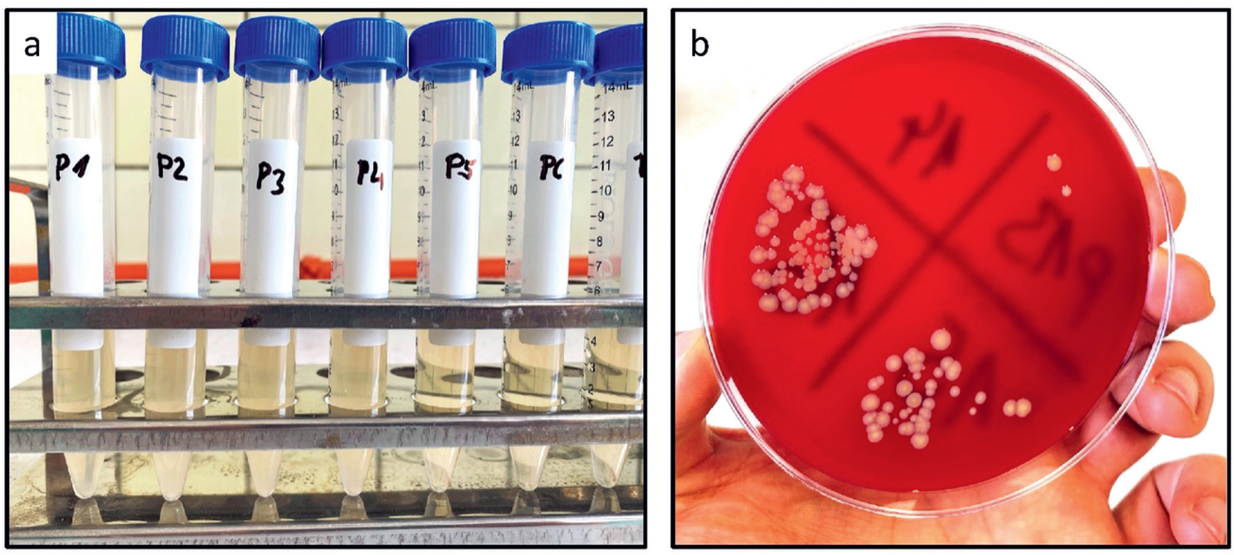

Fig. 5. Illustration of (a) the broth dilution method and (b) the agar plate method. In the dilution method (a), the increasing concentration of ATB is expected to result in the bacterial growth inhibition: Samples in tubes P1–P2 and P3–P4 released 1 mg/L and 2 mg/L of ATB, respectively – this concentration was not sufficient to inhibit bacterial growth and the solutions were turbid, whereas samples P5–P6 released 4 mg/mL of ATB, which inhibited the growth and the solutions were transparent. In agar plate method (b), we used disks covered with blood plasma: In each of the four quarters of the disk, we injected a solution after broth dilution method – the lower two quarters show bacterial growth indicating that some bacteria survived in the solution (even if the growth was inhibited), the rightmost quarter shows moderate growth (almost no bacteria survived) and the uppermost quarter shows no bacterial growth (no bacteria survived, which corresponds to complete bacteriocidic effect).

(ii) the alternative ATB carriers, which are employed occasionally, and
(iii) novel, biodegradable polymer-based materials with optimized properties and ATB release rates, which are subject of contemporary research.

All above-listed materials are discussed in the following subsections. The comparison of different ATB carriers is not easy, as explained at the end of this section. That is why we employed the most reproducible, *in vitro* microbiological experiments in the current study.

The two most common types of ATB carriers in orthopedics

Polymethylmethacrylate (PMMA, frequently referred to as *bone cement*) has been used as a fixative in cemented prostheses and as a non-biodegradable antibiotic carrier to treat and prevent implant infections orthopedic surgery since the 1970s (34). PMMA with an antibiotic is used in the treatment of joint infections in the form of beads or pellets (sometimes connected by a surgical wire to facilitate subsequent removal) and for the preparation of a temporary joint replacement (spacer) in the two-stage management of joint replacement infections (23). Bone cement is prepared by mixing a liquid initiator with a powder pre-polymerized MMA. The polymerization to PMMA occurs in an exothermic reaction, during which temperatures of 82–86 °C are reached in the body (33). Antibiotics are either pre-mixed with the bone cement powder by its manufacturer or added to the bone cement powder during the material preparation just before the surgery. If less than 2g of antibiotic is used for each standard pack (40g) then the mechanical properties are not adversely affected (33). The antibiotics used in bone cements are usually gentamicin, tobramycin, erythromycin, cefuroxime or vancomycin (33). In elution studies, synergistic effects can be observed if suitable combination of antibiotics is used: For instance, combination of gentamicin with tobramycin increased the elution of tobramycin by 68% compared to the control with only one ATB, and the combination of gentamicin with vancomycin increased vancomycin elution by 103% compared to the control (26).

Porous calcium sulfate (CS, chemical formula $CaSO_4$) is known under its trade name Stimulan®. Recent studies show that CS pellets can release ATB locally in high concentrations (1). When evaluating the efficacy of this ATB carrier in the treatment of knee and hip replacement infections, different studies yielded different results depending on the indication for revision surgery - lower reinfection rates (2.4% of patients) were seen in studies that included patients who did not meet the criteria for joint replacement infection (e.g. revision for aseptic loosening). In contrast, studies involving only revision surgeries in prosthetic joint infection using the DAIR (debridement, antibiotics, implant retention) method with use of CS showed reinfection in 48% of patients (1,22). Hypercalcaemia, heterotopic ossification and wound secretion are potential risks of CS implantation, but these conditions were symptomatic in only a minority of cases (1).

Alternative ATB carriers in orthopedics

Non-biodegradable carriers. Probably the most common non-biodegradable carrier is hydroxyapatite (HA, chemically $Ca_{10}(PO_4)_6(OH)_2$). It is an osteoconductive material that is used on implant surfaces due to its ability to form a strong chemical bond with bone. However, it can also be used as a carrier for antibiotics, e.g. in the treatment of knee replacement infections (23). It is also used experimentally in various ***composite materials***, e.g. HA composite with collagen and added ATB has been tested for the treatment of osteomyelitis in animal models. A number of studies show that suitable composite materials may offer improved properties and the possibility of influencing the release of the antibiotic in vivo (3). ***Apatite-wollastonite glass ceramic*** (AW-GC) is a non-biodegradable bioactive material of high strength, which is able to form a strong bond with bones without forming ligamentous scars. The reported composition of AW-CG is 28% silica (SiO_2), 38% apatite [$Ca_{10}(PO_4)_6(O, F_2)$] and 34% wollastonite (SiO_2×CaO). AW-GC was used as an ATB carrier in animal tests *in vivo* and in patients as early as the 1990s, with good effect for the treatment of osteomyelitis (13).

Biodegradable carriers. Alternative biodegradable carriers include calcium phosphate, collagen carriers and polylactides. ***Calcium phosphate*** (tricalcium phosphate, TCP, chemical formula $Ca_3(PO_4)_2$) is one of the biodegradable inorganic materials used to replace bone defects. It exhibits osteoinductive and osteoconductive properties (23). Both TCP and CS (calcium sulfate, which was described in the previous subsection) have been used for the filling of bone defects for decades. The combination of TCP and CS together with antibiotics (combination of vancomycin and tobramycin; combination of vancomycin and gentamicin) showed good protection against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and blocked biofilm formation (10). Another widely used ATB carrier are various ***collagen formulations***. Collagen is a naturally occurring component of human connective tissue and is preferably used as a drug carrier because of its perfect biocompatibility. The advantage of using collagen as a carrier is the possibility of modifying its structure (by changing intra and intermolecular bonds) and thus its properties, especially in terms of pharmacokinetics (27). In clinical practice, collagen-based carriers (such as gelatin sponge; gelatin is a partially degraded form of collagen) with gentamicin (e.g. Garamycin®, Septocoll®) are used (4, 23). An excellent effect is shown by gelatin sponge with gentamicin, e.g. in the prevention of surgical wound infections of the sternum; its application in the wound reduces the risk of infection by 40% (15). ***Calcium sulfate-calcium carbonate composite*** (e.g. Herafill® beads G) contains gentamicin and represents yet another biodegradable ATB carrier employed in the field of bone and joint infections. It is also used to fill bone defects; in this application area, it was proved to release high concentrations of gentamicin while maintaining safety and minimizing the risk of overall antibiotic toxicity. Moreover, it is generally well tolerated by patients, as shown in a study on patients with osteomyelitis (5).

Modern polymer-based ATB carriers

A modern approach in contemporary orthopedics are polymer-based ATB carriers. These materials allow for controlled rate of ATB release due to their chemical variability. Moreover, their biocompatibility and mechanical performance can be optimized for specific applications. Typical medical biopolymers include: polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL) or thermoplasticized starch (TPS) (17, 25). It has been shown in animal models that polymer-based systems can be used to treat osteomyelitis in dogs and rabbits (2, 8). All above-listed polymers are biodegradable. They are frequently combined into composite materials that improve their mechanical and/or antimicrobial properties (14). Our own polymer-based multicomponent system TPS/PCL/ATB, which is a mixture of two biopolymers with different biodegradation rates, shows very promising bacteriostatic properties according to preliminary *in vitro* experiments and the ATB release can be controlled by its composition and structure (7, 30, 32).

Difficulties concerning evaluation and comparison of ATB carriers

The reliable and reproducible comparison of ATB carriers is a difficult task. In principle there are three options: (a) clinical studies, (b) animal studies, and (c) *in vitro* studies.

As for **clinical studies**, it is problematic to compare the efficacy of different therapeutic approaches in different clinical trials due to variability in selection of patients, surgical methods and adjuvant antibiotic therapy (1). Animal studies or *in vitro* microbiological methods offer a higher degree of standardization.

An example of **animal study** is the work of Mendel et al. (24), which compared the effect of two ATB-releasing systems (gentamicin-PMMA beads and gentamicin-collagen sponge) in the treatment of chronic osteomyelitis caused by *Staphylococcus aureus* in rats. Both ATB-releasing systems and systemic treatment with cefazolin (a first-generation cephalosporin antibiotic) were tested within the same study in order to avoid problems with reproducibility. The authors concluded that each of the treatment modalities exhibited a significant therapeutic effect. Better results were obtained with the flexible gentamicin containing collagen sponge than with rigid gentamicin-PMMA system. Nevertheless, the best results were attained when gentamicin-PMMA beads were combined with systemic cefazolin treatment.

***In vitro* studies** employing standardized microbiological experiments (such as broth dilution method and/or plate tests) are the most common methods for testing of newly developed ATB carriers (11). They may just simulate the *in vivo* conditions, but they offer higher reproducibility and lower cost in comparison with animal or clinical studies. This makes them suitable for pilot tests. In this work, we selected two standard *in vitro* microbiological tests: *the broth dilution method* and *the agar plate method*, which enabled us to characterize bacteriostatic and bacteriocidic properties of all investigated samples.

CONCLUSIONS

We tested 132 of specimens releasing different amounts of ATB and the main conclusions could be summarized as follows:

1. All systems, i.e. all non-biodegradable bone cements (with the exception of Palacos®) and the bioresorbable porous calcium sulfate, exhibited excellent *bacteriostatic properties* (as evidenced clearly in Figure 3).
2. The evaluation of *bacteriocidic properties* yielded more scattered results in comparison with the evaluation of bacteriostatic properties (as documented by the comparison of Figures 3 and 4), but it indicated several important facts and trends:
   a. The bacteriocidic properties were closely connected with the material properties and preparation protocols (as suggested by comparison of Figure 4 and Table 2): The best bacteriocidic activity was achieved for Vancogenx®, which was easy to prepare (due to low viscosity of the mixture during preparation and sufficient time for mixing before solidification) and contained pre-mixed ATB (i.e. the manufacturer supplied the original powder containing homogeneously dispersed ATB). The worst bacteriostatic activity was found for Palacos® (high viscosity, short time for mixing and not-premixed ATB in the system) and Stimulan® (low viscosity, but ATB not-premixed in the system).
   b. The homogeneity of ATB dispersion in the system seemed to play a key role in bacteriocidic efficiency. Both systems with pre-mixed ATB (Palacos R+G® and Vancogenx®) exhibited better results than the other two systems without pre-mixed ATB (Palacos® and Stimulan®).
   c. The reproducibility of agar method itself is probably lower in comparison with the broth dilution method. This accords with the available literature, as most studies prefers broth dilution method, while agar method occurs in recent research papers scarcely.
3. From the point of view of clinical practice, the whole study confirmed that the two most common commercial systems used in the orthopedic surgery (bone cements and porous calcium sulfate) prevent bacterial growth (bacteriostatic effect), but they may not be 100% efficient in complete elimination of bacteria (bacteriocidic effect). The scattered results in the case of bacteriocidic tests seem to be connected with the homogeneity of ATB dispersion in the systems and probably also with the lower reproducibility of agar method. The comparison of the homogeneity of ATB dispersion in the investigated materials by means of infrared microspectroscopy and the verification of agar method by means of higher number of experiments to achieve better statistics is a subject of our ongoing research.

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