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Original article

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In vitro evaluation of antimycobacterial activity of bioresorbable implants impregnated with silver nanoparticles and antimycobacterial drugs in the treatment of pulmonary tuberculosis

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Abstract:
Objective: To evaluate the antimycobacterial activity of bioresorbable implants based on polylactide (PLA), polycaprolactone (PCL), and chitosan, impregnated with silver nanoparticles and antituberculosis drugs (ATDs).
Materials and methods. The in vitro study included two experiments, each repeated three times. We studied bioresorbable implants based on PLA/PCL and chitosan, impregnated with silver nanoparticles, silver proteinate, or one of seven ATDs (delamanid, linezolid, bedaquiline, moxifloxacin, capreomycin, isoniazid, amikacin). Antimycobacterial activity was assessed by agar diffusion, in the BD BACTEC™ MGIT™ Mycobacterial System, and by broth microdilution using the *Mycobacterium tuberculosis* H37Rv reference strain.
Results. High concentrations of silver nanoparticles (0.034-0.042 nmol/mL) demonstrated bactericidal activity lasting for 35 days. Silver proteinate exerted a slight inhibitory effect on the growth of *M. tuberculosis*, delaying its growth for only 5 days vs. the control. ATDs, with the exception of delamanid, were released within the first 1-3 days and exhibited bactericidal activity against *M. tuberculosis*, after which their antimycobacterial activity decreased.
Conclusions. Implants impregnated with silver nanoparticles provided a prolonged bactericidal effect against *M. tuberculosis*, which justifies further study of these implants.

Keywords: pulmonary tuberculosis, bioresorbable implant, silver nanoparticles, antituberculosis drug, extrapleural plombage.

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Introduction

Surgery remains an integral component of a comprehensive approach to the treatment of pulmonary tuberculosis. The spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* strains noticeably diminishes the efficacy of standard chemotherapy, which, according to global reports from the World Health Organization, does not exceed 71% [1]. Under these conditions, surgical treatment methods are particularly important, achieving success rates of up to 96.2% [2].

Destructive forms of pulmonary tuberculosis are often accompanied by substantial destruction of lung tissue, with the formation of cavities (caverns) that cannot be treated without surgery. Extrapleural plombage, a procedure in which an implant is placed into the created extrapleural cavity causing collapse of the affected lung area, is a promising approach to thoracic surgery. This method is more effective and less traumatic than conventional thoracoplasty: it provides the required extent of collapse without significant cosmetic defects in the chest wall [3, 4].

However, the widespread adoption of extrapleural plombage in clinical practice is hindered by the lack of an optimal plombage material. Historically, various substances have been used for this purpose (from paraffin oils and silicone balls to autograft), each with significant drawbacks, including rapid resorption, implant migration, infectious complications, and graft rejection [5]. An ideal plombage material must meet several requirements, including biocompatibility, biodegradability, nontoxicity, the ability to maintain the required volume for a specified period, and, most importantly, its local antimicrobial effect on the pathogen of pulmonary tuberculosis [6].

Contemporary advances in biomaterials and regenerative medicine offer new opportunities for solving this problem. Bioresorbable porous materials based on polylactide (PLA), polycaprolactone (PCL), and chitosan have been developed. They exhibited high biocompatibility and controlled degradation in in vivo studies [5, 7]. The porous structure of

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such materials facilitates their impregnation with drugs with subsequent prolonged release at the implantation site. Creating a local antimycobacterial environment can significantly improve treatment efficacy and reduce the risk of postoperative complications [6, 7].

Along with traditional antituberculosis drugs (ATDs), silver preparations are considered potential antimicrobial agents. Amid the global antibiotic resistance crisis, silver nanoparticles attract the attention of researchers due to their broad spectrum of antimicrobial activity and proven activity against mycobacteria. Their mechanism of action involves disruption of cell wall integrity, generation of reactive oxygen species, and interaction with intracellular structures, thereby making the development of resistance unlikely [8].

Objective: To evaluate the antimycobacterial activity of bioresorbable implants based on PLA, PCL, and chitosan, impregnated with silver nanoparticles and ATDs.

Materials and methods

The study was conducted at the National Medical Research Center for Phthisiology, Pulmonology and Infectious Diseases of the Russian Federation Ministry of Healthcare in collaboration with the Kurchatov Institute as part of a research program on developing 3D implants for thoracic surgery.

Manufacture of implant samples with silver nanoparticles. At the Kurchatov Institute, samples of the outer shell of 3D porous composite materials made of PLA/PCL with a chitosan shell were synthesized. Silver nanoparticles were obtained by in situ reduction from a chitosan solution. To implement this method, a 1% solution of Primex 43000 chitosan with 1.04% silver nitrate ($AgNO_3$) (ReaChem 99.95, GOST 1277-75) in 2% acetic acid was prepared and incubated at 60 °C for 5 days. This procedure yielded silver nanoparticles, mostly spherical in shape, with a predominant fraction of 2-20 nm in size, and aggregates of 25–200 nm in size, stabilized by a polysaccharide shell.

We chose silver proteinate as a water-soluble compound for comparative evaluation of the antimycobacterial activity of various forms of silver. To obtain silver proteinate samples, 3 mL of an aqueous silver proteinate solution with concentrations of 66.7 mg/mL (Chitosan Pr, final concentration of 20 mg/mL) and 133.3 mg/mL (Chitosan Pr2, final concentration of 40 mg/mL) were added to a chitosan-glutaraldehyde solution. The shell without added antimicrobial agents served as a control. The morphology of the resulting materials was studied by scanning electron microscopy, confirming the preservation of the porous structure for all material types. Silver nanoparticle concentrations are expressed in nmol/mL due to the colloidal nature of the dispersion, while mg/mL units are used for silver proteinate, as it represents a molecular compound.

Manufacture of implant samples with antituberculosis drugs. The study utilized chemically pure substances of 7 ATDs manufactured by Nanjing Pars Biochem Co., Ltd. (China): delamanid, linezolid, bedaquiline, moxifloxacin, capreomycin, isoniazid, and amikacin. The ATDs were impregnated into a PLA/PCL copolymer implant core to achieve prolonged or modified release of the active ingredients at various concentrations, as presented in *Table* 1.

The choice of relatively low concentrations of antimicrobial agents was driven by two factors. First, the small volume of the implants and the high porosity of the core material (to ensure biocompatibility and controlled biodegradation) limited the maximum possible dose of the impregnated substance without compromising the structural integrity of the porous matrix. Second, the use of two different concentrations for each drug was methodologically justified by the need to study the relationship between the impregnated substance dose and the duration of its release, with the lower concentration serving as a control to assess the retention of antimycobacterial activity at minimal doses.

Modeling drug release conditions. Implant samples with ATDS were placed in phosphate-buffered saline (PanEco, Russia) to simulate physiological conditions in lung tissue. For each drug, there were three test samples and one control. The buffer in the test samples was changed daily, with an aliquot (eluate) collection to assess antimycobacterial activity. The control sample buffer was not changed to assess the stability of the drug in solution. The control points were Days 1, 3, 7, 14, 21, and 28. The experiment lasted 28 days.

Microbiological methods. To determine antimycobacterial activity, a pure culture of the control strain, *M. tuberculosis* H37Rv, sensitive to all investigated drugs, was employed. A suspension was prepared in 0.9% sodium chloride according to a McFarland turbidity standard of 0.5 units (1×10^7 CFU/mL).

Agar diffusion method. We dispensed 25 mL of Middlebrook 7H11 agar medium (BD, USA) into Petri dishes. After agar medium solidification, 100 µL of the *M. tuberculosis* H37Rv suspension was applied evenly over the entire agar surface into a continuous lawn using a Drigalski spatula. Silver nanoparticle-embedded implant samples were placed on the agar surface. The chitosan shell samples were disc-shaped, 4 mm in diameter and 2 mm thick. They were incubated at 35±2 °C for at least 14 days. Results were recorded during bacterial lawn formation by measuring the diameters of growth inhibition zones in millimeters.

Table 1. Concentrations of antituberculosis drugs (ATDs) impregnated into polylactide/polycaprolactone-based implants

ATD	Concentration, mcg	
	1	2
Delamanid	0.015	0.005
Linezolid	0.03	0.015
Bedaquiline	0.015	0.005
Moxifloxacin	0.045	0.03
Capreomycin	0.0125	0.01875
Isoniazid	0.00375	0.005
Amikacin	0.00625	0.0125

The concentration is indicated as the mass of the drug in 1 implant.

BACTEC MGIT 960 liquid culture system for determining antimycobacterial activity. We used test tubes containing 10 mL of Middlebrook 7H9 liquid medium and 750 µL of OADC growth supplement (Middlebrook OADC Enrichment, BD, USA). Implant samples with ATDs and silver nanoparticles, along with 100 µL of *M. tuberculosis* H37Rv suspension, were added to the test tubes. For tubes with eluate, we used a 1:5 dilution of the inoculum (1×10^7 CFU/mL), and a 1:500 dilution (1×10^5 CFU/mL) for the control tube. Incubation was continued until the control tube had 400 growth units. The result was assessed by the growth unit number: ≤100 = sensitive (S), >100 = resistant (R).

Broth microdilution method. We were adding 100 µL of Middlebrook 7H9/OADC medium (BD, USA), the eluate to be tested, and the prepared inoculum to each well of a 96-well plate. The plates were incubated at 35±2 °C for 14-21 days, followed by visual assessment of the microbial colony growth.

For all methods, we established the following controls: negative control of inoculation conditions regarding possible contamination, positive control of the *M. tuberculosis* H37Rv strain growth, and carrier control regarding the absence of antimicrobial activity (chitosan or PLA/PCL without antimicrobial agent).

Results

Antimycobacterial activity of composite implants with silver nanoparticles

A preliminary assessment of the controls confirmed the validity of our experimental results. No microbial growth was observed in any of negative controls, indicating entirely aseptic conditions. Uniform growth of *M. tuberculosis* H37Rv was noted in all positive controls, while culture growth was not inhibited on plates containing the implant without the active agent, thereby confirming the absence of the carrier's intrinsic antimycobacterial activity. Formation of a visible bacterial lawn in the positive control on Middlebrook 7H11 medium occurred on Day 14 of incubation. In the BACTEC MGIT 960 system, the control tube acquired 400 growth units by Day 6.

Using agar diffusion method for implants impregnated with silver nanoparticles revealed growth inhibition zones of 7 mm for both concentrations (0.017-0.021 and 0.034-0.042 nmol/mL). It should be noted that the growth inhibition zones for implants impregnated with silver nanoparticles were characterized by more distinct boundaries vs. implants containing silver proteinate.

We obtained very important results for antimycobacterial activity in the BACTEC MGIT 960 liquid culture system. In tubes containing a control implant without the active agent, culture growth began on Day 6. When using implants with a low concentration of silver nanoparticles (0.017–0.021 nmol/mL), the growth onset shifted to Day 16, indicating a significant antimicrobial effect. The most pronounced result was demonstrated by implants with a high concentration of silver nanoparticles (0.034–0.042 nmol/mL) with complete inhibition of *M. tuberculosis* H37Rv culture growth observed within the standard observation timeframe.

To assess the duration of antimycobacterial activity, tubes containing implants with a high concentration of silver nanoparticles were left in the BACTEC MGIT 960 system for extended observation. A positive result in the form of the beginning of culture growth was recorded on Day 35, which indicates a prolonged bactericidal effect and gradual elution of silver from the implant for over a month (*Table 2*).

Antimycobacterial activity of silver proteinate implants. A study of implants impregnated with silver proteinate at concentrations of 20 and 40 mg/mL demonstrated a growth inhibition zone of 7 mm for both concentrations, thereby indicating weak antimycobacterial activity. When tested in the BACTEC MGIT 960 system, the onset of *M. tuberculosis* H37Rv growth was detected on Day 11, regardless of the drug concentration (vs. on Day 6 in the control). Hence, silver proteinate provided only a slight growth delay (5 days), compared with the control, which precludes its use as an effective antimycobacterial agent for implant impregnation without a significant increase in concentration.

Release of antituberculosis drugs from polylactide/polycaprolactone-based implants. The antimycobacterial activity of the eluates was assessed by two methods: broth microdilution and the BACTEC MGIT 960 system. Results obtained by both methods were consistent at all control points for each of the tested drugs. Analysis of the eluates obtained from implants impregnated with antimycobacterial drugs revealed an important pattern: for all tested drugs, antimycobacterial activity in the eluates was detectable within the first 1-3 days of the experiment. In control samples, where the buffer remained unchanged throughout the experiment, antimycobacterial activity was maintained throughout the entire 28-day observation period (*Table 3*).

Table 2. Antimycobacterial activity of implants with various forms of silver

Specimen	Growth inhibition zone, mm	Onset of growth, days BACTEC)
Chitosan (control)	0	6
Silver nanoparticles, nmol/mL		
0.017–0.021	7	16
0.034–0.042	7	35*
Silver proteinate, mg/mL		
20	7	11
40	7	11

* Complete suppression of bacterial growth within the standard time frame (28 days); growth was recorded in the course of extended observation.

Table 3. Dynamics of antimycobacterial activity of eluates released from polylactide/polycaprolactone-based implants

ATD	Experiment, day						Control, day 28
	1	3	7	14	21	28	
Capreomycin	+	+	–	–	–	–	+
Isoniazid	+	+	–	–	–	–	+
Amikacin	+	+	–	–	–	–	+
Moxifloxacin	+	+	–	–	–	–	+
Bedaquiline	+	–	–	–	–	–	+
Linezolid	+	+/–*	–	–	–	–	+
Delamanid	–	–	–	–	–	–	–

Antimycobacterial activity: +, presence; –, absence; *, activity depended on the concentration of impregnated agent; 0.03 µg – active, 0.015 µg – inactive.

Unlike other drugs, delamanid exhibited no antimycobacterial activity at any of the control points, including the control sample without buffer change. This point to the fact that this drug is inappropriate for impregnation into implants, likely due to its chemical structure.

An additional study using reduced doses of the drugs (0.005 μg of amikacin, 0.01 μg of capreomycin, 0.003 μg of isoniazid) did not improve the results, and the elution profile remained similar, implying that activity was detectable only in the first 3-7 days. This finding confirmed that the limiting factor is not only the drug concentration but also the structural properties of the implants.

Discussion

The results of our study clearly demonstrate fundamental differences in the efficacy of the two studied antimicrobial agent delivery systems for potential use in pulmonary tuberculosis surgery.

Implants containing silver nanoparticles yielded the most pronounced results. Prolonged antimycobacterial activity over 35 days using high nanoparticle concentrations (0.034–0.042 nmol/mL) is of high clinical interest, since this period covers a critical phase of the postoperative stage, when the risk of infectious complications is highest. Several studies have shown that the mechanism of antimycobacterial action of silver nanoparticles is mediated by damage to the cell wall and membrane of mycobacteria, the production of reactive oxygen species, interaction with thiol groups of proteins, and the inhibition of DNA replication [8, 9]. The multiple targets of action make the development of resistance unlikely, which is especially important with respect to treating MDR and XDR tuberculosis.

A significant advantage of chitosan as a carrier is its intrinsic biological activity. Chitosan has hemostatic, regenerative, and immunomodulatory properties, which may further facilitate healing at the implantation site [10]. Moreover, our study confirmed the absence of intrinsic antimycobacterial activity in chitosan, which contributed to an accurate assessment of the input of impregnated substances to the overall therapeutic effect.

In our study, silver proteinate, despite its antiseptic properties and widespread use in medicine, demonstrated weaker antimycobacterial activity, compared with the control. A 5-day delay in culture growth (11 days vs. 6 days in the control) is insufficient for considering silver proteinate an effective antimycobacterial agent for implant impregnation. This result was probably caused by the differences in the mechanisms of action, as silver proteinate is a protein complex that limits the release of silver ions and reduces their bioavailability, compared with free nanoparticles [11].

Results of studies of implants with ATDs revealed rapid and almost complete elution of the impregnated agents within the first 1–3 days. Such elution profile is especially important during the first 24 hours after surgery for preventing early specific postoperative complications, but otherwise is insufficient for treatment and requires systemic chemotherapy [12].

The observed phenomenon may be explained by several factors. First, the high porosity of the material, which ensures its biocompatibility and biodegradability, simultaneously creates conditions for rapid diffusion of impregnated drugs into the environment. Second, hydrophilic ATDs may not bind sufficiently strongly to the hydrophobic PLA/PCL polymer matrix. Third, the pore size and their interconnectedness may contribute to rapid washing out of drugs upon contact with an aqueous environment [13, 14].

A complete lack of delamanid activity was observed even in control samples without buffer change. Delamanid is a nitroimidazole derivative with pronounced hydrophobicity, which likely leads to its strong sorption on the polymer matrix without subsequent release into the aqueous medium. An alternative hypothesis is chemical degradation of the drug upon contact with the implant material. However, the latter hypothesis requires further study using analytical chemistry methods [15].

A comparative analysis of the studied systems demonstrated a greater potential of implants containing silver nanoparticles for further study in regard to the surgical treatment of pulmonary tuberculosis.

This study is pilot in its nature and has several limitations. For instance, just a single reference strain, *M. tuberculosis* H37Rv, was used, and the in vitro drug release conditions did not fully simulate pharmacokinetics in lung tissue.

Promising areas for further research include the development of composite materials with barrier layers that slow the diffusion of active agents; microencapsulation of drugs before impregnation; chemical modification of the pore surface to enhance drug binding to it; and development of core-shell structures with different layer degradation kinetics.

Conclusion

Our study evaluated the antimycobacterial activity of bioresorbable implants based on PLA, PCL, and chitosan impregnated with silver nanoparticles and antimicrobial agents. Implants impregnated with silver nanoparticles exhibited a more prolonged activity against *M. tuberculosis*. The use of linezolid, bedaquiline, moxifloxacin, capreomycin, isoniazid, and amikacin for impregnation resulted in the rapid establishment of a bactericidal concentration outside the implant within the first three days, which is crucial for preventing specific complications associated with the progression of tuberculosis in the early postoperative period.

Our results showed fundamental differences in the antimycobacterial activity of the studied systems and allowed identifying directions for further research, including optimization of the silver nanoparticle concentration, the development of structures with barrier layers for prolonged release of ATDs, and the study of the combined use of silver nanoparticles and ATDs.

Conflict of interest

None declared by the authors.

Author contributions

All authors contributed equally to the preparation of the manuscript.

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Compliance with ethical principles

The study protocol was approved by the local Ethics Committee in accordance with the principles of the Declaration of Helsinki.

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