Microbiological and pharmacological properties of bone cement VancogenX

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ABSTRACT

PURPOSE OF THE STUDY
Prosthetic joint infection is a complication feared in total hip arthroplasty. The use of antibiotic-impregnated bone cement is an important part of preventive and therapeutic strategies. At present a number of commercial bone cements are available and support of their use by the results of experimental trials and clinical studies has varied. In relation to this issue we studied essential microbiological and pharmacological characteristics of VancogenX in comparison with gentamicin-loaded bone cement used conventionally.

MATERIAL AND METHODS
Based on a previously described protocol, we tested four commercial pellets of Bone Cements, namely, Hi-Fatigue G, Palacos R + G, VancogenX, and Palacos R as a control. Bone cement was prepared in a vacuum-mixing system. The bacterial strains used for load included Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Pseudomonas aeruginosa and Escherichia coli. Each cement was tested for its antimicrobial and antibiofilm activities and the results were evaluated by standard methods. In addition, we investigated time-related release of gentamicin and vancomycin from the Bone Cements tested.

RESULTS
All antibiotic-loaded cement pellets were able to prevent growth of the bacterial strains tested. The bactericidal effect lasted for several days in relation to the bacterial species and cement used, with the exception of S. epidermidis whose growth was inhibited by gentamicin-loaded cement only for one day. The antibiotic-loaded pellets also prevented the formation of a biofilm for 24 hours at least. The major amount of vancomycin (32,915 mg/l) was released from the VancogenX MH medium within 24 hours and the last measurable Chatter (4327 mg/l) was recorded at 48 hours after the start of the experiment. Physiological saline in the highest level of vancomycin was 139,852 mg/ml measured at 24 hours, and the antibiotic was detectably at a level of 2.334 mg/ml as late as 8 days after the experiment started. Release of gentamicin from VancogenX was as follows: the 24-hour level was in MH medium higher than physiological saline (178 versus 131.4 mg/ml); referring to: gentamicin was still detectably in physiological saline at 192 hours after the start of the experiment while no gentamicin was found in MH medium after 72 hours.

DISCUSSION
The antimicrobial effect of VancogenX bone cement was not an unexpected finding since both gentamicin and vancomycin have been used with bone cement for a long time and their combination is optimal in terms of preventing prosthetic joint infection. Referring to: there is a disputable issue of poor release of vancomycin from bone cement. In MH medium we were able to detect the vancomycin released from VancogenX only for two days after the initial rapid elution while its release into physiological saline seemed to be slower but much longer. It is possible that more vancomycin is released from bone cement, but this is of amount immediately bound to proteins in the vicinity cement and this process is not detectably by any analytical method.

CONCLUSIONS
The bone cement VancogenX showed excellent antibiofilm and antimicrobial properties and can be recommended for use in orthopedic practice. Therapy of prosthetic joint infection is the main indication. Extension of the indication to the prevention of prosthetic joint infection in high-risk patient's should be preceded by biomechanical studies demonstrating the cement that is appropriate for long-term implant fixation.

Key words: antibiotic-loaded PMMA, vacuum mixed, prosthetic joint infection, gentamicin, vancomycin,
dilution, prevention, VancogenX

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INTRODUCTION

Infection joint replacement (hereinafter IKN) is one of the most common and most feared complications endoprotese. It is estimated that the IKN may occur in up to 5% of patients with hip or knee prosthesis (28). "Higher incidence should be at revision surgery and in patients with impaired immune responses. Importantly, recent epidemiological models suggest a further increase in these infections in the coming decades. (21) If you strip away the personal suffering of patients, it is clear that in the context mentioned forecasts a significant increase of costs associated with treatment IKN (20, 30). These reasons it is essential to choosing the right preventive strategies to minimize the risk of IKN. From the perspective of prevention are most effective processes that affect perioperative and early postoperative period, which forms a biofilm (14).

There is evidence that the risk of IKN reduces systemic antibiotics (9). Similarly, it seems to be sufficiently proven protective effects of antibiotic bone cements, which in combination with systemic administration creates a "double block" significantly reduce the risk of IKN (19). According to one recent study should be cement-free hip even up to 50% more prone to infection, joint replacement compared with cemented primary substitutions (7). The overall administration of antibiotics are chosen mostly acting mainly on gram-positive bacteria forming skin microflora, where it is assumed that could contaminate the wound. The local variation is extensive experience with gentamicin, at which fixation cement added at a concentration of 0.5 to 1.0 g to 40 g of bone cement. Higher concentrations of antibiotics (over lg to 40 g cement) deteriorate the mechanical properties of bone cement, and therefore in the prevention indication routinely used (18). Bone cement to be added certain other antibiotics (Table 1).

In our previous study, we in vitro showed that the bone cement, to which we added gentamicin and vancomycin at a dose of lg per 40 g of bone cement, effectively prevent the growth of strains of Staphylococcus aureus as compared to commercially produced cements containing only gentamicin (12). He was recently the launch of a new bone cement VancogenX (companies Tecres Spa, Italy). The manufacturer states that the cement should be used for temporary fixation and fixation spaces in revision prostheses originally infectious terrain, especially if the offender is sensitive to gentamicin and vancomycin. We were interested in this context, how will the new commercially manufactured bone cement behave in accordance with the theoretical and experimental assumptions against staphylococci and other potential pathogens IKN.

MATERIALS AND METHODS

Preparation of samples of bone cement

In the operating room as we prepared exactly the same conditions on the same day samples tested bone cements:

First Hi-Fatigue G Bone Cement with gentamicin (AAP Biomaterials GmbH, Germany);
second Palacos R + G with gentamicin (Heraeus Medical GmbH, Germany);
third VancogenX with gentamicin and vancomycin (Tecres Spa, Italy);
fourth Palacos R without antibiotics (Heraeus Medical GmbH, Germany).

All tested bone cement to the VancogenX We normally use. Each test bone cement was mixed well experienced theater nurse in a vacuum and then it was in the working phase two teams prepared pellets with a diameter of about 0.5 x 0.3 cm (average weight 39.4 ± 8.39 mg). Samples were placed in a sterile container (Fig. 1) and sent to the microbiology laboratory.
Selection of the test strains

Based on our previous publications (11, 13) and literature (8, 24) we selected bacterial isolates from the archives of the Institute of Microbiology, University Hospital Olomouc following reference strains of bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, oxacillin-resistant *Staphylococcus epidermidis* A/5879, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 35218th

Determination of antimicrobial effect

Each sample of cement, including the control without antibiotics, was tested with all four bacterial strains. Approximately 4-5 bacterial colonies were inoculated with a bacterial loop in 2 ml of MH broth (TRIOS) and after an hour of aerobic incubation at 37 °C are mixed in a Petri dish with 10 ml of distilled sterile water. The suspension was then inoculated with the vaccine hedgehog into microtiter plates with 150 ml of BHI broth (Himed) and cement. After aerobic incubation for 24 hours at 37 °C, the samples were transferred into the cement next to new wells with BHI broth, and then inoculated with the appropriate bacterial strain and incubated. This process was repeated for a total of eight days. The evaluation was made on the basis of bacterial growth in the well microtiter plate, which is reflected in the positive case, clearly visible turbidity. At the same time after each incubation was vocykovan BHI broth on blood agar (TRIOS) to determine the bactericidal / bacteriostatic effect.

Determination of biofilm formation

Individual samples of cement were aseptically placed in wells containing 150 ml of BHI broth. The plates were inoculated with bacterial strains and cultured aerobically at 37 °C for 24 and 48 hours. Cement samples were then aseptically transferred into a dry sterile tubes and kept in an incubator at 37 °C for 24 hours and then placed in sterile BHI broth and incubated at 37 °C for 24 hours. Positivity was evaluated using growth potential and turbidity was also BHI broth after incubation vocykovan on blood agar for confirmation of negative growth of tested bacteria. In the case of a positive bacterial growth was determined identity of the bacterial strains, that is inoculated into broth at the beginning of the experiment and subsequently obtained from the dried cement.

Establishing the identity of bacteria

Similarly, respectively, identity of isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* was analyzed by comparing the genome-wide DNA restriction fragments separated by pulsed field gel electrophoresis (PFGE). Bacterial DNA was isolated as described previously and digested with restriction enzymes *Xba I* (*Escherichia coli*), *Spe I* (*Pseudomonas aeruginosa*) and *Sma I* (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*) (16). PFGE was performed in 1.2% agarose gel. Separation of DNA fragments obtained by cleavage endonuclease *Xba I* ran 24 h at 6 V.cm⁻¹, pulse times of 2-35 with 14 °C. For DNA digested endonuclease *Spe I* are the parameters 23 h, 6 V.cm⁻¹, pulse times of 5 to 60 the temperature 12 °C. DNA staphylococci and enterococci were separated 24 h at a voltage of 5 V.cm⁻¹, pulse times of 1-75 with 14 °C. Subsequently, gels were stained with ethidium bromide and photographed. The resulting restriction profiles were compared by means of a computer program GelComp Vet (Applied Maths, Kortrijk, Belgium).

Elution of antibiotics tested cements

The dissolution study was conducted in saline (FR) (Fresenius Kabi, Italy) and in MH medium. Cementitious product (pellets) was inserted either in MH medium (1 ml) or saline (1 ml) was incubated 24 h at 37 °C. After 24 h the pellets with the tested product are removed from the media, respectively. From FR that was frozen at -70 °C, and then inserted into the new media, or FR. Thus, the procedure for a total of six cement with gentamicin (samples after 24 h, 48, 72, 96, 120 and 192 ha eight of cement with vancomycin (samples after 24 h, 48, 72, 96, 120, 144, 168 and 192 h). Following thawing, the samples were further processed and analyzed by a suitable analytical method. were tested with the same procedure cement control samples free of antibiotics.

First Vankontycinu release in saline

Samples were prepared immediately after thawing for analysis. The concentration of vancomycin in the saline samples were determined according to the modified method of Abu-Shandi (1). The analysis carried out on the liquid chromatograph Prominence LC20 (Shimada-zu, Kyoto, Japan). As a stationary phase column was used LiChroCART ® 250-4 LiChrospher 100 RP-18 (Merck, Darmstadt, Germany). Analyte was
detected spectrophotometrically at a wavelength of 210 nm. The elution of vancomycin from the column by isocratic conducted using the mobile phase consisting of 25 mM K/P04 (phosphate buffer) at pH = 6.5 and 100% acetonitrile in the ratio 85:15 (v / v). Analysis carried out at normal room temperature. Flow rate of mobile phase was 1 ml / min. The concentration of vancomycin were evaluated by an external standard. The method was linear over the concentration range mg/1-500 vancomycin 1 mg / l

**Second Release of vancomycin in MH medium**

For very complex biological matrix (microbiological media) it was to determine the concentration of vancomycin in this case is relatively complicated. The analytical method described in the previous paragraph had to be modified and extended by the extraction method, which preceded it. To modify biological matrix method was selected solid phase extraction (SPE extraction) extraction using Oasis ® HLB boxes lcc (Waters, Massachusetts, USA). The boxes were conditioned first 1 ml of methanol and subsequently washed with 1 ml deionized water. Subsequently, it was applied to the column media sample volume of 1 ml. The column was successively washed with 500 μl of water and 500 ml of 5% methanol in water (v / v). Vancomycin was eluted from the boxes 600 ul mixture of acetonitrile: water (50:50 v / v). The eluate was evaporated under nitrogen and reconstituted in 200 ml of mobile phase A. The analysis carried out on the liquid chromatography Prominence LC20 (Shimadzu, Kyoto, Japan). As the stationary phase was chosen Kinetex column for HILIC 100A 2.6 150 x 4.6 nm (Phenomenex, California, USA). Analyte was detected spectrophotometrically at a wavelength of 210 nm. The elution of vancomycin from the column with a gradient conducted using a combination of mobile phase consisting of buffer A - 2.5 mM ammonium formate, pH = 5.8 (adjustment with formic acid) and mobile phase B, which was 100% acetonitrile. Gradient was set as follows: 0 min - 70% B, 6 min 10% 7th min - 70% B, 15 min - B 70%. Analysis carried out at normal room temperature. Flow rate of mobile phase was 1 ml / min. The concentration of vancomycin were evaluated by an external standard. The method was linear over the concentration range mg/1-200 vancomycin 1 mg / l

**Third Release of gentamicin in MH medium and FR**

Due to its specific structure is routine analytical methods gentamicin difficult to ascertain. Among several possible procedures (derivatisation with specific derivatization reagent and subsequent determination of the derivative of gentamicin using fluorescence or UV detection, liquid chromatography) was to determine gentamicin in MH medium and FR was chosen Immunological method (AxSYM Gentamicin reagent kit, Abbot Diagnostics, Czech Republic). Analysis of samples was conducted in cooperation with the Department of Clinical Biochemistry, University Hospital.

**RESULTS**

**Antimicrobial activity of bone cements**

All tested bone cement containing antibiotics could prevent the growth and multiplication of bacterial strains tested, which can be considered as proof of their antimicrobial effect. After wyozkőni bouillons on blood agar followed by negative growth, it was clear that it was a bactericidal activity (Table 2). Bactericidal effect was maintained for several days depending on the bacterial species and the type of cement, but at least two days with the exception of *P. epidermidis*, in which the efficacy of cements containing only one type of antibiotic shortened to one day (Table 3).

**Biofilm formation on the surface of bone cement**

Tested bone cements containing antibiotics could prevent the formation of biofilm at least 48 hours in all tested bacteria and vice versa all the tested bacteria formed a biofilm on the surface of the control cement without antibiotics (Table 4). The identity of the strains obtained from the tested cements odsušení after the
Elution of antibiotics from bone cement

The results of the analyzes for vancomycin are shown in Table 5 and in Figure 2. The results of the analyzes for gentamicin are shown in Table 6 and in Figure 3.

Vancomycin is released from the preparation VancogenX far better than in FR MH broth to the test, while in the case of gentamicin was the other way around.

Vancomycin is released from the specimen VancogenX MH media to the greatest extent 24 h after the start of the experiment, when the concentration of 32.915 mg / L.

Conversely the last measurable concentration of vancomycin in the test medium was 48 h from the beginning of the experiment (4, 327 mg / l). If used as a test medium saline solution, the concentration of vancomycin in the solution measured several times higher than in the case of MH medium (139 852 mg / ml). In addition, vancomycin was detected in saline still 8th day after the start of the experiment (2.3341 mg / l).

Gentamicin is "easier" excreted in complex media, and slightly worse in saline. Conversely vancomycin is easily released into the saline, and the "complex" solution secreted only a short time and in much lower concentrations.

DISCUSSION

In our study, we found that all tested bone cement containing gentamicin, respectively gentamicin and vancomycin against test bacteria showed a bactericidal effect. Bactericidal effect persisted for at least 24 hours after inoculation, but usually a few days. All the tested cements antibiotic also managed to prevent the formation of biofilm on the surface of the tested pellets. Release of gentamicin into the medium and physiological saline was carried out in cement VancogenX other than the release of vancomycin. Gentamicin is "easier" excreted in complex media, and slightly worse in saline. Conversely vancomycin is easily released into the saline, and the "complex" solution secreted only a short time and in much lower concentrations.

In our previous study, we found an excellent bactericidal behavior of bone cement mixed with gentamicin and vancomycin against staphylococci (12). In the current study, we wanted to examine the behavior of commercially manufactured bone cement containing gentamicin and vancomycin against a wider range of potential pathogens. According to some studies offers a commercially produced antibiotic cements with a slightly larger and more regular than the zone of inhibition of bone cements, in which the same antibiotic adds to the operating room (10). On the other hand, could be affected by antibiotic elution method of preparation commercially manufactured bone cement. Today's standard is vacuum mixing of cement, which should significantly reduce the porosity of bone cement and seemingly prevent the release of antibiotics. In one recent study, however, showed that the worst release rule can not be applied across the board to all bone cements (22).

For example Palacos R + G mixed in vacuum exhibited the longest antimicrobial activity in all tested cements and even fourth day release at a concentration of 32 mg / l, while stirring under atmospheric pressure at that value had been 2nd Day.

Conversely SmartSet GMV is somewhat better behaved with stirring at atmospheric pressure than vacuum. However, in one test situation reached at concentrations of gentamicin SmartSet GMVpo 48 hours at 32 mg / l Better release antibiotics when mixing in vacuum explain some authors much greater than the number of micropores in the cement prepared at normal pressure (23). In our study, we evaluated only vacuum-mixed bone cement and we did not observe appreciable
differences in the width and antibiofilmové antimicrobial activity among the tested cements.

The pellets made from bone cement containing antibiotics, we found an excellent bactericidal effect and the ability to eliminate the biofilm formation in contrast to the pellets of the bone cement without antibiotics. Effect was almost the same for cements with gentamicin as cement, which contained in addition vancomycin. The question thus arises, what is the potential target of vancomycin in that combination. Vancomycin importance lies in extending the activity spectrum and the multidrug-resistant gram-positive bacteria such as methicillin-resistant mainly staphylococci (25). Given the current high level of resistance in coagulase-negative staphylococci to vancomycin can be considered a combination of gentamicin with vancomycin for a useful perspective.

The basic condition for effective operation of antibiotics is long enough to be released from the surface of bone cement in sufficiently high concentrations. This is a function primarily of the total concentration of the antibiotic in the cement and the uniformity in the distribution because it is only released from the superficial layer of bone cement (27). Regardless of the dose and type of added antibiotic, the highest level recorded during the first few hours after insertion into the test medium, followed by a rapid decrease in concentration of usually up to measurable values, which is consistent with our results of measurement of gentamicin and vancomycin. For routine prophylactic indication is however used cements blended with a maximum of 1 g antibiotics. Higher doses of antibiotics, especially added to the room, and disrupt the polymerization of the mechanical properties of bone cement (3). An important role is also played by the type of bone cement, and it is not clear exactly what causes the differences in the dilution curves of the same antibiotics (18, 22). Also, we found differences in the concentrations of gentamicin-releasing between cements tested using the same analytical methods (Table 6).

Some work suggests that it might be advantageous to mix the two antibiotics, because other "create" better conditions for release from bone cement and extend the spectrum efficiency, which is especially true for cements with a high dose of antibiotics (2). It is also possible that there are specific of each antibiotic dilution factor. According to some works with vancomycin released from bone cement inconsistent and worse (greater dependence on concentration) than other antistafylokoková antibiotics (17). In terms of maximum achieved concentration can not confirm it, because after 24-hour incubation, we measured in saline averaged 140 mg / L in MH medium, more than 30 mg / L vancomycin. That value can be regarded as sufficiently high, ensuring the majority of Gram-positive bacteria, including methicillin-resistant staphylococci, sufficient antimicrobial effect (26). Similarly, other authors measured relatively high levels of vancomycin released from the cement, to which was added from 0.5 grams to several antibiotics (Table 7), (5, 15, 17, 29).

Gentamicin is considered "standard" antibiotic in bone cement, which is an effective and well of cement releases (4). According to some of the work should also be less dependent on the presence of a second antibiotic in cement (6). In our study, gentamicin released from bone cement VancogenX relatively well in MH medium and slightly worse in saline, although much better than vancomycin. As in the case of vancomycin can be explained by variability in the literature and our observations at least partially the selected measuring method. Due to its specific structure (gentamicin is not pure chemical entity, but consists of several functional isomers) is because gentamicin conventional analytical methods difficult to ascertain.

The following section briefly mention some of the limitations of our study. First, it is difficult to model in vitro conditions that would be similar to the real environment of the joint. Most of the surface of bone cement is directed to bone bed, respectively, the component. Only a small portion of bone sticky surface facing toward the inside of the joint and is in contact with joint fluid (blood initially, which gradually discolour and then with articular effusion). In our study, however, samples of cement tested in a simple and stable liquid medium, but were significant differences in the release of both tested antibiotics. But these are not reflected in antibiofilmové antimicrobial activity. Certain objects can also make use of archival microbial strains which may behave differently than wild or multidrug-resistant clinical strains. Another limitation is related to the applied analytical methods that must not only be sufficiently validated, but in the case of measurement of gentamicin levels would be appropriate to introduce a method using liquid chromatography with mass spectrometer. Determination of the concentration of antibiotics is also influenced by the type of test media. During the development of methods for the determination of vancomycin in MH medium showed that this medium with starch and a number of other components can adsorb vancomycin obviously part and this fraction can then "escape" detection method. Therefore, it was necessary to adjust the sample using extraction methods on the solid phase.
CONCLUSION

In the present study, we found that bone cement VancogenX has antimicrobial and potential antibiofilmový quite comparable or even better when compared to "traditional" bone cement with antibiotics. From this perspective, it is therefore possible to recommend its use in clinical practice. The main indication is the treatment of infections of joint replacements. A possible extension of indication to prevent infections of joint replacements (including relapses) in patients at particular risk must be preceded by a solid biomechanical studies that demonstrated the strength and fatigue properties of cement VancogenX. We also found that vancomycin and gentamicin are released from the cement VancogenX stirred under vacuum depending on the type of media in which the test pellet located. Variability of findings at least partially be explained by the used analytical methods.

Literature


