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Assessment of Antibacterial and Antifungal Activity of a Biocomposite Based on Nanocellulose in Vitro

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Abstract

Introduction: Biomaterials which used in the treatment of osteomyelitis, should not only fill in bone defects, but also be a "tool" for local delivery of antibiotics. The bone substitute is the biomaterial of human, animal, plant or synthetic genesis, implanted into the body to restore and strengthen bone substance. The creation of the latest nanocompositional materials today is the main direction of the development of science and technology. Nanocellulose (NC) and biocomposits based on it became one of the most promising "green" materials due to its unique properties. Our hypothesis: nanocellulose-based biocomposites with added antibiotics show antimicrobial effectiveness in vitro.

Methods: For preclinical testing, three samples of the NC were provided: nanofibrillar, nanocrystalline, microfibrillar nanocellulose. The microbiological purity was studied, the minimum-inhibitory concentration of the antibiotic, and the study of the antibacterial and antifungicide effect of biocomposite samples by the method of museum tests of Staphylococcus aureus (ATCC 6538), Escherichia coli (ATCC 25922), Bacillus subtilis (ATCC 6633), Candida Albicans (ATCC 10231) by measuring the diameter of the growth retardation zone.

Results: Biocomposites based on nanocellulose, saturated with antibiotics, demonstrate high antibacterial and antifungicidal potential. Further experimental studies are required by their ability to uniform and slow controlled release of the active substance, along with the effective suppression of the growth of microorganisms and a prolonged effect, which would allow them to be considered as an effective local transport system. In the future, it is planned to implant biocomposites with an antibiotic in the treatment of osteomyelitis in vivo.

Keywords: nanocellulose, biocomposite, chronic osteomyelitis, Staphylococcus aureus, treatment, bone substitutes.

Introduction

The problem of osteomyelitis treatment consists in the absence of such a filler, which, in addition to bone-forming properties, was a system for drug delivery. High indicators of the success of the antimicrobial treatment of most infections have not yet been achieved with bone infections due to the physiological and anatomical characteristics of the bones [1]. One of the current areas of reconstructive orthopedics is biotechnology. Multiple variability from autocracy to synthetic biopolymers indicates the enduring importance of finding such an implant that would meet all relevant characteristics [2].

The bone substitute is the biomaterial of human, animal, plant or synthetic genesis, implanted into the body to restore and strengthen bone substance [2]. The biomaterials used in the treatment of osteomyelitis should not only fill in bone defects, but also be a "tool" for local delivery of antibiotics [3]. The creation of the latest nanocompositional materials today is the

main direction of the development of science and technology [4]. Nanocellulose (NC) and biocomposites based on it became one of the most promising "green" materials due to its unique properties [5]. This polymer is divided into three types depending on the origin: nanocrystalline, nanofibrillar and bacterial. From natural cellulose, nanofiber (nanofibrillar cellulose - NFC) can be distinguished by multistage chemical and mechanical processing. At the same time, nanofibrils are formed with a diameter of 5 to 100 nm and a length of 200 nm to several tens of microns. The specific area of the surface of such nanofibers can reach 100-200 m2/g. It is the high specific surface, as well as the unique mechanical strength of individual fibers (the Yung module reaches 200 GPa, such values of the module are characteristic of steel) allows the use of nanofibrillar cellulose as a reinforcing component of composite materials [6].

Cellulose, due to its outstanding characteristics in the seal, is traditionally used in pharmaceuticals, where it acts as an effective auxiliary component for the formation of dense matrix structures with drugs, as well as in the production of high - quality pills for oral use. Various forms of nanocellulous carriers of drugs are classified according to three groups: microspheres (microparticles), hydrogels (gels) and nanomembranes (films) [7]. The use of natural nanocellulose in the drug delivery system is a promising direction, however, there are many unresolved issues, especially relating to the influence and control of the drug release, as well as the interaction between drug molecules and nanocellulose.

There are still open questions about the effectiveness of nanocellulose in relation to bone tissue infection, there is no literature data on its use in the treatment of osteomyelitis in vivo. This study is one of the stages of preclinical testing and aims to evaluate the antibacterial and antifungal activity of a nanocellulose-based biocomposite in vitro.

Methods

The object of the study was nanocellulose-based biocomposites provided by the Department of Physical and Colloidal Chemistry of the Federal State Autonomous Educational Institution of Higher Education «Russian State University of Oil and Gas (National Research University) named after I.M. Gubkin», Moscow. Biocomposites are the result of multi-stage chemical and mechanical processing of nanocellulose.

The study of the microbiological purity of the biocomposite

The microbiological purity of the biocomposite was estimated by direct sowing: 1 g of the substance was mixed with 9 ml of physiological solution, then a series of at least 3 serial tenfold dilutions of tested samples were prepared, of which, subsequently, 1 ml of the resulting mixture was taken by the dispenser. Sowing was made in sterile cups of Petri. As nutritional mediums used: yellow-salt agar (identification of Staphylococcus aureus), agar McConkey in order to release gram negative microorganisms, a thioglycol medium for anaerobes (incubation was carried out in the CO2 incubator "Memmert", chromogenic agar for mushrooms. Cup incubation was carried out in a thermostat at 36 ± 1ºC for 24 hours. To determine the mushrooms, the cup was cultivated at a temperature of 26 ± 1°C for 5 days.

Determination of the minimum inhibitory concentration of a biocomposite with the addition of antibiotics in relation to Staphylococcus aureus

The most common pathogen of chronic bone infection, according to literary data, is Staphylococcus aureus, which is associated with pronounced virulence factors:

• α-toxin, which destroys the owner's macrophages membrane, which initiates their death;

• leukotoxins lukSE, HlgAB and HlgCB, LukED and LukAB, leading to the polarization of macrophages, which further leads to the formation of a weakly inflammatory phenotype and the corresponding immune response;

• staphylococcal complement inhibitor (SCIN), which blocks classic complement paths;

• protein inhibiting Staphylococcal chemotaxis (CHIPS);

• Squeezing factor A (ClfA) and cell adhesion protein (Eap);

• Change in the genotype due to "quorum sensing" [8].

The determination of the minimum-inhibitory concentration of gentamycin was carried out in relation to the Staphylococcus aureus of the museum ATCC 6538 and clinical strains (65245, 65246, 64238), provided by the research laboratory, as follows: 1 ml of culture and various antibiotic concentrations – 20 µl, 40 µl, 60 µl, 80 µl, 100 µl (0.1 ml), 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml were introduced to 3 ml of meat-peptone broth. Control was the broth and broth with a culture without an antibiotic. In the future, tubes were incubated in the thermostat at 36 ± 1 ºC during for 24 hours. The results were analyzed using spectrophotometry "Tecan" with the measurement of optical density.

Preparation of samples

As subjects, the materials were provided: nanofibrillar (I), nanocrystalline (II), microfibrillar nanocelluloses (III). They were studied both in monovariant (1 gr) and with the addition of antibiotics (0,1 ml of gentamycin, 0,1 nistatin, also calcium gluconate (0.5 g of the studied sample + 0.5 g of calcium gluconate, t. e. in the ratio of 1:1). Calcium is the main mineral that gives bone tissue strength: from 1 kg of calcium contained in our body, 99% are in bones. Compositions of the studied samples with designations presented in Table №1. Standard discs with gentamicin and nystatin were used as controls.

Table 1 Compositions of the studied samples with designations

№	The composition of the studied sample	Designation
1	Nanofibrillar NC	I
2	Nanofibrillar NC + gentamycin	I + G
3	Nanofibrillar NC + gentamycin + Ca	I + G + Ca
4	Nanofibrillar NC + Ca	**I + Ca**
5	Nanofibrillar NC + nystatin	I + Ns
6	Nanofibrillar NC + nystatin + Ca	I + Ns + Ca
7	Nanocrystalline NC	II
8	Nanocrystalline NC + gentamycin	II + G
9	Nanocrystalline NC + gentamycin + Ca	II + G + Ca
10	Nanocrystalline NC + Ca	II + Ca
11	Nanocrystalline NC + nystatin	II + Ns
12	Nanocrystalline NC + nystatin + Ca	II + Ns + Ca
13	Microfibrillar NC	III
14	Microfibrillar NC + gentamycin	III + G
15	Microfibrillar NC + gentamycin + Ca	III + G + Ca
16	Microfibrillar NC + Ca	III + Ca
17	Microfibrillar NC + nystatin	III + Ns
18	Microfibrillar NC + nystatin + Ca	III + Ns + Ca
19	Gentamycin	G
20	Nystatin	Ns

Determination of antimicrobial activity of biocomposites in relation to museum tests

The study of antimicrobial and antifungal activities of biocomposites was determined by Staphylococcus aureus (ATCC 6538), Escherichia Coli (ATCC 25922), Bacillus Subtilis (ATCC 6633), Candida albicans (ATCC 10231), according to the State Pharmacopoeia Republic of Kazakhstan.

Gentamicin was chosen as an antibacterial agent, both a bactericidal antibiotic of a wide spectrum of action against both many grampositive and gramnegative bacteria.

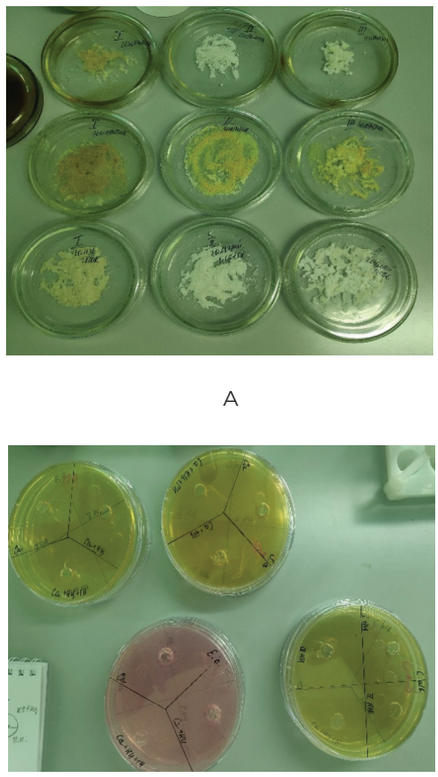
Nystatin - a polyene macrolide, was chosen as a fungistatic and fungicidal drug for yeast and yeast -like mushrooms, including Candida albicans.

The microbial suspension of test culture was prepared on a physiological solution in a concentration corresponding to the turbidity standard 0.5 Mac Farland. Next, the suspension was evenly applied to the surface of the agar Muller-Hinton in the Petri cup and dried. The studied samples of 1 g, imprinted 0.1 ml of gentamycin and 0,1 nystatin, were placed in holes (0.6*0.5 cm) of infected agar (Figure 1 a) and b). Cups were incubated in a thermostat at 36 ± 1 ºC for 24 hours (and at 24 ± 1 ºC with Candida albicans for 5 days). The result was evaluated by the presence of the diameter of the delay in the growth of the test culture around the test sample. The results were recorded on the basis of measuring the diameter of the zone (if any) from the edge of the sample to the edge of the growth retardation zone using a line-show designed for these purposes (Himedia PW297, India).

Statistical analysis

For statistical processing, the STATISTICA 6 and IBM SPSS Statistics, Version 24 program were used. The analysis of the data was carried out at the level of significance α = 0.05. The verification of the normal distribution of quantitative data was carried out using the criterion of the Shapiro – Wilk (S-W). According to the Shapiro-Wilk criterion, p <0.01, which is less than the significance level (0.05), which indicates that the distribution of the indicator is abnormal. The Shapiro-Wilk criterion was chosen because the sample size is less than 50 (n>50).

The description of quantitative data was carried out on the basis of: average value and apartment. For comparison of independent groups, the Mann-Whitney U criterion. The Mann-Whitney criterion was chosen as the research method, since the data are quantitative, the distribution is different from normal, the samples are independent, and the significance level is α=0.05.



A

B

Figure 1 – The microbial suspension of test culture placed in holes (0.6*0.5 cm) of infected agar

Results

The results of the study of the microbiological purity of biocomposites

With a threefold direct sowing of ten -time serial dilutions of subjects, the results showed that the presented samples are microbiologically pure. According to the State Pharmacopoeia of the Republic of Kazakhstan, requirements are applied to implants as drugs for parenteral use.

Medicines for parenteral use (parenteral drugs) are sterile drugs designed for administration by injection, infusion or implantations into the human or animal body.

The submitted samples are sterile and can be used as bone implants to fill osteodefects.

The results of determining the minimum inhibitory concentration of a biocomposite with the addition of antibiotics in relation to Staphylococcus aureus

When determining the minimum inhibitory concentration of gentamycin (20, 40, 60, 80 µl), a statistically higher optical density indicator (p = 0.012) was noted, respectively, with an increase in the concentration of gentamycin (0.1, 0.2, 0.4, 0.6 0.8 ml) a statistically lower optical density indicator (p = 0.012) was noted (Figure 2).

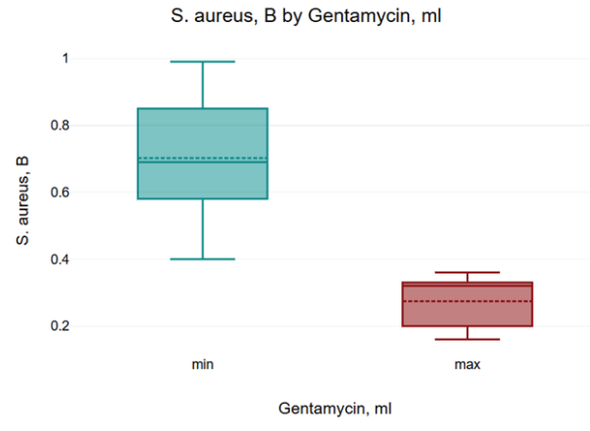


Figure 2 – Frequency of Hepatitis

Determination of antimicrobial activity of biocomposites in relation to museum tests

According to experimental data, the tested samples do not have a bactericidal effect as a monocomponent (without an antibiotic) in relation to all studied microorganisms (samples I,

Table 2 Diameter of the biocomposite growth retardation zone in relation to museum test strains

№	Examined samples	The diameter of the growth delay zone, mm (M±m)			
		S. aureus ATCC 6538	E. coli ATCC 25922	B. subtilis ATCC 6633	C. albicans ATCC 10231
1	I	0±0,0	0±0,0	0±0,0	0±0,0
2	I + G	35,5±1,5	26,8±1,2	35,5±1,5	0±0,0
3	I + G + Ca	35,4±1,6	27,5±0,5	38,1±0,9	0±0,0
4	I + Ca	0±0,0	0±0,0	0±0,0	0±0,0
5	I + Ns	0±0,0	0±0,0	0±0,0	30±1,0
6	I + Ns + Ca	0±0,0	0±0,0	0±0,0	32,1±0,9
7	II	0±0,0	0±0,0	0±0,0	0±0,0
8	II + G	36,4±1,6	27,1±1,9	34,4±1,6	0±0,0
9	II + G + Ca	32,2±1,8	27,5±0,5	38,3±0,7	0±0,0
10	II + Ca	0±0,0	0±0,0	0±0,0	0±0,0
11	II + Ns	0±0,0	0±0,0	0±0,0	31,3±0,7
12	II + Ns + Ca	0±0,0	0±0,0	0±0,0	31,3±0,7
13	III	0±0,0	0±0,0	0±0,0	0±0,0
14	III + G	34,5±0,5	25,4±1,6	37,1±0,9	0±0,0
15	III + G + Ca	37,4±0,6	27,5±0,5	40,3±0,7	0±0,0
16	III + Ca	0±0,0	0±0,0	0±0,0	0±0,0
17	III + Ns	0±0,0	0±0,0	0±0,0	31,3±0,7
18	III + Ns + Ca	0±0,0	0±0,0	0±0,0	31,3±0,7
19	G	15±0,8	16±0,5	19±0,9	0±0,0
20	Ns	0±0,0	0±0,0	0±0,0	12±0,6

II, III, I+SA, II+SA, III+SA). Statistical processing of the results was carried out by the method of parametric analysis Student's t-test with the calculation of the arithmetic and standard error (Table №2).

However, biocomposites impregnated with an antibiotic showed high antibacterial (samples I+ G, II+ G, III+ G) and antifungicidal (samples I+Ns, II+Ns, III+Ns) effect. The addition of calcium in some cases enhances the effect of drugs (samples I+ G +Ca, II+ G +Ca, III+ G +Ca in relation to E. soli, B. subtilis; biocomposite I+Ns+Ca against C. albicans) (Table №2). As a result, nanocellulose composites with antibiotics showed higher potential of 1.5-2 times, than standard control discs.

Discussion

In the study of microbiological purity, the studied samples of biocomposites based on nanocellulose were sterile, which was not expressed in the absence of the growth of microorganisms on nutrient media. The sterility of the samples makes it possible to use them in the operating room for filling out osteodefects in vivo.

As a mono-matter, samples of nanocellulose did not have bactericidal properties, which corresponds to the data of affordable literature. This is due to its physico-chemical properties [9, 10]. This can be achieved by functionalizing it or incorporating antimicrobial agents.

As the results of our study showed, biocomposites based on nanocellulose, saturated with antibiotics, demonstrate high antibacterial and antifungicidal potential, which is confirmed by a number of scientific publications (Table №3).

Table 3 NC variations for biomedical applications

Substance	Pathogen	Application	Reference
A composite material based on poly(3-hydroxybutyrate) and nanocellulose coated with zinc oxide (ZnO) nanoparticles	Escherichia coli and Staphylococcus aureus	Packaging Applications	10
Mixing colloidal silver nanoparticles with cellulose nanofiber (CNF) and depositing this hybrid coating (CNF/Ag)	Escherichia coli (Gram-negative) and Staphylococcus aureus (Gram-positive) bacteria	Layer on different paper substrates.	12
NC + silver nanoparticle	Staphylococcus aureus	Antimicrobial wound dressing	13
Chitosan and cellulose nanocrystals	Escherichia coli	Antimicrobial tissue paper	14
Nanocellulose	COVID-19	Antimicrobial tissue paper	15
Allicin-conjugated nanocellulose and lysozyme-conjugated nanocellulose	Aspergillus niger, C. albicans, S. aureus and E. coli	Antimicrobial agent in food packaging, inside foodstuffs, and in textile materials	16
Nanofibrillar NC, Nanocrystalline NC, Microfibrillar NC + antibiotics	S. aureus, E. coli, B. subtilis, C. albicans	The authors suggest the use of biocomposites in the treatment of bone tissue infections	This study

So, it is known that in the work of Panaitesca et al. (2018) created a composite material based on poly(3-hydroxybutyrate) and nanocellulose coated with zinc oxide (ZnO) nanoparticles. This approach significantly improved the physical properties of the material and demonstrated antibacterial activity against Escherichia coli and Staphylococcus aureus [10].

The combination of silver nanoparticles with nanocellosis showed a good antimicrobial effect on B. Subtilis, E. Coli, S. Aureus and P. Aeruginosa [11–13]. In other studies of chitosan film with nanocellulose, plasma treated showed a high inhibit zone and 99% decrease in E. coli growth [14, 15].

Antifungicidal and antibacterial potential showed an NC in combination with allicin (organic compound formed during the mechanical destruction of garlic cells) and lysozyme against Aspergillus niger, C. albicans, S. aureus and E. coli [16].

Data of available literature showed that nanocellulose is a favorable material for the delivery of drugs and shows sufficient antibacterial potential in relation to a number of microorganisms (Table № 3).

Conclusion

The microbiological purity of the examples studied makes it possible to use them as substances for filling in vivo osteodefects.

Biocomposites based on nanocellulose, saturated with antibiotics, demonstrate high antibacterial and antifungicidal

potential. Further experimental studies are required by their ability to uniform and slow controlled release of the active substance, along with the effective suppression of the growth of microorganisms and a prolonged effect, which would allow them to be considered as an effective local transport system. The presented biocomposites will be used in the treatment of osteomyelitis in vivo.

Author Contributions: Conceptualization, S.A., B.T. and V.V.; methodology, S.A. and Zh.T.; formal analysis, S.A., B.T. and V.V.; resources, V.V.; writing – original draft preparation, M.R.; writing – review and editing, S.A., Zh.T. and M.R.; visualization, M.R.; supervision, S.A., B.T. and V.V.; project administration, funding acquisition, A.K.; statistical analysis, D.T. All authors have read and agreed to the published version of the manuscript.

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Data availability statement: The corresponding author can provide the data supporting the study's conclusions upon request. Due to ethical and privacy constraints, the data are not publicly accessible.

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