In vitro comparison between commercially and manually mixed antibiotic-loaded bone cements

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ABSTRACT
Purpose: The purpose of this study is the evaluation of the differences and, eventually, of the advantages or disadvantages of manual formulations with respect to industrial ones.
Methods: Medical-grade bone cements (Palacos R® and Palacos LV®), based on poly-methyl methacrylate (PMMA) and used clinically in several cemented prosthetic devices were manually enriched with gentamicin sulphate during preparation and then compared with a commercially-available, antibiotic-loaded cement (Palacos R+G®) by means of an in vitro antibacterial test (inhibition zone evaluation). The purpose of this study was to evaluate the differences and advantages or disadvantages, if any, of manual formulations compared to commercial ones. The use of a different antibiotic (vancomycin) alone or in addition to gentamicin-containing bone cements was also considered.
Results and conclusion: The commercial formulation produces an inhibition zone that is a bit larger and more regular than the manually mixed preparation. The vancomycin halo is smaller but clearer than the gentamicin halo. The addition of vancomycin to gentamicin-containing bone cements does not significantly increase the halo dimensions but could be an interesting strategy in the prevention of multiple and resistant infections.

Key words: Bone cement, Antibiotic, Cemented prostheses, Infection

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INTRODUCTION

The development of infection after orthopedic prosthetic surgery is a serious problem that often causes implant failure, prolonged hospitalization periods, patient diseases and increased medical costs (1-3). Infections are favored and enhanced by the formation of a biofilm on the material surface. Biofilm is a three-dimensional structure that protects bacteria against the patient’s own defense (immune system) and also from systemic antibiotic therapy, making it particularly difficult to treat (1, 4-8). In order to prevent prosthetic infections, different solutions such as strict antiseptic operative procedures, including laminar flow and systemic antibiotic prophylaxis, have been proposed but even if the infection rate has been significantly reduced, the problem in joint arthroplasty still remains to be solved.

The idea of introducing antibiotics directly into PMMA-based bone cements to prevent bacterial proliferation at the interface between bone and prosthesis began in the 1970s (9-12). In the cited studies a significant reduction of infection rate by the use of gentamicin-loaded bone cement compared to plain PMMA cement was reported. Josefsson et al (13) and Espehaug et al (14) contradicted these results in their own studies. Antibiotic-loaded bone cements are widely used (15), but the increasing percentage of total joint infections with multi-resistant bacteria (16) makes adequate prophylaxis against these organisms still necessary.

A lot of studies have been carried out to demonstrate the efficacy of antibiotic-loaded cements, by comparing different antibiotics and bone cements. The best technique applicable for introducing antibiotic into cement mixture is still an unresolved question, since drug powder can be added to the polymer matrix directly at the surgical site using a variety of mixing procedures, or it can be added to the solid fraction of the cement during industrial production.

In Europe the use of commercially-available, antibiotic-loaded bone cements is quite common, while in the United States manual addition of antibiotic powder to a traditional bone cement during surgery is preferred. Despite the high number of data in literature about bone ce-
ments, little information about the characteristics and efficacy of different formulations, with industrial or manual addition of the drug, are available. In this work, for the first time in the literature, a systematic comparison between different antibiotic-loaded bone cements has been performed. The aim of this study is thus an in vitro comparison between commercial antibiotic-loaded bone cements and manually mixed ones through the evaluation of their antibacterial behavior based on inhibition zones.

MATERIALS AND METHODS

Bone cements

Three different commercial bone cements were used during this study (Heraeus, Wehrheim, Germany):
- Palacos R® (high viscosity)
- Palacos LV® (low viscosity)
- Palacos R + G® (added with 0.5 g of gentamicin, 40.8 g pack)

For manually loaded samples, gentamicin sulphate powder was mixed for about 3 minutes with a solid fraction of commercial cements (both Palacos R® and Palacos LV®) in the same proportions as commercial antibiotic-loaded cements (Palacos R+G®), i.e., 1.24% wt (considering gentamicin compared to polymer weight).

In order to prepare a double antibiotic-loaded bone cement, 0.5 g of vancomycin powder was added to 40.8 g of gentamicin-loaded (manually or industrially) bone cement. Finally, 0.5 g of vancomycin powder was added to 40.3 g of solid fraction of both Palacos R® and Palacos LV® to test the efficacy of this antibiotic alone.

Antibiotics

Gentamicin sulphate (Farmalabor) was chosen since it presents a broad spectrum of activity, high temperature resistance and a low tendency to cause allergic reactions. It is also one of the most widely employed antibiotics in the treatment of prosthetic infections and the most used in commercial formulations of antibiotic-loaded bone cements. This made it possible to make a direct comparison between commercial products and those mixed in situ.

The possibility of introducing a second drug to the industrially or manually gentamicin-loaded cements was also investigated. The combination of two different antibiotics in the same cement has been proposed by several authors as a possibility for a higher release, an increase in antibiotic spectrum activity, and a lower risk of resistance development (17-21). In this research study, vancomycin (Vanco 500 mg JO1XA01; Bayer®, Leverkusen, Germany) was chosen as second antibiotic because it has been demonstrated that cement added with both gentamicin and vancomycin (Palamed G® with 1 g vancomycin added) is effective against methicillin-resistant staphylococci (MRSA) (20, 21).

Cements with the addition of only vancomycin were tested to compare their inhibition zone to those of gentamicin. Vancomycin-loaded bone cement can offer a good solution when resistance to other antibiotics (such as gentamicin or tobramycin, which are the most diffused) has developed (18), despite the observation that the vancomycin release rate is lower if compared to that of gentamicin and tobramycin (19, 23).

In this experiment, all antibiotics were in powder form because in the literature it has been reported that liquid formulations introduced into bone cements have detrimental effects on mechanical properties (24).

Cement sample preparation

All cements were prepared by manual mixing of the solid and liquid fractions using a spatula and following manufacturer proportions and instructions similar to clinical use by surgeons. Before completing polymerization, the plastic cement was transferred into a polished aluminum mould (100 x 100 x 4 mm) with 25 holes of 10 mm in diameter and closed between two polished aluminum plates in order to prepare 25 identical cement samples (Fig. 1).
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Using this procedure, samples of about 0.5 g in weight and 282.74 mm² of external surface were prepared for various cements, differing from each other in formulation and the added drug:

Palacos R® as control samples for high-viscosity cement
Palacos LV® as control samples for low-viscosity cement
Palacos R+G®
Palacos R® gentamicin added
Palacos R® vancomycin added
Palacos LV® gentamicin added
Palacos LV® vancomycin added
Palacos R+G® vancomycin added
Palacos® gentamicin and vancomycin added.

Bacterial strains

All antibacterial characterization was carried out on *Staphylococcus aureus* because it is one of the main bacteria involved in prosthetic infections. Bacterial broth was prepared dissolving a lyophilized disc (*S. aureus* ATCC29213 BD Microtrol discs; Becton Dickinson, Franklin Lakes, NJ USA) into 5 mL of Brain Heart Infusion (Becton Dickinson) and incubated overnight at 35°C to 37°C. Ten µL of this suspension was then spread on Blood-Agar plates and incubated overnight at 35°C to 37°C. Some colonies were spread onto another Blood-Agar plate (Becton Dickinson) and incubated in the same conditions in order to favor bacterial proliferation. Finally some colonies were added to Mueller Hinton Broth (Becton Dickinson) to reach a standard bacterial concentration (0.5 McFarland). This suspension was used for inhibition zone testing as described in the following section.

Antibacterial tests

In order to obtain a simple, visible and direct comparison between different cement formulations, the inhibition zone evaluation was used as an antibacterial test method. The procedure for this qualitative test (known also as the Kirby Bauer Method) is standardized (NCCLS M2-A9 “Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard – Ninth Edition”). It includes firstly the preparation of a standard bacterial suspension (0.5 McFarland) and its spreading on a Mueller Hinton Agar plate (Becton Dickinson) and finally positioning samples on plates. After overnight incubation at 35°C to 37°C inhibition zones around antibacterial samples could be observed. Inhibition zone is a halo around samples where bacteria haven’t grown up. In order to obtain semi-quantitative information, the distance between samples edges and halo border were measured together with the entire halo diameter after sample removal (a schematic representation of the test is shown in Fig. 2).

In order to compare the behavior of different cement formulations and arrive at conclusions, the results for inhibition zone dimensions were statistically evaluated using one way analysis of variance (one-way ANOVA) and p values less than 0.05 were considered statistically significant.

RESULTS

In this research study, all tested samples (except controls) produced halos greater than 3 mm, indicative of *Staphylococcus aureus* susceptibility to both gentamicin (commercial and manually enriched) and vancomycin.

Figures 3 to 8 show inhibition zones for different tested cements. In each plate the upper sample is a standard cement control (Palacos R or Palacos LV). As expected, control samples are not able to produce any inhibition zone. Figure 3 shows a typical inhibition halo produced
by an antibiotic-loaded sample. Figures 4 and 5 report a comparison between the inhibition halo of industrially and manually gentamicin-loaded Palacos® bone cements. It is evident that commercial Palacos R+G® is able to produce a larger halo if compared to Palacos R with manually loaded gentamicin. The difference (about 1.48 mm without and 1.83 with a partial inhibition area) is statistically significant (p<0.05). Furthermore, the commercial formulation presents a more regular halo if compared to the manually enriched one.

Comparing gentamicin and vancomycin, Figures 7 and 8 clearly show that gentamicin-loaded cements are able to produce larger inhibition zones both in the high-(Fig. 7) and low-viscosity (Fig. 8) formulations than vancomycin-loaded samples. The comparison is also shown in the graph of Figure 9. The difference is statistically significant (p<0.05) and it is attributable to the characteristics of...
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Vancomycin added to Palacos R+G® produces a moderate increase in halo diameter (0.36 mm for the clean zone and 0.63 mm for the total area) which is not statistically significant, as shown in Figure 10. Despite this, the addition of a second antibiotic could be extremely interesting in multiple bacterial infections, resistant strains, unknown pathogens and generally to increase drug spectrum activity.

The comparison between Palacos R+G® with vancomycin added and Palacos R manually loaded with both gentamicin and vancomycin shows similar results, since the inhibition zone for the commercial formulation is a bit larger and the increase introduced by the vancomycin addition is not statistically significant either for industrially or manually gentamicin-loaded bone cements.

Finally, it can be observed that the difference between halo dimensions of high- and low-viscosity gentamicin-loaded Palacos is not statistically significant (p<0.05). Vancomycin-loaded Palacos in high- and low-viscosity formulations produces the same result. Consequently, cement viscosity does not influence halo dimensions either for gentamicin or vancomycin.

Table 1 provides a summary of inhibition zone measurements for all tested samples, mean values and standard deviations (SD).

**Table 1 - Inhibition Zone Measurements for all Tested Samples, Mean Values and Standard Deviations (SD)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition zone (without partial inhibition portion)</th>
<th>Inhibition zone (with partial inhibition portion)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (mm) ± SD (mm)</td>
<td>Mean (mm) ± SD (mm)</td>
</tr>
<tr>
<td>Palacos R+G® (n = 6)</td>
<td>10.0 ± 0.3</td>
<td>11.5 ± 0.4</td>
</tr>
<tr>
<td>Palacos R® gentamicin added (n = 4)</td>
<td>8.1 ± 0.5</td>
<td>9.7 ± 0.7</td>
</tr>
<tr>
<td>Palacos LV® gentamicin added (n = 3)</td>
<td>8.8 ± 1.2</td>
<td>9.8 ± 1.4</td>
</tr>
<tr>
<td>Palacos R® vancomycin added (n = 3)</td>
<td>4.0 ± 0.2</td>
<td>–</td>
</tr>
<tr>
<td>Palacos LV® vancomycin added (n = 3)</td>
<td>3.9 ± 0.3</td>
<td>–</td>
</tr>
<tr>
<td>Palacos R+G® vancomycin added (n = 5)</td>
<td>10.4 ± 0.7</td>
<td>12.1 ± 0.8</td>
</tr>
<tr>
<td>Palacos R® gentamicin and vancomycin added (n = 3)</td>
<td>9.5 ± 0.4</td>
<td>10.5 ± 0.5</td>
</tr>
</tbody>
</table>

**Fig. 9 - Image showing the inhibition halos of A) control sample Palacos R®, B) Palacos R+G®, C) Palacos R+G® vancomycin added. The addition of a second antibiotic produces a moderate increase of the inhibition zone.**

**Fig. 10 - Comparison between high (Palacos R®) and low viscosity (Palacos LV®) bone cements added with gentamicin or vancomycin. Bars indicate the respective inhibition zone extents.**
DISCUSSION

No vacuum mixing techniques were considered in this study. Even if it is well known that vacuum mixing often provides better mechanical properties to the final cement, for most orthopedic surgeons, manual mixing using a spatula is a very common practice, since the antibiotic release rather than mechanical properties is the main objective for revisions and for any application where a temporary device is needed. Moreover, vacuum mixing is a very expensive method and during the polymerization of vacuum-mixed cements, a volume reduction 1/3 greater compared to manual mixing is commonly observed. This is considered by most of surgeons to be a negative feature. Several opinions on the effect of vacuum mixing on both shrinkage and porosity are reported in the literature (25-27). This work is mainly focused on the antibacterial properties of the different cements and we did not consider mechanical properties at this stage of our research.

In the literature (28-32) the inhibition zone is defined as the distance between the edge of the sample and the borderline of the bacterial proliferation area. It could also be described in terms of the diameter of the total halo after sample removal (33).

In this study, the halo diameter was measured in two different directions and the mean value of this data was finally reported in terms of distance between sample edge and halo border in order to free the data from the particular dimensions of the samples. In some cases, an intermediate zone was observed between the clear halo and the bacterial proliferation zone (this can be observed, for example, in the Fig. 3 magnification); this area has been widely described in literature (31-34) as a partial inhibition zone that is often omitted in diameter measurement. In this work, measurements with and without partial inhibition are reported so that they can be compared with different studies in the literature.

One of the main problems related to the evaluation of the inhibition zone as an antibacterial test method is the low correspondence between in vitro and in vivo behavior: if a bacterial strain is resistant to an antibiotic in vitro, it will be resistant in vivo as well, but if it appears sensitive in vitro it is not necessarily also sensitive in vivo. In the literature, different models have been described to simulate physiological conditions for bone cements (34-38), but none has become standard for in vitro testing of antibiotic-loaded bone cements. In this study, the samples were tested for 24 hours but it can be interesting to consider a longer period to evaluate the long-term efficacy of these materials. Some authors (30-31) have carried out tests for a larger time period but it is difficult to make a comparison because there are several differences in experimental conditions (sample shape and dimensions, mixing techniques, etc.). On the other hand, all in vitro studies need in vivo tests confirmations and clinical trials in order to give more realistic results. Consequently, several analyses have been reported in the literature (36, 39-42) describing short- and long-term behavior and the advantages of different formulations of antibiotic-loaded bone cements, but no statistically significant differences among different preparation techniques have been noted.

In spite of this, inhibition zone test is widely employed in in vitro drug testing because in vitro susceptibility is an index of efficacy of therapy: halo dimensions are proportional to the antibiotic diffusion rate into agar and are strictly connected to the antibacterial power of the drug. The inhibition zone dimension is proportional to the drug diffusion rate into the cement matrix and to the antibacterial efficacy of the antibiotic. The antibiotic molecular weight and charge can also affect halo dimensions. Considering a particular drug, the inhibition zone diameter is an index of antibiotic diffusion through the cement.

In this work some differences in the halo dimension were observed in the various cement formulations. There are many possible explanations for these differences:

1) Manual mixing of cement solid fraction and antibiotic powder is less effective than the industrial mixing since it causes the formation of gentamicin clusters (which are clearly visible from inspection of the samples) that affect antibiotic activity. In the literature, these aggregates are also described as detrimental factors for mechanical properties (35, 38). This hypothesis can be confirmed by looking at the inhibition zones: indeed, we found that in the presence of gentamicin clusters, an irregularity in the halo can be observed along with an increase in its dimensions. In Figure 6, two inhibition zones (with different dimensions) for Palacos R® with manually-loaded gentamicin are reported.

2) The antibiotic powder employed in manual mixing is a commercial gentamicin sulphate which is a mix of different substances produced by Micromonospora purpurea bacterium. It includes: C1 gentamicin sulphate (25.75%), C1a gentamicin sulphate (34.40%), C2 gentamicin sulphate (38.85%) and on the whole is a 99% pure drug. No information is available for the antibiotic used in industrial formulations, therefore no direct comparison between the two compositions is possible.

The use of a liquid antibiotic could be a solution to increase the homogeneity of manually mixed formulations. It has not been considered, however, because some studies have stressed that it negatively affects the mechanical properties of the final bone cement (24).

A difference in halo characteristics for the two antibiotic types was also observed: while vancomycin halo is clean, the gentamicin one shows a partial inhibition zone. This is due to differences in molecular weight, dimensions, elution and diffusion properties for the two drugs. The gentamicin molecular weight is 477.596 g/mol, whereas that of vancomycin is 1449.3 g/mol. This difference could explain the poor elution properties and
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consequent diffusion into the agar of vancomycin, as reported by other studies (43-45).

Conventionally, a bacterial strain is considered susceptible to a certain antibiotic if the halo produced is greater than 3 mm, while it is considered resistant if no halo is produced. Intermediate dimensions indicate moderate susceptibility (32).

Even if the increase in halo dimension is not statistically significant, at this step of the research the investigation of a possible synergistic effect of two commonly used antibiotics on the most common bacterial strain is important for future comparisons with different antibiotic combinations and different strains.

CONCLUSIONS

In this research study, it was observed that the manual addition of antibiotics to PMMA-based bone cement produces inhibition zones that are moderately smaller and more irregular if compared to commercial formulations of the same antibiotic-loaded bone cements. It would thus seem that in clinical use, commercial antibiotic-loaded bone cements are preferable because they do not require particular mixing accuracy and are independent of the manual ability of the person preparing the cement.

Despite this consideration, the difference in the inhibition zone dimension has to be studied, also taking into consideration the mechanical and economical effects of manual preparations. In addition, quantitative antibacterial tests could be performed in order to have a more detailed description of the problem. This would call for the design of a more realistic in vitro model, to better describe in vivo bone cement applications.

This research is a preliminary study and the statistical significance of the results is limited because of the small number of samples, but it can give a qualitative description of the efficacy of different antibiotic-loaded bone cements and their dependence on the mixing technique used to prepare them.

Finally, antibiotic-loaded bone cements present an effective antibacterial activity which is a bit higher and reproducible in commercial formulations. Gentamicin is a good drug for this kind of application, while vancomycin may be of interest as a second, additional antibiotic for multiple bacterial infections and resistant strains. These basic conclusions may be relevant for orthopedic surgeons in choosing the kind of cement, antibiotic, and mixing technique for the treatment of infected hip and knee revisions.

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