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Effect of aeration of antibiotic-loaded bone cement on its properties and bactericidal effectiveness

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Abstract

Antibiotic-loaded bone cement are now widely used in medicine. They are used for local delivery of antibiotic particles and they allow treat or protect against infection. It is assumed that the bactericidal effectiveness of bioactive bone cements depends on the parameters of its production. Hence, the aim of this study was to check the effect of aeration of bone cement before mixing the components on its properties as well as its effectiveness in combating infections. The results show that this additionally step is an easy method that enables the improvement of the open-pore structure of bone cement, its porosity and in effect better inhibition of bacterial growth. However, longer aeration time resulted in defects in the structure that may contribute to fatigue breaks. Future research should undertake a broader investigation of mechanical properties, determination of the released dose of antibiotic and potential optimization of the aeration process of bone cement.

Key words

antibiotic-loaded bone cement; production process, aeration, bactericidal effectiveness.

1 Introduction

Bone cements, well known biomaterials nowadays, have been developed since J. Charnley in the 1960s proposed them as synthetic biomaterials in orthopedics. They are used mainly as interfacial phase between the metallic implant and the bone. In that case bone cements are responsible for transferring the loads of the joint to the bone. Moreover, cements can be used to stabilize fractures or fill bone defects [1-3]. Different requirements are posed to cements, i.e.: biocompatibility, bioconductivity or many kinds of biomechanical properties (such as bone-like Young Modulus, compressive and bending strength and high durability) [4,5].

The biggest threat during implantation are infections. Despite the effort to ensure the highest sterility of the surgery, opening the body always carries the risk of introducing microbes into the operating site. In the case of orthopedics, the main strains responsible for infections are: *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* [6,7]. Hence the new types of bioactive bone cements are still the subject of many research. This bioactivity in the case of cements is provided by their porous structure, which allows embedding antibiotic particles into the pores and their local release [8,9]. There are two well-known groups that are able to fight orthopedic infections: aminoglycosides and glycopeptides. Generally, gentamycin is one of the most commonly used antibiotics, which is added to bone cement – manually (during surgery) or industrially. Many studies of antibiotic-loaded have shown that antibiotic modification has a preventive and/or therapeutic effect [10-12].

However, the bactericidal effectiveness of antibiotic-loaded bone cements depends on the release of antibiotic particles from polymer matrix, and this parameter depends on [13-15]:

- the porosity of bone cement (pores size, open or close porosity);
- the viscosity of bone cement;
- the type and the dose of antibiotic;
- the size of the antibiotic particles;
- the circulation of body fluids.

In the literature, studies on the influence of bone cement production parameters on its antibacterial properties have been found [13-17]. The research concerns mainly the speed of mixing bone cement and the type of production (manual or vacuum). Kongkit Pithankuakul et al. and Nugent et al. showed that increases the porosity obtained during high-speed hand mixing affect the antibiotic release [16,17].

The aim of this study was to check whether the aeration of the cement powder affects the porosity, its selected properties and bactericidal effectiveness. The effect of this study was to enable potential optimization of the process of manufacturing antibiotic-loaded bone cement.

2 Experimental

2.1. Materials and methods

2.1.1 Bone cements preparation

In the research, commercially available bone cement – Cemex Genta (Tecres, Italy) was used. The cement was prepared following the procedure by the manufacturer's recommendation [18], but the preparation of cements was conducted with a preceding step. Firstly, the powder was aerated for 2, 5 and 10 minutes mixing with average speed – 2 revolutions per second. The bone cements were prepared by combining the powder component with liquid in a bowl and hand-mixing at an average speed of 2 revolutions per second. Next, the obtained paste was applied on the yarn and allowed to cure for 1 hour in ambient conditions (Fig. 1). The specimens intended for surface tests were wet ground using 1500 grit silicon carbide paper and cured for 24 h. The composition of bone cement used in this work is presented in Tab. 1.

Tab. 1. The chemical composition of bone cements used for research.

Bone Cement	
Powder component:	
Polymethyl methacrylate	82.78% w/w
Barium sulphate	10.00% w/w
Benzoyl peroxide	3.00% w/w
Gentamicin sulphate	4.22% w/w
Liquid component:	
Methyl Methacrylate	98.20% w/w
N,N-dimethyl-p-toluidine	1.80% w/w
Hydroquinone	75 ppm

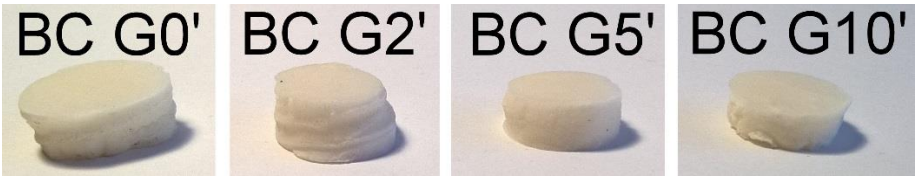


Fig. 1. An example of specimens of the bone cement used in the research

2.1.2 Research on the properties of bone cements

In order to evaluate the properties of tested bone cements, the following studies were carried out: wettability, microhardness, porosity, a microstructure and topography analysis. The number of tested specimens was 5 (n=5). To determine the porosity, microstructure and surface topography, a scanning electron microscope JSM-7800F (Joel, Japan) was used. The microhardness test was carried out using Vickers hardness tester FM-800 (Future-Tech, Japan). The indentation press time was 10 s and the press load was 10 N. To examine the surface hydrophilicity, an optical tensiometer (Attention Theta Life, Biolin Scientific, USA) was used. The measurements were carried out using the falling drop method with demineralized water.

2.1.2 Research on the bactericidal effectiveness of bone cements

The bactericidal effectiveness of bone cement was determined based on the study of bacterial growth inhibition in 2 methods: by the turbidity assessment of the bacterial solution according to McFarland standards [19] and by disc-diffusion antibiotic sensitivity test according to Kirby-Bauer test [20]. Before testing, the specimens were sterilized in an autoclave at 120°C for 30 min.

For disc-diffusion antibiotic sensitivity test, a combination of three clinically isolated bacterial strains was used: *Staphylococus aureus, Pseudomonas aeruginosa and Escherichia coli* (supplied by the Specialist Hospital in Kościerzyna, Poland). These strains of bacteria were selected for being the most common sources of medical infections [7]. Each strain of bacteria was incubated separately and then added to a sterile 0.9% NaCl solution. For the study of the bacterial growth inhibition zone, 100 µl of this suspension was taken and seeded on the Mueller-Hinton agar plates. The final concentration of bacteria was $1.5x10^8$ CFU ml^{-1}. The inhibition zone test consisted of placing the specimens (10 mm diameter, 2 mm thickness) on plates with the resulting bacterial suspension and incubation at 37°C. The experiment was performed using three specimens for each type of bone cement (n=3). The whole experiment lasted 7 days, and the measurements of the inhibition zone were carried out after: 24, 48 and 168

hours. The bacterial growth inhibition zone was determined as an area without bacterial growth. The area of bactericidal activity was assessed by naked eye, but additionally, a biological microscope (Axio Observer D1, ZEISS, Germany) was used to analyze the bacterial medium.

The study according to McFarland turbidity assessment consisted of incubating the tested specimens in a bacterial broth and measuring its optical density. The *Staphylococcus aureus* strain (ATCC 29213) was used for this tests and the initial concentration of bacteria was $1.5x10^8$ CFU/ml, which corresponds to 0.5 McFarland Index (MSx). The experiment was performed using three specimens for each type of bone cement (n=3) in disk form (5 mm diameter, 2 mm thick) for 2.5 ml of bacterial solution. For bacterial cultivation, Tripticase soy broth (Sigma Aldrich, Germany) was used and temperature of 37°C. The DensiChEK Plus (BioMerieux, USA) was used for measurements and reading were made after 0.5, 2, 4, 6 and 24h. The maximum measuring range of this device is 4 MSx. According to the assumptions of the McFarland Standard [21], the following conversion factor was assumed for the number of bacteria and is included in Tab. 2. In pursuance of the recommendations for optical densitometry methods, some measurements were rejected as positively false if the material affected the color of the solution.

Tab. 2. The McFarland index conversion for the approx. cell density and number of bacteria [21]

McFarland Index No.	0.5	1	2	3	4
Approx. cell density ($1 \cdot 10^8$ CFU/mL)	1.5	3.0	6.0	9.0	12.0

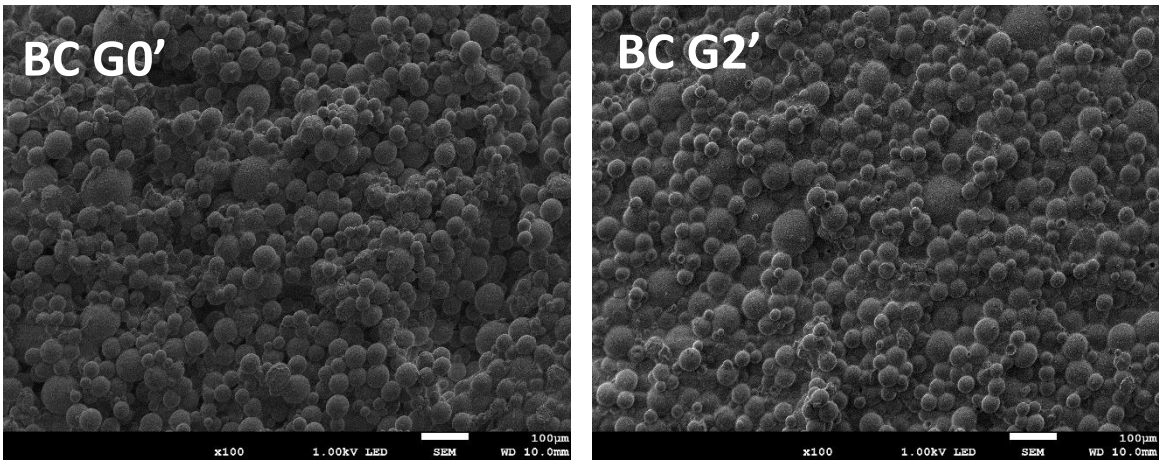
2.1.3 Statistical analysis

Statistical analysis of the data was performed using commercial software (SigmaPlot 14.0, Systat Software, USA). All of the results were presented as mean ± standard deviation (SD) and were statistically analyzed using one-way analysis of variance (one-way ANOVA). The Shapiro-Wilk test was used to assess normal distribution of the data. Multiple comparisons versus control group between means was performed using Bonferroni t-test with statistical significance set at $P < 0.05$.

2.2. Results

2.2.1 Research on the properties of bone cements

Topography of antibiotic-loaded bone cement specimens with different aeration time is shown in Fig. 2. The obtained cements are characterized by high roughness with a porous internal structure. This structure is typical for polymeric materials based on PMMA. With the extension of the aeration time of cements, the relaxation of the bond of the MMA monomer chains was observed. It is assumed that the structure will improve in terms of so-called open pores.



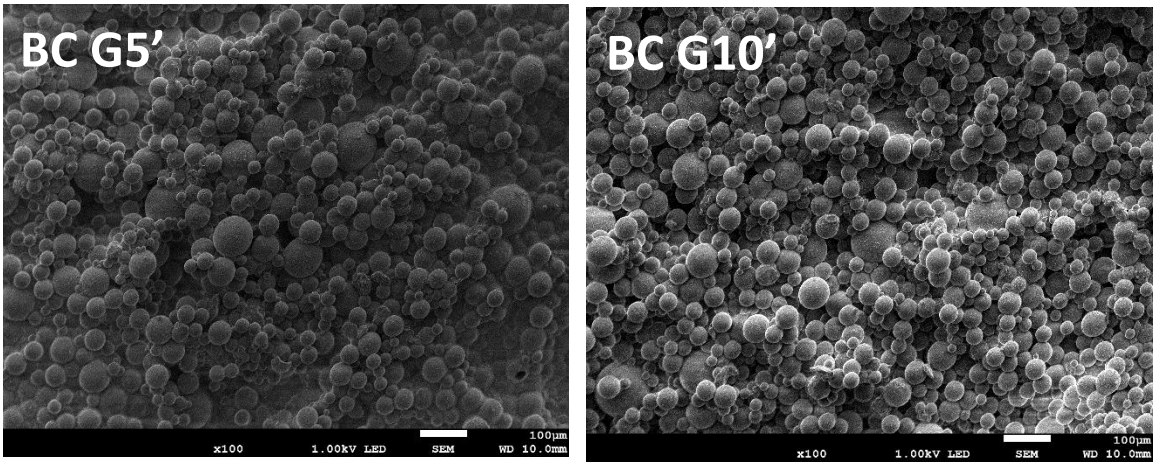


Fig. 2. Comparison of the topography of the obtained specimens with different aeration time (SEM 100x)

The pore size of the obtained cements was evaluated after polishing the specimens using SEM microscopy. An exemplary image along with the marked pore size is shown in Fig. 3.

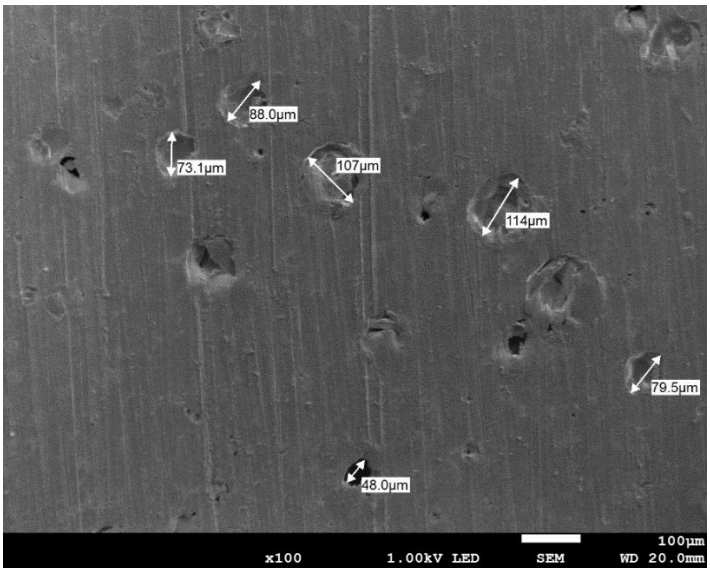


Fig. 3. An example of SEM image used to assess the size of the surface pores of the tested antibiotic-loaded cement – G10’ (SEM 1000x)

The average pore size of tested antibiotic-loaded bone cement with different aeration time is collected in Tab. 3. An increase in pore size was observed along with a longer aeration time. The tested cements were characterized by a large discrepancy in the aspect of pore size, which is well illustrated in Fig. 3. It is stated that all obtained cements have a microporous structure.

Tab. 3. Average pore size of tested cements (n=3)

	Average pore size [µm]
BC 0’	43.5 ± 24.3
BC 2’	68.1 ± 32.3^
BC 5’	97.7 ± 27.2*^
BC 10’	119.4 ± 41.6*^
* Statistical analysis between groups and control (BC G0’) was performed, the group, where the statistically significant difference occurred was marked. ^ Statistical analysis was performed between groups, and the groups, where the statistically significant difference occurred were marked.	

However, the SEM studies also shown that as a result of high aeration there noticed some collapses and cracks (FIG. 4). Concentrations of occurrence of these damages increase with the time of aeration.

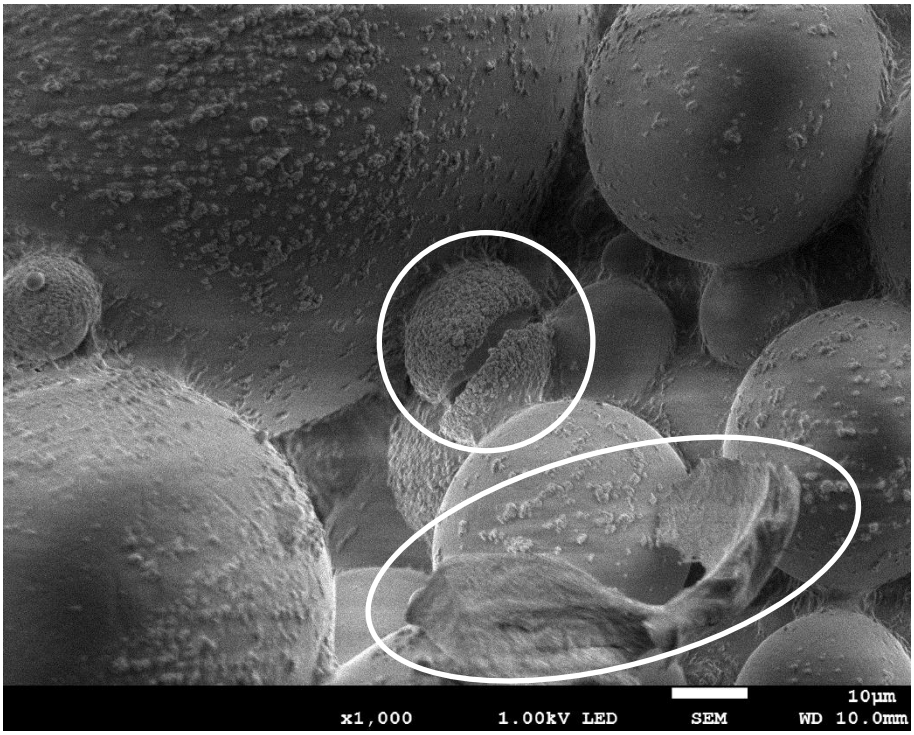


Fig. 4. An example of damage in the structure of aerated antibiotic-loaded cements – G10' (SEM 1000x)

The effect of aeration on the surface wetting of obtained specimens was assessed. It has been observed that aeration increases the value of contact angle, i.e. worsens wettability (Tab. 4). However, all tested cements have hydrophilic properties (contact angle < 90°). An exemplary measurement of contact angle is shown in Fig. 5.

Tab. 4. Results of the wettability of the tested specimens (n=5)

The value of contact angle [°]			
BC G0'	**BC G2'**	**BC G5'**	**BC G10'**
56 ± 5.2	66 ± 4.5*	62 ± 3.1^	74 ± 4.8*^
* Statistical analysis between groups and control (BC G0') was performed, the group, where the statistically significant difference occurred was marked. ^ Statistical analysis was performed between groups, and the groups, where the statistically significant difference occurred were marked.			

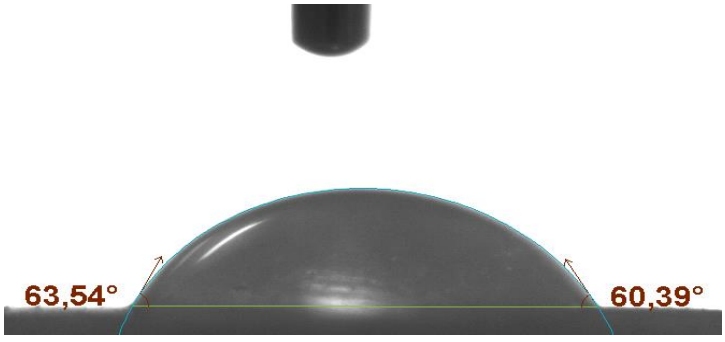


Fig. 5. An example of contact angle measurement for tested specimen (BC G10')

The microhardness values of tested antibiotic-loaded bone cements are showed in Tab. 5. It was observed that cement without aeration is characterized by the highest hardness (22.3 µHV). Aeration worsens the hardness of

the specimens, however, this is not statistically significant. All of obtained values of microhardness oscillates around 20 μHV.

Tab. 5. Results of the microhardness of the tested specimens (n=5)

The value of microhardness [μHV]			
BC G0'	**BC G2'**	**BC G5'**	**BC G10'**
22.3 ± 2.1	21.1 ± 3.6	19.7 ± 3.5	20.8 ± 3.2
^ Statistical analysis was performed between groups, but there were no statistically significant differences between the groups.			

2.2.2 Research on the bactericidal effectiveness of bone cements

Antibacterial tests confirmed the effectiveness of antibiotic-loaded cements. The results of the bacterial growth inhibition zone showed that the cements containing gentamycin effectively reduced the growth of bacteria on the agar plates Fig. 6. Bacterial growth inhibition zone didn't change significantly through the duration of the study (Tab. 6). There weren't observed any significant differences between the time of aeration of specimens.

Tab. 6. Results of the study of bacterial growth inhibition zone for the tested specimens after 24, 72 hours and 7 days (n=3)

	Bacterial growth inhibition zone [mm]		
	24 h	**72h**	**7d**
BC	0		
BC G0'	28.7 ± 1.5*	28.3 ± 1.0*	27.5 ± 1.8*
BC G2'	29.3 ± 2.1*	30.2 ± 0.8*	30.0 ± 0.5*
BC G5'	29.3 ± 1.0*	29.3 ± 1.0*	29.5 ± 1.0*
BC G10'	29.8 ± 1.2*	29.3 ± 1.2*	27.5 ± 0.8*
* Statistical analysis was performed between groups and control (BC), the group, where the statistically significant difference occurred was marked. ^ Statistical analysis was performed between groups, but there were no statistically significant differences between the groups.			

The bactericidal effect of the cements with gentamicin was confirmed within 7 days. At that time, for all specimens, the zone of bacterial growth inhibition was close to 28-30 mm.

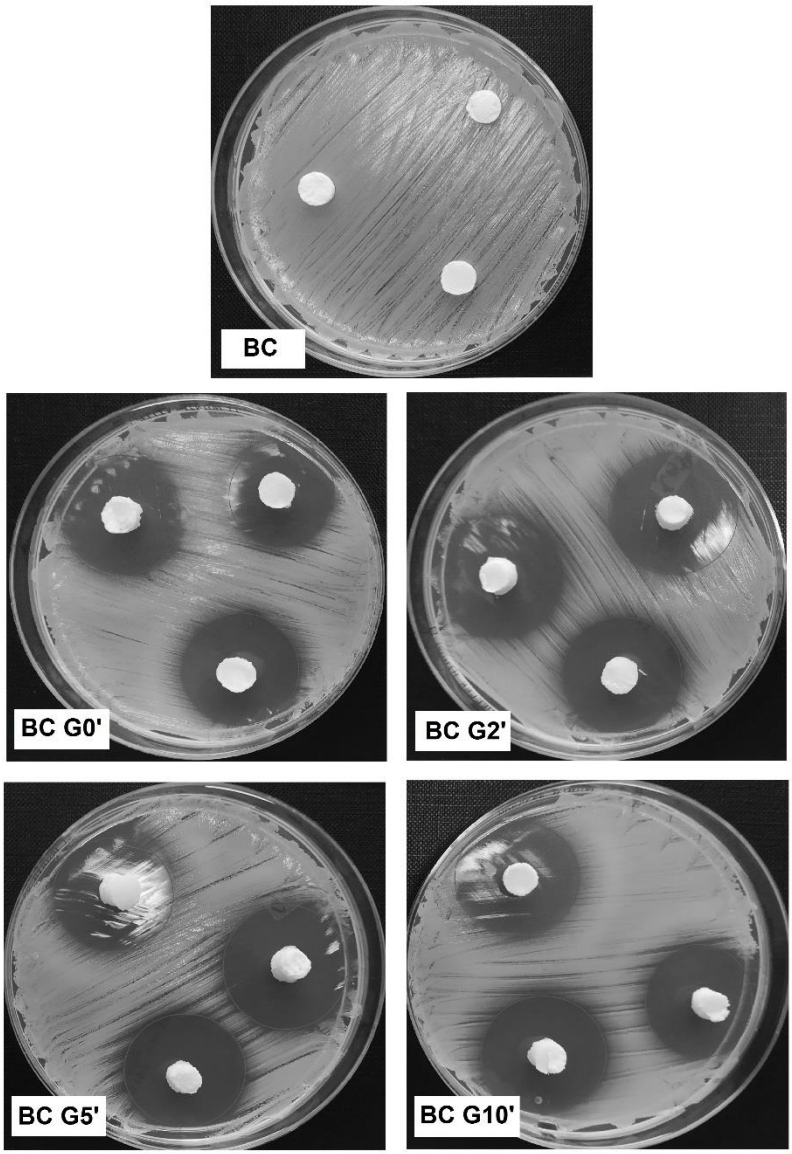


Fig. 6. Comparison of the result of the bacterial growth inhibition zone for the antibiotic-loaded bone cement with different time of aeration after 72 hours of incubation

The effectiveness of antibacterial properties of released antibiotic particles from cement during 24h was evaluate. This time is to correspond to the initial postimplantation phase, which is most exposed to postoperative infection. Significant inhibition of bacterial growth in the case of antibiotic-loaded cements was confirmed. The results of McFarland standard are shown in Tab. 7.

Tab. 7 Results of McFarland index values specifying the number of bacteria *Staphylococcus aureus* during incubation with tested cement with different aeration time (n=3)

	McFarland Index[#]					
	Control	**BC**	**BC G0'**	**BC G2'**	**BC G5'**	**BC G10'**
0	0,5	0,5	0,5	0,5	0,5	0,5
2h	1,22	1,07	0,98	0,94	0,92	0,90
6h	>4,00	>4,00	1,09	1,15	1,09	1,00
8h			1,18	1,30	1,11	1,12
12h			1,21	1,31	1,15	1,13
24h			1,36*^	1,32*	1,29*	1,19*^

[#] max. SD ± 0.05

* Statistical analysis was performed between groups and control after 24h, the group, where the statistically significant difference occurred was marked.

^ Statistical analysis was performed between groups, and the groups, where the statistically significant difference occurred were marked.

After 24 hours, the number of bacteria incubated with cements containing gentamicin was about $4 \cdot 10^8$ CFU/mL, while in the case of control, after 6h it exceeded the measuring range of the device - $12 \cdot 10^8$ CFU/mL. The rapid increase in the number of bacteria in the first 2 hours of incubation is associated with a small amount of release antibiotic particles from cements. It was observed that the aeration of bone cements improved its antibacterial properties (statistically significant for the time of 10 min). The difference between the lack of aeration (BC G0') and aeration for 10 min (BC G10') is 0.17 MSx, which corresponds to the number of bacteria – $0.5 \cdot 10^8$ CFU/mL and approximately means a reduction in the number of bacteria by 12%.

2.3. Discussion

Acrylic antibiotic-loaded bone cements are nowadays a basic standard in the prophylaxis, as well as, treatment of orthopedic infections [22,23]. They enable local delivery of the antibiotic in the surgical site. Numerous items related to the assessment of the influence of the antibiotic type, the type of cement and the mixing method on the release of the substance were found in the literature. Our study was aimed to assess whether the aeration of cement powder will affect the bactericidal effectiveness. This aeration process would be a simple, cost-free and quick step before producing bone cement paste.

The addition of an antibiotic can protects against infection, however it may also cause deterioration of mechanical properties. Most authors agree that a low dose (below 5 wt/wt%) does not significantly affect these properties, but ensures adequate bactericidal effectiveness. In the research commercially available bone cement – Cemex Genta (Tecres, Italy) containing 4.22% of gentamicin sulphate was used. Three aeration times were used to prepare it: 2, 5 and 10 min, but also a control without aeration process [24-26].

The structure and topography of the obtained cements has been assessed. It is typical that the production parameters will affect the quality of the biomaterial, hence the effect of aeration was analyzed. It was observed that the prolongation of the aeration time loosens the structure of MMA chains bonds and improves the open-pore structure, moreover, the average pore size has also increased significantly ($P<0.05$). Porosity is an important parameter, because of the potential binding to bone tissue, and on the other hand it is supposed to allow a better release of the antibiotic. It is generally stated that porosity is the most important factor of antibiotic release, even more importantly than the antibiotic dose use [27].

Another aspect important for osseointegration is wettability. Porous materials with a rough hydrophilic structure are considered the best for cell deposition and proliferation [28-31]. The tests have shown that aeration slightly worsens wettability, but the surface is still hydrophilic.

In order to assess whether the aeration process is aggravated by mechanical properties, microhardness was assessed. There were no statistically significant changes ($P<0.05$). Obtain microhardness for all of specimens oscillated about 20 HV.

However, the occurrence of microcracks and voids. This defects can have a significant impact on the fatigue strength of the material, especially in the human body environment. The number of defects increases with the aeration time.

The bactericidal effectiveness of cements has been confirmed. In the initial postimplantaion phase, the inhibition of bacterial growth was observed, statistically significantly better for the aeration time of 10 min and reduction in the number of bacteria by 12%. In the case of a long-term (7 days) study, the zone of bacterial growth inhibition on discs did not show statistically significant differences after longer aeration time. It is possible to assume even a deterioration of bactericidal effectiveness for BC 10' after 7 days (about 8% vs. BC G2'). This may be related to the faster release of the active substance from the porous structure and deterioration of efficacy after this period.

The main limitation of our study and the possibility of continuing future research are: no quantification of the released antibiotic dose (only qualitative efficacy tests was performed) and lack of research on fatigue strength.

3 Conclusions

- The aeration time improves the porosity and pore-open structure of antibiotic-loaded bone cements,
- No significant negative impact of aeration on microhardness and wettability of cements was observed,
- Longer aeration time (5 min +) causes defects in the structure that can cause a fatigue fracture of the cements,

- The aeration time improved bactericidal effectiveness in the initial implantation phase (by 12% for 10 min), however worsened for a longer period – 7 days.
- It is assumed that optimal aeration (about 5 minutes) can improve bactericidal effectiveness without adversely affecting the properties of antibiotic-loaded bone cements.

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