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In vitro assessment of antimicrobial activity of modified bone xenomaterials

M.V. Stogov[1], D.V. Smolentsev[2], Z.S. Naumenko[1], N.V. Godovykh[1], M.V. Gurin[3], E.A. Kireeva[1], A.E. Lukianov[4], O.V. Diuriagina[1], N.V. Tushina[1]

[1]Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation;
[2]Med-Inzh-Bio Ltd., Penza, Russian Federation;
[3]Cardioplant Ltd., Penza, Russian Federation;
[4]National Research Tomsk Polytechnic University, Tomsk, Russian Federation

Objective To assess antimicrobial characteristics of original bone xenomaterial implants with vancomycin impregnated with different technologies. **Material and methods** Bone xenomatrix was modified with two technologies of vancomycin adsorbed on the surface of the material and vancomycin adsorbed in the volume of the material through intermediate carrier. Antibiotic was impregnated using supercritical fluid extraction with carbon dioxide. Antibiotic release from modified xenomaterial was evaluated and antimicrobial activity against *S. aureus* assessed *in vitro*. **Results** Elution of vancomycin over 24 hours from the material produced with absorption technology was 98 % of baseline content in the matrix. Residual content of antibiotic was 1.75 % on average. The use of intermediate carrier (L/D polylactide isomer) allows for obtaining material with gradual prolong vancomycin release. Major release (68.16 % from baseline content) of vancomycin occurred smoothly over the first 14 days. Bone block eluted 22 % of the residual antibiotic load by 30 days of incubation. The products impregnated with antibiotic using two different technologies exhibited evident antimicrobial activity against *S. aureus*. **Conclusion** Technologies developed to impregnate vancomycin in xenogenic bone matrix are practical to obtain new modified bone grafting material with evident antimicrobial activity.

Keywords: bone xenomaterial, biotechnology, antimicrobial activity, antibiotic release kinetics

INTRODUCTION

A plethora of implant materials of different origin are currently used in a wide range of orthopaedic and trauma applications [1]. New materials are being continuously developed for the needs of clinical practice [2, 3]. Current researches focus on possibilities with materials of natural origin (autologous, allogeneic and xenogeneic) in a variety of modifications including hybrid materials combined with native bone modified with biologically active substances to be applied in trauma and orthopaedics [4–9]. In our opinion, application of xenogeneic materials is underestimated [10–13]. However, manufacturing technologies of the materials allow quality improvement due to decrease in antigen activity with increased mechanical strength and approximate xenogeneic material to autologous, allogeneic materials by osteoinductive characteristics [14, 15]. Additional substances stimulating osteogenesis can be impregnated in bone xenomaterial to improve bioactivity [16–18]. Possibilities with xenomaterial modified with antibacterial agents are not well studied to allow its application in arrest and/or prevention of infection at implantation site along with bone substitution.

Objective To assess in vitro antimicrobial characteristics of original bone xenomaterial implants with vancomycin impregnated with different technologies.

MATERIAL AND METHODS

Technology of xenomaterial manufacturing Bone xenomaterial was obtained from bones of bovine animals under 6 months of age following GOST P ISO 22442 guidelines. Experimental samples were produced from cancellous bone cut in blocks of 20 × 15 × 5 mm with tendons manually removed with a knife. The blocks were pre-washed in running tap water for 2–3 min., processed in 7 % NaCl solution for 12 hours and ultrasound bath with 0.1 % hydrogen peroxide solution for 48 hours. The

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samples were washed in running tap water after each stage of processing and dried on absorbent paper for three hours in plastic packages before extraction. Final deep cleaning was produced with supercritical fluid extraction using CO_2. The extraction parameters used included: P = 350 atm, t = 50 °C, flow 20–22 scfh (cf. 16.9 liquid CO_2 G/min.) and intermittence of time cycles consisting of dynamic and static modes of 25 and 5 minutes, respectively. Lipid extraction was visually recorded and exposed to 25 min. of streaming for accuracy at gas output registration. The samples were weighed and cleaning process was complete with absent changes in weight and the approach warranted the cleaning quality. Osteoplastic material manufacturing with the above technique is described in patent application № 2609201 dtd 14.08.2015. Then experimental bone blocks were impregnated with vancomycin using two different technologies to provide different kinetics of elution from bone material. Control blocks were produced with similar methodology and none of antibiotic impregnation.

Technology of vancomycin impregnation – T1 The technology involved antibiotic impregnation by filling pores of the material with the drug aqueous solution (antibiotic adsorption). Antibiotic solution was prepared using 50 mL of distilled water with concentration of 5 mg/mL. Then bone blocks measuring 20 × 15 × 5 mm were placed in the solution and container with the solution was put into Waters' supercritical fluid extraction reactor (U.S.A.). Carbon dioxide was supplied and parameters set at P = 250 atm, t = 25 °C. Static mode was provided and the blocks were exposed for 3 hours and lyophilized in VaCO-2 dryer (Zirbus, Germaby). Then bone blocks underwent ethylene oxide sterilization followed by vacuum and aeration processing for two days. Final vancomycin content in the bone block was 35 μg.

Technology of vancomycin impregnation – T2 L,D-polylactide was used as an intermediate carrier of the drug to provide delayed elution of vancomycin from bone block with antibiotic absorbed in the volume of material. The carrier smoothly fixed the drug on the surface of bone matrix covering pore walls. Amorphous L,D-polylactide with molecular weight of 18 kDa and inherent viscosity of 0.2 dL/g was used. Polylactide powder (1.8 g polymer) was added to 50 mL ethanol solution with the resulting solution of vancomycin 2 g in 50 mL to provide combined coverage on the surface of bone matrix. Bone blocks were placed in the viscous solution. Supercritical fluid extraction with carbon dioxide was employed to remove the remaining ethanol solution and provide polymer plastification. Container with the solution was placed in Waters' supercritical fluid extraction reactor with carbon dioxide supplied and parameters set at P = 25 MPa, T = 32 °C, flow rate 10 g/min. for 5 minutes to remove ethnol. The static mode was set at P = 120 MPa, T = 32 °C for 60 minutes for polymer plastification and swelling. Finally, the pressure was reduced to atmospheric pressure and the material was frozen and lyophilized. The resultant blocks underwent ethylene oxide sterilization followed by vacuum and aeration processing for two days. Final vancomycin content in the bone block was 100 μg.

Material with fixed antibiotic was placed in phosphate buffered saline (pH 7.4) on rotatory shaker to determine kinetics of eluted antibiotic. The solution was replaced at 30, 60 minutes, 2, 4, 6, 12, 24, 48 hours and every three days onwards and optical density measured at 280 Nm using UVmini – 1240 spectrophotometer (Shimadzu, Japan) [19]. Standard buffered solutions were used to determine calibration relationship between optical density and vancomycin concentration.

Techniques for assessing in vitro antimicrobial activity of bone material Disk diffusion antimicrobial susceptibility test was modified to standardize testing of in vitro antimicrobial activity of bone material (Clinical guidelines "Antimicrobial susceptibility testing", version 2018-03).

Test-microorganism Standard microbe cultures were used to assess antimicrobial matrix characteristics. *Staphylococcus aureus* subsp. *aureus* ATCC 29213 was employed to test antimicrobial characteristics of matrix with vancomycin. Test-microorganisms were accomplished on meat infusion agar by allowing the bacteria to grow for 18 to 24 hours at 37 °C.

Control of microbial cultures Test-microorganism culture underwent quality control. There was the

need to ensure that the strain grown in media was not contaminated by foreign microflora prior to investigation. Culture purity was assessed by inoculations' appearance, pattern and intensity of growth, changes in media color. Gram-stained smears from cultures were tested for purity by microscopic examination. Examination method and assessment of antimicrobial characteristics of osteoplastic matrix was performed according to Clinical Guidelines "Antimicrobial susceptibility testing" (version 2018-03).

Parameters of the method Microbiological growth medium: Mueller-Hinton agar. Inoculum was prepared by direct suspension of pure 18-24-hour bacteria cultures grown on solid non-selective media in sterile isotonic solution. The broth was adjusted to equal the turbidity of a 0.5 MaFarland standard. Incubation: 35 ± 1 ºC, 18 ± 2 hours under normal atmospheric conditions. Assessment of the results. Bactericidal activity of matrix with antibiotic was considered to be evident if the zone of inhibition of test-microorganism growth measured not less than 25 × 30 mm around matrix with antibiotics. The test was conducted in reflective light. The zone of complete inhibition of visible growth was considered while measuring growth inhibition area.

There were total three in vitro series performed. Control samples not impregnated with antibiotics and two series of experimental samples impregnated with antibiotic using T1 and T2 technologies underwent testing procedures. Six bone blocks were tested in each series. Non-parametric χ^2–test was used for statistical analysis of experimental and control series. For calculations, a significance level of 0.05 was adopted.

RESULTS

Kinetics of vancomycin elution from bone blocks Vancomycin eluted from material produced with T1 technology after 24 hours of incubation constituted more than 98 % of baseline matrix content. Antibiotic residues averaged to 1.75 %. Therefore, this impregnation technology allowed obtaining of bone blocks with 'rapid' antibiotic elution. Quantitative parameters of vancomycin elution from bone blocks produced with T2 technology showed considerable differences in that of the samples of the first series (Table 1).

The use of intermediate carrier (L/D-polylactide isomer) with T2 technology was found to provide material with gradual sustained release of vancomycin. Antibiotic elution was recorded on a high equable level during the first 14 days (68.16 % of overall impregnated antibiotic). Antibiotic residues in the bone block averaged to 22 % after 30 day incubation. It can be indicative of a two-phase antibiotic elution: from superficial pores first with degrading polylactide film formed on the bone block surface during processing and retaining the drug and then from deep pores with bioresorbing material.

Assessment of the in vitro antimicrobial activity of bone matrix Bactericidal activity of bone matrix impregnated with antibiotic using T1 and T2 technologies is presented in Table 2. Evident antimicrobial effect against *S. aureus* was seen in the materials impregnated with antibiotic using two different technologies. It should be noted that the zone of inhibited growth of *S. aureus* was significantly greater for T2 bone matrix than that in a similar experiment with T1 blocks.

Table 1

Kinetics of vancomycin elution from the materials produced with T2 technology

Days of incubation	Average release of vancomycin in % from baseline content
2	19.11
5	16.71
8	16.10
11	14.53
14	1.71
17	4.13
20	0.45
23	0.37
26	3.70
30	1.02
Residual content, %	22.00

Table 2

Bactericidal activity of bone matrix impregnated with antibiotic using T1 and T2 technologies against *Staphylococcus aureus*

Bone matrix	Zone of growth inhibition, mm	Bactericidal effect (number of blocks)
Block with vancomycin, T1	28 × 31	evident bactericidal effect (n = 6)*
Block with vancomycin, T2	28 × 35**	evident bactericidal effect (n = 6)*
Control block without antibiotic	0	no bactericidal effect (n = 6)

Note: * – significant differences from control blocks at $p < 0.001$; ** – significant differences from T1 blocks at $p < 0.05$.

DISCUSSION

Xenomaterial is a good alternative to autologous and allogeneic grafts in bone plasty [1, 4]. Improvement of the product is related to highly purified xenogeneic bone and the material with bioactive substances in the matrix facilitating biological and antimicrobial characteristics [14]. Introduction of bioactive substances in bone matrix without loss of activity is complicated and can be addressed with techniques relying on their physical and chemical properties and specific bone matrix structure. Development of the material with T1 technology is based on naturally porous texture of xenogeneic bone to allow antibiotics adsorption from aqueous solutions. Our series showed that the method allowed a certain amount of antibiotic to be impregnated in bone blocks that rapidly eluted from the bone in hydrophilic environment within 24 hours.

Understanding the principle of impregnation as saturation of the natural pores with antibiotic that quickly releases, the question of increasing rate of substance release from the bone and, hence, the efficacy of a sustained action of the product requires further solution. The technology is devised to produce xenomaterial impregnated with antibiotic using supercritical carbon dioxide impregnation technique to provide antibiotic adsorption in the whole volume of the material. Low solubility in a polar compound medium including aqueous antibiotic solution is a major problem encountered with impregnation of biologically active substances using the above technology. Our findings showed that the use of intermediate carrier rather than aqueous solution was a potential solution in the scenario to facilitate slow drug elution. L,D–polylactide was employed as an intermediate carrier of pharmaceutical agent to obtain material with gradual and sustained elution of vancomycin.

The in vitro testing of the material demonstrated kinetics of vancomycin eluted from T1 and T2 materials being 98 % and 19 % in the first 24 hours, respectively, and contributed to effective suppression of growth of *S. aureus*. However, the T2 sample was shown to be more beneficial for clinical goals with sustained antibiotic release and high level of residues (22 % of baseline content). Nevertheless, T1 material could be useful for the cases with high antibiotic concentration to be provided at the site of implantation within a short period of time, for instance, filling in infected bone defects. It is evident that final assessment of biocompatibility and antimicrobial activity of xenomaterial modified with the techniques offered requires further experimental in vivo studies.

CONCLUSION

Technologies developed to impregnate vancomycin in xenogenic bone matrix are practical to obtain new modified bone grafting material with evident antimicrobial activity. Different kinetics of antibiotic release from the material results in differentiated indications to the practical usage improving clinical efficacy of the product.

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Information about the authors:

1. Maksim V. Stogov, Ph.D. of Biological Sciences,
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation,
Email: stogo_off@list.ru
2. Dmitry V. Smolentsev,
Med-Inzh-Bio Ltd., Penza, Russian Federation
3. Zinaida S. Naumenko, Ph.D. of Biological Sciences,
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation
4. Natalia V. Godovykh,
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation
5. Maksim V. Gurin,
Cardioplant Ltd., Penza, Russian Federation
6. Elena A. Kireeva, Ph.D. of Biological Sciences,
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation
7. Alexander E. Lukyanov,
National Research Tomsk Polytechnic University, Tomsk, Russian Federation
8. Olga V. Dyuryagina, Ph.D. of Veterinary Sciences,
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation
9. Natalia V. Tushina, Ph.D. of Biological Sciences,
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation