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Experimental study of impregnation conditions for sustained antimicrobial activity of the original osteoplastic material based on cancellous bone allograft

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Abstract

Introduction Local antibiotic therapy is used to prevent and treat periprosthetic joint infection, but the available antibiotic delivery systems have some limitations.

The **objective** was to determine optimal parameters of pressure, exposure time and type of solvent to ensure prolonged elution of vancomycin from the original osteosubstituting material based on cancellous allograft bone using an *in vitro* experiment.

Material and methods Seven impregnation techniques with different combinations of parameters were examined including pressure: from atmospheric to reduced (7–10 hPa), time: from 5 minutes to 24 hours, solvent (distilled water, 50 % ethanol solution, a combination of 50 % ethanol and 5 % polyvinylpyrrolidone (PVP)). The efficacy was assessed by changes in the diameter of the *S. aureus* ATCC 43300 inhibition zone using the bacteriological method and the dynamics of vancomycin concentration in the eluate and high-performance liquid chromatography (HPLC). Statistical analysis was performed using the ANOVA method, Tukey's post-hoc test, Spearman's rank correlation and calculation of the area under the pharmacokinetic curve.

Results The best efficiency was demonstrated by the method employing reduced pressure, 60-minute exposure and an alcohol solution with PVP, which provided prolonged release of vancomycin for 14 days with the maximum area under the elution curve (301364.70) and a high correlation between the concentration of the antibiotic and the growth inhibition zone ($r = 0.908$, $p < 0.001$). The pressure was found to be the most significant factor ($F = 19.9916$, $p < 0.0001$), followed by solvent type ($F = 7.7485$, $p = 0.0006$) and impregnation time ($F = 6.8084$, $p = 0.0014$).

Discussion The technique with use of reduced pressure and an alcohol solution with PVP provides prolonged release of vancomycin for 14 days as opposed to conventional local antibiotic therapy with limited effectiveness of 3 to 7 days. The advantage of the approach includes uniform elution kinetics compared to polymethyl methacrylate and biodegradable carriers, which demonstrate a sharp initial release of the antibiotic. The complementary use of the microbiological method and HPLC indicated antimicrobial activity of vancomycin maintained after impregnation being essential for the therapeutic effect.

Conclusion It has been experimentally established that reduced pressure (7–10 hPa), an exposure time of 60 min and the use of 50 % ethanol with 5 % PVP as a solvent appeared to be the optimal parameters for ensuring prolonged elution of vancomycin from an osteosubstituting material based on cancellous allograft bone.

Keywords: bone-substituting material, impregnation, vancomycin, local antibiotic therapy, antibiotic elution, periprosthetic joint infection, MRSA

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INTRODUCTION

Osteosubstitute materials (bone grafts, calcium phosphate materials, bioactive glasses, biopolymers, composite materials) are used to fill defects formed during total joint replacement, spinal arthrodesis, after radical treatment of chronic osteomyelitis and in orthopedic oncology [1]. With modern antiseptic and antibacterial agents used intra- and postoperatively infection is one of the most serious and devastating complications following orthopedic surgery [2].

Staphylococcus aureus is the most common causative agent of periprosthetic joint infection [2, 3]. The infectious process caused by this microorganism passes through several stages: penetration into the body, evasion of immune system factors, adhesion to the implant surface and formation of a biofilm [4, 5]. Cell wall components, enzymes and exotoxins of *S. aureus* contribute to the virulence by ensuring invasion and stable persistence of the pathogen in the bone [1]. Moreover, staphylococcal cells in biofilms demonstrate reduced sensitivity to antibiotics [4, 6] which leads to chronic infection, repeated surgical interventions and long-term etiotropic antibacterial therapy [7].

Systemic antibacterial therapy to be administered in the perioperative period as a standard of medical care during the interventions is used to prevent infections during implantation of a significant volume of allogeneic osteosubstituting materials which is the [8]. However, systemic antibiotic therapy can be ineffective due to insufficient blood supply at the site of the replaced defect [9]. Physical adsorption of antibiotics on the surface of osteoplastic materials is a promising method of local antibiotic therapy in the treatment of infections. Key factors of therapeutic effectiveness are to be identified in the development of osteosubstituting materials with antibacterial properties. The ability to ensure stable maintenance of a local antibiotic concentration exceeding the minimum inhibitory concentration (MIC) over a long period of time is the fundamental criterion to be considered. This condition is necessary to achieve an antibiofilm effect and prevent the selection of resistant strains of microorganisms [10]. The uniformity of antibiotic release from the osteosubstituting material is an important parameter. Previous studies have shown that a significant portion of the antibiotic elutes during the first day after implantation, which reduces the effectiveness of antibacterial therapy [11].

The **objective** was to determine optimal parameters of pressure, exposure time and type of solvent to ensure prolonged elution of vancomycin from the original osteosubstituting material based on cancellous allograft bone using an in vitro experiment.

MATERIAL AND METHODS

The biological material was obtained from the femoral heads resected during primary hip arthroplasty and immediately placed in a three-layer sterile package. The patients were examined preoperatively for antibodies to human immunodeficiency viruses HIV-1 and HIV-2, hepatitis B and C, total IgM/IgG antibodies to *Treponema pallidum* (ELISA) in the blood and signed informed voluntary consent for intraoperative bone collection. The material was frozen at –80 °C, a standard temperature for long-term storage of biological tissues to slow down protein and other molecules degradation.

Freshly frozen femoral heads were manually cleaned off the cartilage and cortical bone under sterile conditions. The remaining cancellous bone was sawn into 5×5×5 mm blocks (n = 21) to standardize the samples and facilitate placement in 10 ml test tubes. A combination of chemical and physical effects was used to delipidize and purify the material according to the method described in patent RU 2722266 C1 “Lyophilized biological biodegradable mineralized bone-plastic material and the manufacturing method” (Fig. 1).

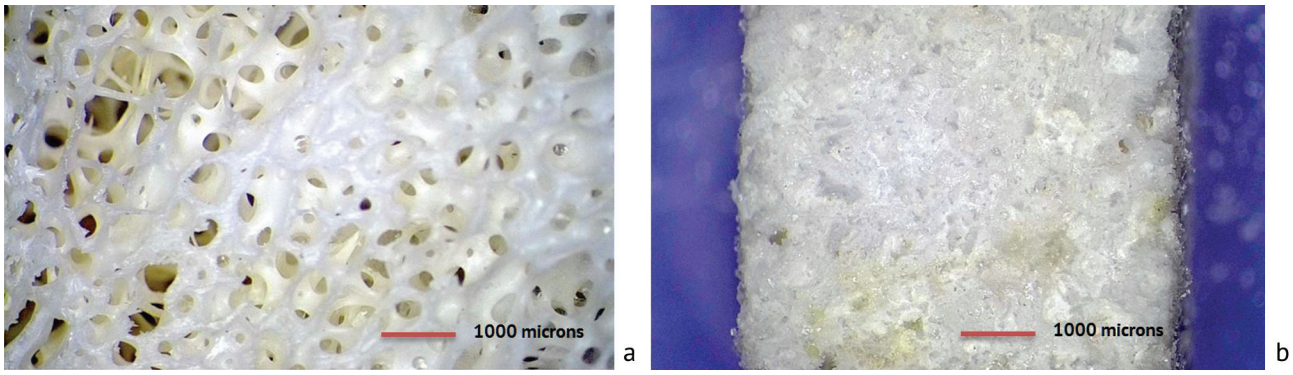

Fig. 1 Stereomicroscopic image of purified delipidized bone material prior to block cutting (a) and after block cutting and impregnation with AB vancomycin (b). Magnification 2× (Lomo, Russia)

Pressure, impregnation time and type of solvent as the three key parameters were explored to optimize impregnation of osteosubstituting material with vancomycin (Vancobact, Pharmasyntez JSC, Russia).

The pressure during impregnation varied from atmospheric (≈ 1013 hPa) to reduced (7–10 hPa) at a solution temperature of +2 °C to +8 °C. The pressure allowed for conditions under which the water was in a pre-boiling state to ensure the best penetration of the antibiotic into the porous structure of the material and the effective impregnation.

The impregnation time period ranged from 5 min to 24 h. Short impregnation periods (5 and 60 min) were used to assess the possibility of rapid saturation of the material with antibiotic, which is important for the practical application of the method and the potential reduction in the overall manufacturing time of the final product. Long-term exposure (24 hours) allowed us to estimate the maximum possible degree of material saturation under various conditions.

The following solvents were used:

1) distilled water (H_2O) as a base solvent;

2) 50 % ethanol solution with better penetrating ability due to lower surface tension;

3) a combination of 50 % ethanol with 5 % polyvinylpyrrolidone (PVP), where the polymer additive was supposed to provide better stabilization and retention of the antibiotic in the transplant structure.

The volume of the solution depends on the calculated density of the solvents, based on the need to achieve a mass percentage of the antibiotic of 5 %. The aqueous solution has a density of approximately 1 g/ml, while a 50 % alcohol solution has a lower density (~0.93 g/ml) and the density of the solution slightly increases (~0.94 g/ml) with use of a 50 % alcohol solution with the addition of PVP. Three cancellous bone samples were used for each method. A comparative analysis of the methods was produced by sequential changing of one parameter with fixed values of the others (Table 1).

Table 1

Methods of impregnation of samples of original osteosubstituting material with vancomycin

No	Solvent	V of the solution, mL	Pressure, hPa	Impregnation time
1	H_2O	20	≈1013	60 min.
2	H_2O	20	≈1013	24 h
3	H_2O	20	7–10	5 min.
4	H_2O	20	7–10	60 min.
5	H_2O	20	7–10	24 h
6	Ethanol 50 %	21.5	7–10	60 min.
7	Ethanol 50 % + PVP 5 %	21.2	7–10	60 min.

For a comparative study of the duration of antibacterial activity, each impregnated sample was placed in a separate sterile tube containing 3 ml of physiological solution. A sample without vancomycin was used as a negative control. Incubated at 37 °C for 18–24 hours. The samples were transferred to fresh saline after 24 hours and continued to be incubated under the same conditions. A suspension of the test isolates with an optical density of 0.5 by McF was prepared, applied with a cotton swab to Mueller-Hinton agar and distributed over the surface. Reference strains of *S. aureus* ATCC 43300 (MRSA) with a vancomycin MIC of 1.5 mg/ml were used as test strains. A suspension of the daily bacterial culture (0.5 McF) was distributed over the surface of Mueller-Hinton agar. After each day of incubation, 10 µl of the incubation solution with samples were applied to the bacterial lawn in three replicates and incubated for 24 hours at 37 °C. The formation of a growth inhibition zone at the site of application of a drop of supernatant was assessed as the presence of a sufficient concentration of vancomycin to inhibit bacterial growth. The dynamics of vancomycin elution were assessed visually by the presence and size (diameter, mm) of the MRSA growth inhibition zone (Fig. 2). The physiological solution was replaced daily and fresh saline was added to the sample tube. The procedure was repeated until there was no visible growth inhibition zone in the Petri dishes.

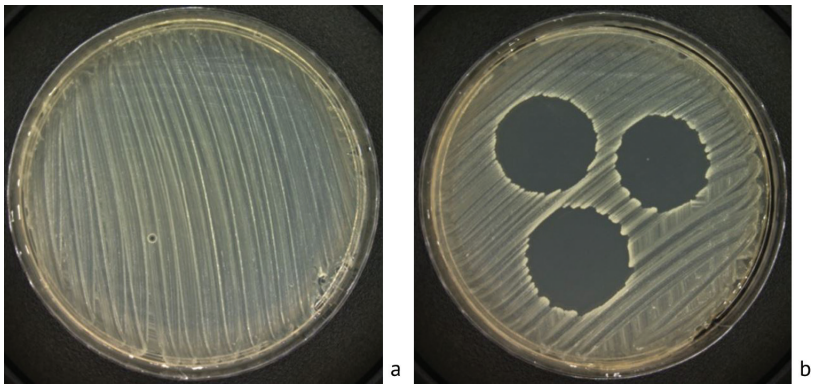

Fig. 2 Petri dishes with *S. aureus* ATCC 43300 (MRSA) culture 10 days after the start of the experiment: (a) no growth inhibition zone, method 1; (b) growth inhibition zone at the site of drop application, method 7

The concentration of vancomycin in the incubation solution was determined by high-performance liquid chromatography (HPLC) on a SHIMADZU device, Shim-pac HR-ODS column (Japan). From test tubes with samples, 1 ml of the daily incubation solution was transferred to Eppendorf tubes and centrifuged for 5 min., 13,000 rpm. Then the supernatant was transferred to a vial and placed in the chromatograph. The volume of the injected sample was 100 µl. Flow rate was 0.45 ml/min. The test lasted for 25 min. The vancomycin retention time on the chromatogram was 8.5 min. During the first 7 days, the incubation solution was diluted 1000 times, and the concentrations were multiplied by the dilution factor. Standard vancomycin solutions in concentrations from 0.1 to 10 mg/ml were used for HPLC calibration. The calibration curve was plotted based on the peak area corresponding to the vancomycin retention time (8.5 min). Chromatographic analysis was performed in LabSolutions software, ensuring accuracy and reproducibility of measurements by performing triplet analyses of each sample.

Statistical analysis was used to process the data in order to determine significant differences between vancomycin impregnation methods. Primary data were recorded in Microsoft Excel 2019, Version 16.72. The normality of distribution was tested using the Shapiro – Wilk test as the most powerful for small and medium-sized samples ($n < 50$). All quantitative indicators demonstrated normal distribution ($p > 0.05$). Quantitative data were presented as mean ± SD. One-way analysis of variance (ANOVA) was used to compare means between groups, followed by Tukey's post-hoc test to detect pairwise comparisons because:

— all groups had equal sample size;

— pairwise comparisons between all groups were needed;

— Tukey's test provided good control of Type I error maintaining sufficient statistical power.

The area under the pharmacokinetic curve (AUC, Area Under Curve) was calculated as an integral pharmacokinetic indicator of vancomycin elution during the observation period using the trapezoid method. The calculation was produced using GraphPad Prism 9.0 software (GraphPad Software, USA) based on the experimentally obtained drug concentrations at various time points (from 1 to 14 days). The Spearman correlation coefficient was used to assess correlation between vancomycin concentration and the zone of growth inhibition. The significance threshold was set at $p < 0.05$. The determination coefficient (R^2) was used to assess the degree of compliance of the experimental data with the exponential model. All statistical analyses were performed using Microsoft Excel 2019, Version 16.72, IBM SPSS Statistics, Version 23.0.0.0 (USA), MacOS, Monterey 12.2.1.

RESULTS

Analysis of the antibacterial activity of various impregnation methods for *S. aureus* (MRSA) demonstrated comparable efficiency with growth inhibition zones in the range of 20.7–24.7 mm (Table 2) on the first day of observation. Some groups of samples reduced the activity after two days.

Table 2

Dynamics in the diameter of MRSA growth inhibition zones in groups of samples impregnated with different methods

Day	Diameters of MRSA growth inhibition zones using different methods, M ± SD, mm						
	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6	Method 7
1	22.0 ± 0.0	21.7 ± 0.6	24.0 ± 0.0	23.3 ± 1.6	24.7 ± 0.6	20.7 ± 0.6	22.3 ± 1.2
2	16.7 ± 2.3	18.3 ± 0.6	20.0 ± 0.0	20.7 ± 0.6	20.7 ± 0.6	20.7 ± 0.6	22.3 ± 1.2
3	15.3 ± 0.6	16.0 ± 1.7	16.3 ± 1.2	16.7 ± 0.6	15.3 ± 1.5	15.3 ± 0.6	22.0 ± 0.6
4	9.0 ± 0.0	12.7 ± 1.2	14.0 ± 3.5	14.0 ± 3.6	16.7 ± 1.5	15.7 ± 2.5	16.7 ± 1.5
7	b/d*	b/d	12.0 ± 0.0	13.3 ± 1.2	15.3 ± 1.2	15.3 ± 1.2	16.0 ± 0.0
8	b/d	b/d	8.0 ± 0.0	8.7 ± 0.6	13.3 ± 1.2	13.7 ± 0.6	15.0 ± 1.0
9	b/d	b/d	b/d	8.0 ± 0.0	9.3 ± 2.3	11.7 ± 2.5	13.0 ± 13.0
10	b/d	b/d	b/d	b/d	9.0 ± 0.0	8.7 ± 0.6	10.7 ± 2.3
11	b/d	b/d	b/d	b/d	b/d	5.3 ± 4.6	8.0 ± 0.0
14	b/d	b/d	b/d	b/d	b/d	b/d	8.0 ± 0.0

Note: b/d — values below the detection limit of the method (0.0 ± 0.0); data are presented as mean ± standard deviation (M ± SD), $n = 3$ for each group.

Statistical analysis of the results revealed significant differences in the effectiveness of the antimicrobial treatment methods. One-way analysis of variance (ANOVA) showed statistically significant differences in the dynamics of the diameter of MRSA growth zone suppression in samples impregnated with different methods ($F = 4.8192$, $p = 0.0001$). Impregnation in an aqueous solution of antibiotic at atmospheric pressure was the least effective, and increasing the time from 60 min to 24 h had no impact on the duration of antimicrobial activity (AMA) of samples impregnated by methods 1 and 2. Saturation of samples in an aqueous solution of antibiotic under negative pressure did not lead to a significant prolongation of the antistaphylococcal activity. The use of an alcohol solution for impregnation under vacuum conditions was the most effective in comparison with methods 1 and 2 ($p = 0.0283$ and 0.05). The impregnation of 5 min., 60 min. and 24 hours showed no significant effect of time on the duration of AMA of samples groups 5, 6 and 7, respectively (p from 0.9476 to 1.0 in pairwise comparisons). Statistical analysis of the dynamics in the diameters of the MRSA growth inhibition zones using the Tukey post-hoc test showed that this parameter for samples impregnated with methods 5, 6 and 7 was statistically significantly higher than the indicator for method 1; in addition, the indicators for method 7 were significantly better

than those for method 2 ($p = 0.0023$) (Fig. 3). A detailed study of the influence of impregnation parameters on the duration of antimicrobial activity showed that pressure was the most significant factor ($F = 19.9916$, $p < 0.0001$), followed by the type of solvent used ($F = 7.7485$, $p = 0.0006$) and impregnation time ($F = 6.8084$, $p = 0.0014$). All the parameters showed a statistically significant influence on the efficiency of the impregnation process.

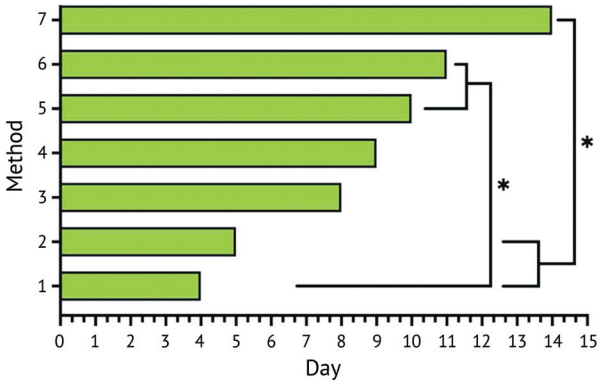

Fig. 3 Duration of in vitro antimicrobial activity of samples with different impregnation methods; * — $p < 0.05$

Analysis of the results of vancomycin elution evaluated by HPLC showed significant differences between the impregnation methods (Table 3). The duration of effective elution ranged from 4 to 14 days, with the least total amount of vancomycin isolated from samples impregnated according to Method 1 using an aqueous solution and atmospheric pressure and the largest amount was isolated from samples saturated in an alcohol solution with a polymer under vacuum conditions (method 7).

Table 3

Dynamics of vancomycin concentration in incubation solution with samples impregnated in different ways

Day	Vancomycin concentration, M ± SD, μg/ml						
	1	2	3	4	5	6	7
1	23658 ± 1183	18747 ± 938	21551 ± 1078	25001 ± 1250	65258 ± 3263	44679 ± 2234	82755 ± 2069
2	258 ± 13	3234 ± 162	4287 ± 215	7839 ± 392	17894 ± 895	17516 ± 876	99578 ± 4979
3	574 ± 29	1234 ± 62	1852 ± 92	2816 ± 141	4648 ± 233	5791 ± 287	92877 ± 4644
4	447 ± 3	246 ± 13	1554 ± 75	1755 ± 88	3780 ± 189	5260 ± 263	45621 ± 2281
5	b/d*	36 ± 2	1354 ± 68	489 ± 25	1957 ± 98	3649 ± 183	9658 ± 483
6	b/d	b/d	823 ± 41	546 ± 28	933 ± 47	2846 ± 143	5710 ± 286
7	b/d	b/d	450 ± 23	480 ± 24	784 ± 39	1120 ± 56	3903 ± 195
8	b/d	b/d	129 ± 7	238 ± 12	417 ± 21	490 ± 25	1244 ± 62
9	b/d	b/d	b/d	38.1 ± 2.0	236.1 ± 12.0	166.9 ± 8.0	987 ± 50
10	b/d	b/d	b/d	b/d	45.0 ± 2.3	69.0 ± 3.5	259 ± 13
11	b/d	b/d	b/d	b/d	b/d	43.0 ± 3.4	54.1 ± 2.7
14	b/d	b/d	b/d	b/d	b/d	b/d	27.9 ± 1.4
Total release	24937 ± 1183.5	23497 ± 954.8	32000 ± 1112.6	39202.1 ± 1314.8	95952.1 ± 3384.9	81629.9 ± 2414.8	342674 ± 7507.3

Note: b/d — values below the detection limit of the method (0.0 ± 0.0); data are presented as mean ± standard deviation (M ± SD), $n = 3$ for each group; total release calculated as the sum of the average values for the observation period, μg/ml.

It was found that increasing the impregnation time in an aqueous solution of the antibiotic at atmospheric pressure from 60 min. to 24 h statistically increased the concentration of vancomycin in the incubation solution throughout the observation period ($p < 0.05$). The most pronounced differences were observed after two and four days ($p = 0.0000$).

Increasing the saturation time of the samples in an aqueous solution under vacuum from 5 to 60 min showed no significant differences in the concentration of the antibiotic isolated on the first day ($p > 0.05$), and the concentration of vancomycin eluted from the samples in method 4 was 1.4–1.8 times higher than in method 3 after 2 to 3 days ($p = 0.0001$). A similar trend was revealed when analyzing the effect of pressure on the intensity of the antibiotic release. A similar trend was revealed when analyzing the effect of pressure on the intensity of antibiotic release. Samples inoculated with methods 1 and 4 showed no significant differences in vancomycin release on the first day ($p = 0.2479$), however, differences in the concentration of the antibiotic in the incubation

solution were statistically significant after two days ($p = 0.0000$). Increasing the impregnation time of samples in an aqueous solution in a vacuum to 24 hours (method 5) showed the highest saturation efficiency of all the methods using distilled water, both in terms of the duration of vancomycin elution and the concentration ($p < 0.05$).

A 60-min impregnation in an alcoholic antibiotic solution (method 6) under vacuum conditions was comparable to 24-h saturation in an aqueous solution (method 5) ($p > 0.05$) in terms of the duration of vancomycin elution and the total mass of antibiotic released. Addition of the polymer to the alcoholic antibiotic solution (method 7) provided the longest elution time (14 days) and the highest amount of released antibiotic among the methods.

The area under the elution curve (AUC) demonstrated significant variability between groups. The minimum value was recorded with method 1 (13108.00), then a consistent increase in the indicator was observed: method 2 (14123.50), method 3 (21224.50), method 4 (26701.60), method 5 (63323.10), method 6 (59333.40). The maximum AUC value was achieved with method 7 (301364.70), which exceeded the indicator by more than 23 times for method 1 and by 5 times for method 6.

The results indicated a significant influence of both impregnation time and pressure on the elution characteristics, with the greatest efficiency achieved when using a combined solvent with the addition of PVP.

Assessment of the correlation between the concentration of vancomycin in the incubation fluid and the zones of inhibition of microorganism growth (Fig. 4) indicated the strongest correlation for samples of group 7 ($r = 0.908$; $p < 0.001$), which indicated a high degree of linear dependence between the parameters. Methods 4, 5 and 6 demonstrated statistically significant correlations ($p < 0.05$), with correlation coefficients of 0.809, 0.822 and 0.723, respectively. Method 3 showed borderline statistical significance ($r = 0.796$; $p = 0.058$), while Methods 1 and 2 did not reach the level of statistical significance ($p > 0.05$) despite high correlation coefficients (0.777 and 0.870, respectively).

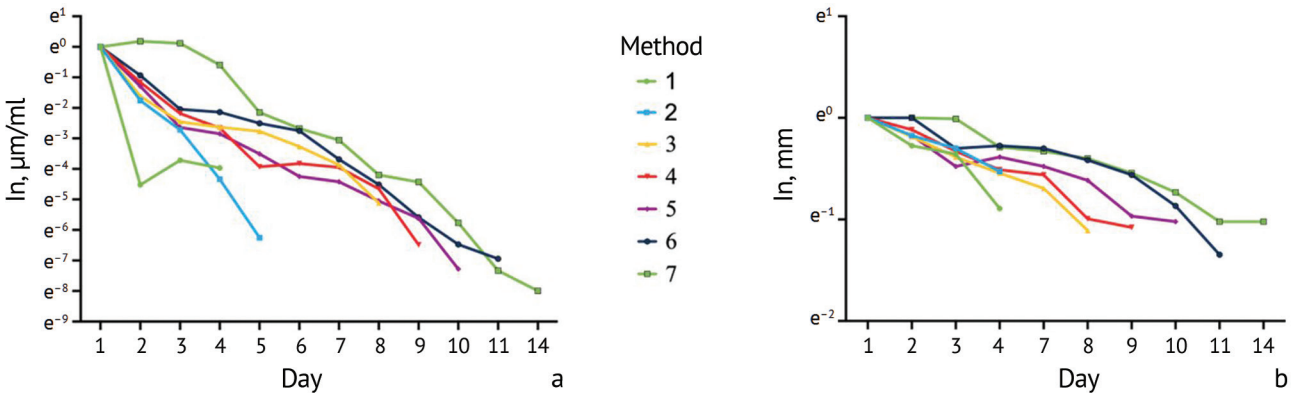

Fig. 4 Dynamics: (a) of vancomycin elution from samples impregnated with different methods; (b) of changes in growth inhibition zones of *S. aureus* (MRSA)

The exponential decay analysis revealed a high degree of confomity of the exponential model for most groups, as confirmed by the values of the coefficient of determination (R^2). Group 2 showed an almost perfect conformity ($R^2 = 0.990$) and the steepest slope of the exponent (–1.509) indicating the fastest decrease in indicators in this group. Groups 3–7 showed similar exponential decay characteristics with $R^2 > 0.91$ and flatter slopes ranging from –0.591 to –0.778. Notably, Group 1 showed the lowest conformity to the exponential model ($R^2 = 0.484$) with a relatively steep slope (–1.111).

The exponential dependence of the antibiotic release kinetics demonstrated the importance of a comprehensive assessment of several key parameters: the duration of antimicrobial activity, the total dose of the released antibiotic and the area under the pharmacokinetic curve (AUC), which together determine the therapeutic efficacy of local antibiotic therapy.

DISCUSSION

Local antibiotic therapy in modern orthopedic surgery demonstrates high efficiency in the prevention and treatment of infectious complications after operations (arthroplasty, vertebrology, maxillofacial surgery, oncology, purulent osteology) [12–15]. Literature analysis indicates significant progress in the development and implementation of various systems for local delivery of antibacterial drugs [16–18].

Polymethyl methacrylate (PMMA), as a traditional material for local antibiotic therapy, provides stable, but not prolonged release of the antibiotic when mixed with vancomycin lasting from 1 to 9 days and up to 14 days in exceptional cases [19–21]. In addition to disadvantages of bone cement including re-intervention [16, 22] and the risk of bacterial colonization with the formation of primary biofilms on the PMMA surface in the first 18 hours [23, 24], only 7 % of the total amount of antibiotic used is eluted into the surrounding tissue [25]. Increased local concentration of the antibiotic through increased mass of the added drug (more than 6 g per 40 g of PMMA) reduces the mechanical strength of the cement, which does not meet ISO standards [26].

As an alternative for creating a local depot, biodegradable carriers (calcium sulfate, biodegradable polyurethane, polyethyl acetate, polylactide-coglycolide and polylactide) are reported [12, 27] with local concentrations being approximately ten times higher than with PMMA but remained below reported cell toxicity thresholds[28]. However, complete resorption of the implants after 6 weeks (range 30–60 days), outpacing the rate of bone formation can lead to the formation of bone cavities, fractures and recurrent infection [29]. The kinetics of antibiotic release from all carriers is characterized by an initial peak release after 48 hours: (9862 ± 1782) ng/ml for PMMA spacers; (38394 ± 7071) ng/ml for PMMA beads, with a subsequent gradual decrease in local concentration [30]. Biodegradable materials demonstrate a faster and more complete release of antibiotics compared to PMMA (CaSO4 during the first three days) [22]. In our series Method 7 demonstrated a smoother exponential decrease ($R^2 > 0.91$, slope from –.591 to –0.778).

Other methods of local antibiotic therapy include direct sprinkling of the wound with antibiotic during surgical treatment or intra-articular administration of antibiotic solutions, which allows for the short-term creation of therapeutic concentrations at the site of surgical intervention (up to 24 hours) without a significant increase in systemic levels of the antibiotic [31, 32].

Allografts impregnated with antibiotics is an alternative method for creating local concentrations of antibiotics, devoid of the above-described disadvantages. The available methods can be divided into several categories depending on the technology of saturating the material with antibacterial drugs. Allograft bone mixed manually in antibiotic solution followed by drying is the simplest and most common method. Impregnation time can vary from a short period of 30–60 min to prolonged 120–180 min. Antibiotic release is uneven, with the maximum concentration in the first 24–48 hours with 40–60 % of the total amount of impregnated antibiotic being released [33–36]. Coraça-Huber et al. reported some of the antibiotic concentrations exceeding the MIC for staphylococci for up to 7 days *in vitro* and for up to 3 days *in vivo* with the allograft immersed in an antibiotic solution [36].

Iontophoresis is an effective method for providing high initial concentrations of antibiotics and maintaining antimicrobial activity for up to 2 weeks [37]. However, the method is characterized by significant variability of results and requires additional studies of the effect on bone structure.

Our study offered an optimized method for impregnating osteosubstituting material as an approach to local antibiotic therapy. As opposed to conventional methods demonstrating limited effectiveness (3–7 days of antimicrobial action), the method provided prolonged release of vancomycin for up to 14 days while maintaining therapeutically significant concentrations using a combination of reduced pressure (7–10 hPa) and an alcohol solution with the addition of PVP. Statistical analysis revealed that the factors explored in the study had impact on the impregnation efficiency.

In our series, we used a complementary approach, combining the microbiological method and HPLC, which allowed us to obtain a more complete picture of the efficiency of the impregnation method developed.

Microbiological analysis plays a role and allows confirmation of the preserved vancomycin AMA after exposure to various physical and chemical factors during the impregnation process.

The presence of zones of inhibition of MRSA growth throughout the observation period suggests that the antibiotic retains its biological activity despite potential structural changes under the influence of pressure, temperature and chemical agents. High correlation between the size of inhibition zones and antibiotic concentration ($r = 0.908$; $p < 0.001$) confirms the release of the active form of the drug from the samples.

HPLC provides a quantitative assessment of the kinetics of vancomycin elution and allows us to determine the exact concentrations of the drug at different time points. This enables detailed pharmacokinetic profiles to be constructed, release uniformity to be assessed and the total antibiotic release to be determined. Analysis of AUC and exponential decay patterns ($R^2 > 0.91$) demonstrates the benefits of the optimized impregnation technique for sustained drug delivery. The osteosubstituting material used in the study is characterized by an optimal porous structure comparable to other materials used in bone grafting. Autografts and allografts have a porosity of 50–90 % with a pore size of 100–500 μm. Synthetic materials demonstrate the following parameters: hydroxyapatite, porosity of 30–90 % with pores of 100–400 μm; tricalcium phosphate, porosity of 35–80 % with pores of 100–400 μm; bioactive glasses, porosity of 20–60 % with pores of 100–500 μm. Biopolymers, such as collagen matrices (porosity of 85–95 %, pores of 50–350 μm) and PLLA scaffolds (porosity of 60–90 %, pores of 100–500 μm) have a developed porous structure [38, 39]. The material architecture provides a large surface area for interaction with antibiotics, which is confirmed by examples of impregnation of synthetic osteosubstituting materials [40–42]. The spongy structure of such a product allows for a high degree of antibiotic adsorption, which ensures stable and controlled release of the drug in combination with optimal impregnation conditions (pressure, solvent, time).

Despite the high local concentrations of the antibiotic achieved during elution, the use of vancomycin remains safe in the context of potential toxicity. At concentrations up to 1000 μg/ml, vancomycin does not have a significant cytotoxic effect on osteoblasts, confirming its safety for bone tissue when applied locally [43]. With high local concentrations of the antibiotic (up to 1400 μg/ml) laboratory tests demonstrate minimal systemic vancomycin levels (less than 1.5 mg/ml) and the absence of nephrotoxicity, confirmed by the absence of statistically significant changes in creatinine and urea levels after surgery ($p > 0.05$) [15]. The exponential nature of the antibiotic release ($R^2 > 0.91$ for methods 3–7) ensures a gradual decrease in local concentrations and increases the material safety.

A longer period of antibiotic release can be expected in clinical scenario when the impregnated allograft is impacted in the bone bed. This is due to the fact that the compression of the material creates additional diffusion barriers that slow down the elution of the drug. This assumption requires further study *in vivo*, but the results obtained allow us to predict a sufficient duration of the antimicrobial effect for the prevention and treatment of peri-implant infection.

The study allowed us to identify the optimal parameters of impregnation of osteosubstituting material based on spongy allobone with vancomycin to ensure prolonged elution of the antibiotic. The obtained results open up prospects for the creation of new protocols of local antibiotic therapy in orthopedic surgery. The method can be used to obtain material for osteosubstitution in infected cases to form a local depot of an antibiotic with controlled release to be employed in the complex treatment of patients with peri-implant infection.

CONCLUSION

Pressure followed by the type of solvent and exposure time were shown to hav the greatest influence on the impregnation efficiency. The combination of reduced pressure, an alcohol solution with the addition of polyvinylpyrrolidone and an optimal exposure time provided the best results being superior to those obtained with conventional methods. The technique allows for a more uniform release of the antibiotic with a gentle slope of the exponential elution curve and ensures the maintenance of therapeutically significant concentrations of vancomycin for two weeks. This exceeds the indicators of standard methods of local antibiotic therapy and creates prerequisites for effective prevention and treatment of peri-implant infection. High correlation between the concentration of the antibiotic and the zones of MRSA growth inhibition confirms the preservation of the biological activity of vancomycin after impregnation, which is essential for clinical use.

Conflict of interest *Not declared.*

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