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ABSTRACT

of the dissertation submitted for the degree of Doctor of Philosophy (PhD)

Topic: **«Effectiveness of using a nanocellulose-based biocomposite with an antibiotic in an in vivo model of chronic osteomyelitis»**

Educational program: «Medicine» 8D10100

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Relevance

The incidence of chronic post-traumatic osteomyelitis ranges from 5 to 30 %, while patient disability reaches 50–90 % [1, 2, 3]. The high disability rate is associated with the formation of necrosis and sequestra, involvement of the surrounding soft tissues in the purulent-inflammatory process, and delayed osteoreparation. The financial cost of treating one patient is approximately 25 thousand dollars [4, 5]. Recurrences after appropriate combined therapy, including surgical debridement and medical treatment, are recorded in 20-30%, and the literature reports reactivation of infection after a 75-year initial period [6]. The rate of amputation of the affected segment is from 20% [7]. Treatment of chronic osteomyelitis is not only a medical but also a socio-economic problem, since patients of working age are most often affected [1, 3, 4].

Treatment of chronic osteomyelitis cannot be imagined without modern fillers. The wide variety from autologous bone to synthetic biopolymers indicates the enduring importance of finding an implant that would meet all relevant characteristics: osteoconductivity, osteoinductivity, bioresorbability, biocompatibility; provision of mechanical strength, absence of cytotoxicity, biological safety [8].

It is known that biomaterials used in the treatment of osteomyelitis should not only fill bone defects, but also serve as a “vehicle” for local delivery of antibiotics, since the local minimum bactericidal concentration and minimum biofilm eradication concentration of antibiotics cannot be achieved by systemic treatment alone because of biofilm formation and poor blood supply to necrotic areas [9, 10].

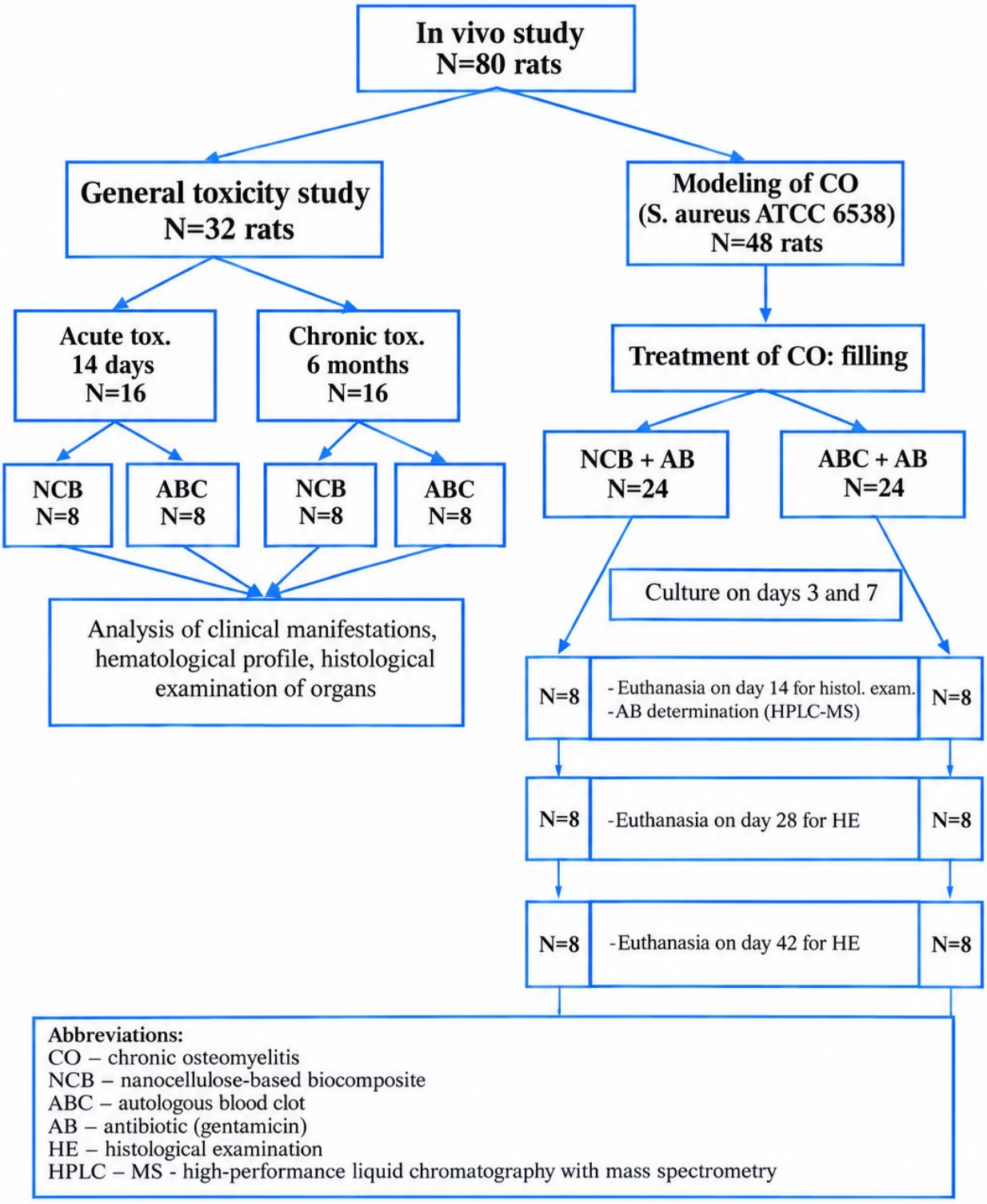
The development of advanced nanocomposite materials is currently a major direction in science and technology [8]. Nanocellulose (NC) and biocomposites based on it have become some of the most promising “green” materials because of their unique properties [11]. However, it is known that when moving from objects of traditional linear dimensions to the nanoscale, the physicochemical properties of substances change dramatically. Due to their physical and chemical properties, nanoparticles can cause tissue damage and impairment of intracellular organelle functions, in particular by inducing inflammatory reactions/cytokines [12, 13, 14]. Assessment of the general toxicity of a new NC-based biocomposite and its study not only as a filler but also as an antibiotic delivery system to the osteomyelitic focus are priority areas of this research work.

Research objective: to evaluate the effectiveness of using a nanocellulose-based biocomposite with an antibiotic in an experimental model of chronic osteomyelitis.

Research objectives:

1. To evaluate the general toxicity of a nanocellulose-based biocomposite *in vivo*.
2. To evaluate the histological pattern of regeneration using an NC-based biocomposite with an antibiotic compared with the traditional method in chronic osteomyelitis.
3. To evaluate the NC-based biocomposite as a substance for local antibiotic delivery *in vitro* and *in vivo*.

Research design



Abbreviations:
CO – chronic osteomyelitis
NCB – nanocellulose-based biocomposite
ABC – autologous blood clot
AB – antibiotic (gentamicin)
HE – histological examination
HPLC – MS - high-performance liquid chromatography with mass spectrometry

Scientific novelty

-For the first time, the general toxicity of a nanocellulose-based biocomposite was evaluated.

-For the first time, the histological characteristics of bone defect regeneration using a biocomposite based on nanofibrillar nanocellulose with an antibiotic in a chronic osteomyelitis model were studied.

-For the first time, the possibility of using an NC-based biocomposite as a local carrier of an antibiotic to the focus of chronic osteomyelitis was evaluated.

Main provisions submitted for defense:

1.Upon intramedullary implantation, the nanocellulose-based biocomposite does not induce acute or chronic systemic toxicity *in vivo* experiments. Throughout the study period, physiological parameters remained within normal limits, and no statistically significant differences were observed between the experimental and control groups regarding clinical-hematological, biochemical, behavioral, or histological indices ($p > 0.05$).

2.The nanocellulose-based biocomposite stimulates reparative bone regeneration in cases of chronic osteomyelitis, facilitating earlier and more pronounced bone formation alongside the regression of inflammatory-fibrotic changes. By days 28 and 42, the volume of newly formed bone in the biocomposite group reached 56 [49; 63] and 80.8 [78; 87], respectively, compared to 28.5 [20.5; 36.7] and 50 [42.5; 60.5] in the control group. Concurrently, a twofold reduction in the severity of the inflammatory response ($p = 0.0008288$) and a twofold increase in bone plate restoration indices ($p = 0.002271$) were observed, indicating that the regenerative process was predominantly directed toward bone tissue restoration.

3.The antibiotic-loaded nanocellulose-based biocomposite exhibits *in vitro* antibacterial activity and ensures the prolonged, predominantly local release of gentamicin *in vivo.* The antibacterial activity of the biocomposite exceeded that of the standard by 5 mm. On day 14, the plasma gentamicin concentration was 0.178 [0.171; 0.253] μg/mL in the biocomposite group versus 0.193 [0.158; 0.202] μg/mL in the control group, representing a 7.8% reduction in systemic concentration, with no statistically significant differences observed ($p > 0.05$). These findings characterize the biocomposite as a system that ensures the sustained release of the antibiotic, with its action localized primarily at the bone defect site and without signs of significant systemic accumulation.

Practical significance: a comprehensive study of the NC-based biocomposite showed no general toxicity, which determines its safety for further use. The results of modeling and treatment of osteomyelitis using a nanocellulose biocomposite with an antibiotic create an experimental basis for filling bone defects under conditions of chronic osteomyelitis with histological and microbiological substantiation. The developed approach can be introduced into experimental and potentially clinical practice of reconstructive bone surgery with confirmation of effectiveness. The results of the study can be integrated into the educational process in the training of medical university students in the disciplines “Microbiology”, “Traumatology and Orthopedics”.

Implementation: 3 implementation acts for the educational process were completed (Appendices M, N, O).

Relationship of the dissertation with other research works: the dissertation was carried out within the grant funding of the Ministry of Education and Science of the Republic of Kazakhstan No. AR19678427 "Study of the interaction of a nanocellulose-based biocomposite in a model of a tubular bone defect in an *in vivo* experiment".

Author's personal contribution: With the advisory support of the scientific supervisors and the research teams of the Departments of Traumatology and Neurosurgery and Molecular Biology, Biochemistry and Microbiology, the author performed experimental studies on laboratory animals, including investigation of manifestations of general toxicity, modeling of chronic osteomyelitis and its treatment using a nanocellulose-based biocomposite with an antibiotic. M. G. Rashova performed intramedullary implantation of the NCB, dynamic observation of the animals, thermometry, weighing, video recording and analysis of behavioral reactions, preparation of bacterial suspensions, study of the antimicrobial activity of the NC-based biocomposite, cultures from postoperative wounds, isolation of pure cultures followed by identification of microorganisms. Systematization, analysis and preparation of the study results were carried out independently by the applicant.

Approbation of the work. The main results of the study were presented at scientific events:

- at the young scientists' conference "Batpenov Readings" (Astana, August 28–29, 2024), held within the IV Congress of Traumatologists-Orthopedists of the Republic of Kazakhstan and the III Congress of the Kazakhstan Association of Traumatologists-Orthopedists;
- at the IX All-Russian Congress on Medical Microbiology, Clinical Mycology and Immunology (XXVIII Kashkin Readings), June 5-6, 2025, St. Petersburg;
- at the international scientific and practical conference "Modern Approaches in Traumatology, Orthopedics and Rehabilitation: Innovations and Practical Application", dedicated to the 110th anniversary of Professor Kh. Zh. Makazhanov and the 75th anniversary of NJSC "Karaganda Medical University" (Karaganda, October 9–10, 2025);
- at the All-Russian Conference of Young Scientists dedicated to the 120th anniversary of the Federal State Budgetary Institution "Vreden National Medical Research Center of Traumatology and Orthopedics" of the Ministry of Health of Russia, "Vreden Games", April 10, 2026, St. Petersburg;
- at the International Scientific and Practical Congress "Multidisciplinary Approaches in Medicine: Integration into Clinical Practice, Science and Education", April 16-17, 2026, Karaganda;
- at the meeting of the Regional Society of Traumatologists and Orthopedists of the Karaganda Region on June 26, 2026, Karaganda;
- at the international anniversary scientific and practical conference "Interdisciplinary Traumatology and Orthopedics: from Diagnosis to Rehabilitation", dedicated to the 35th anniversary of Independence of the Republic of Kazakhstan and the 25th anniversary of the Republican State Enterprise on the Right of Economic

Management “National Scientific Center of Traumatology and Orthopedics named after Academician N.D. Batpenov” of the Ministry of Health of the Republic of Kazakhstan, September 10-11, 2026, Astana;
- at the II International Biomedical Forum “International Biomedical Forum: Research and Innovation”, October 22–23, 2026, Karaganda;
- at an extended meeting of the Department of Surgical Diseases and Biomedicine.

Information on publication activity:

2 articles in Scopus-indexed journals;

4 articles in journals recommended by the Committee for Control in the Sphere of Education and Science;

5 patents and 4 copyright certificates.

Volume and structure of the dissertation. The dissertation consists of an introduction, literature review, main part, conclusion with conclusions and practical significance, and a list of references comprising 155 works. The text is presented on 174 computer-typed pages, including 17 appendices, 36 tables and 21 figures.

Materials: the object of the *in vitro* and *in vivo* studies was a biocomposite based on nanofibrillar nanocellulose and dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$), which was manufactured and provided by the Department of Physical and Colloid Chemistry of the “Gubkin Russian State University of Oil and Gas”, Moscow. The NCB was obtained from bleached cellulose by multistage mechanical treatment on a Masuko MKCA supermass 6-5 grinder, resulting in fibrils with an average diameter of 12 nm and a length of about 1.5 μm. The composite was synthesized by precipitating dicalcium phosphate dihydrate in an NFC suspension followed by filtration, washing and drying at 40 °C.

Animals. The experiment was conducted on 80 outbred male rats weighing 200-250 grams kept in the vivarium of NJSC KMU. During the experiments, the principles of humane treatment of laboratory animals were observed in accordance with the requirements of the European Parliament and the Council of the European Union set out in Directive 2010/63/EU, as well as the provisions of the National Research Council presented in the Guide for the Care and Use of Laboratory Animals. All manipulations related to the procedures and analysis of the obtained data complied with the established standards GOST ISO 10993-11— 2021, GOST ISO 10993-6— 2021, GOST ISO 10993-2— 2025. The study was approved by the Local Bioethics Commission at NJSC KMU (extract from Protocol No. 2 of the Meeting of the Local Bioethics Commission at KMU dated 11.10.22 and Protocol No. 22 dated 30.12.25).

Study sites. The study was conducted at NJSC KMU at the Departments of Traumatology and Orthopedics, Molecular Biology, Biochemistry and Microbiology in the educational microbiology laboratory; the diagnostic part was performed at Dr. Kryukov’s veterinary clinic of traumatology and orthopedics; the histological study was carried out in the pathology block of the NJSC KMU clinic; MALDI-TOF mass spectrometry with species identification of microorganisms was performed in the research laboratory of NJSC KMU. The experimental part was carried out in the vivarium of the medical university.

Methods:

1.Surgical:

A) 32 rats: after administration of Zoletil (international nonproprietary names of the active substances: tiletamine, zolazepam; "Virbac S.A.", France) at a dose of 0.1 mg/kg, preoperative preparation was performed with shaving of the hair in the left thigh area. For implantation, a 10 mm surgical incision was made on the anterior surface of the thigh. A bone tissue defect was formed in the middle third of the femoral diaphysis using a round drill creating a hole 2 mm in diameter and 1.5 mm deep. To assess general toxicity, intramedullary implantation of the NCB was performed in the main group (16 rats), while in the control group the defect was filled with an autologous blood clot (16 rats). Animals were withdrawn on day 14 and at the end of month 6, 8 animals from each group.

B) 48 rats: chronic osteomyelitis was modeled according to the certificate of entry of information in the State Register of Rights to Objects Protected by Copyright No. 13857 dated 11.12.2020, Ministry of Justice of the Republic of Kazakhstan, "Method for modeling chronic localized osteomyelitis in the diaphyseal zone with the presence of a sequestrum" [15]. Allogeneic bone was harvested from the intact side of healthy rats (animals in which toxicity was studied). It was then fragmented with bone forceps into bone chips. A bacterial suspension of the test culture was prepared in saline at a concentration corresponding to the 0.5 McFarland turbidity standard. The microbial suspension was mixed with the bone chips in a container, which was then placed in a thermostat for incubation for 24 h at 37°C. Subsequently, a piece of bone impregnated with the bacterial suspension was placed into a 2*1.5 cm defect formed in the middle third of the left femur. On day 14 after modeling, radiographic examination in direct and lateral projections was performed to verify CO using an AGFADX-D100 X-ray unit (Agfa-Gevaert Group, Belgium).

C) 14 days after modeling CO, treatment was performed: after removal of the allogeneic bone and irrigation of the cavity, intramedullary implantation of the NCB with gentamicin was performed in the main group (24 rats), while in the control group the defect was filled with an autologous blood clot also with gentamicin (24 rats). Eight rats from each group were withdrawn on days 14, 28 and 42 (see research design).

2. Microbiological:

A) Calculation of the antibiotic dose for impregnation: the minimum inhibitory concentration of gentamicin against the reference strain *S. aureus* determined and was 2 μg/ml, which is consistent with European Committee on Antimicrobial Susceptibility Testing data (2 mg/L). A 4 % gentamicin solution (40 mg of substance per 1 ml) was used. V=2 mg:40mg/ml=0.05 ml. Thus, to impregnate 0.006 g of NCB (the average weight of one isolated "unit" of NCB), 0.05 ml of 4% gentamicin was used. The same antibiotic dose was used in the control group.

B) The microbiological purity of the biocomposite was assessed by the direct inoculation method. One gram of the test material was suspended in 9 ml of sterile saline, followed by preparation of a series of tenfold dilutions. 1 ml of suspension was taken from each dilution and seeded on nutrient media: yolk-salt agar for the detection of Staphylococcus aureus, McConkey agar for the detection of gram-

negative bacteria and Saburo for fungi of the genus Candida. To detect anaerobic microorganisms, the material was introduced into a thioglycol medium. Bacterial crops were incubated at a temperature of 36 ± 1 ° C for 48 hours, fungi — at 26 ± 1 °C for 5 days, anaerobic cultures were cultivated under anaerobic conditions. After incubation, the growth of microorganisms was recorded and the number of colony-forming units (CFU) was calculated.

B) The antimicrobial activity of the biocomposite with gentamicin and nystatin was determined by the well diffusion method against reference test strains *S. aureus (ATCC 6538, 29213), Escherichia coli (ATCC 25922), Bacillus subtilis (ATCC 6633), Candida albicans (ATCC 10231).* The results were interpreted in accordance with the criteria of the European Committee on Antimicrobial Susceptibility Testing and the Clinical and Laboratory Standards Institute using established threshold values of inhibition zone diameters for specific microorganism–antimicrobial drug combinations. For the well diffusion antimicrobial activity study, a 0.05 g portion of NCB was impregnated with 0.4 ml of 4% gentamicin solution; for an isolated "unit" of NCB weighing 0.006 – 0.05 ml of 4% gentamicin solution was used.

C) A bacterial culture from the postoperative wound after treatment of osteomyelitis was performed on days 3 and 7

D) Identification of microorganisms using MALDI-TOF MS (Matrix Supported Laser Desorption/Ionization Flight Time Mass Spectrometry, Bruker, Germany).

3. Clinical method: the general condition of the animals was assessed, weighing was performed, and behavioral reactions in the "open field" test were video-recorded using a Canon EOS 250D camera (Taiwan) with the "Open Field for Rats" apparatus (LLC "Scientific and Production Company Open Science", Moscow Region, Krasnogorsk, Russian Federation).

4. Laboratory method:

A) to assess general (acute – on day 14, chronic – at the end of month 6) toxicity, blood was collected for a complete blood count (leukocytes, lymphocytes, monocytes, granulocytes, monocytes, granulocytes, erythrocytes, hemoglobin concentration, hematocrit, etc.). Blood biochemistry parameters: total protein, total bilirubin, creatinine, urea, ALT, AST, GGT, ALP, albumin, phosphorus, potassium, glucose, amylase, triglycerides, magnesium, cholesterol, bile acids, globulins, creatine kinase, calcium, phosphorus (instruments: URIT-2900Vet Plus veterinary hematology analyzer (China) and MNCHIP Celercare V5 automatic veterinary biochemistry analyzer (China).

B) for quantitative determination of gentamicin by HPLC-MS/MS (Agilent 1260 Infinity (Agilent Technologies, USA) in plasma, blood was collected on day 14 before euthanasia. Plasma was obtained by centrifuging the blood tube at 2 000 rpm in an OPn 3-02 centrifuge (manufacturer OJSC "TNK "Dastan", Kyrgyzstan).

5. Histological:

A) to assess general (acute – on day 14, chronic – at the end of month 6) toxicity, after simultaneous withdrawal of animals from the experiment, each animal underwent autopsy with removal of the brain, liver, heart, lungs, liver, kidneys, pancreas, spleen, intestine, testes. Animal tissues were fixed in neutral buffered 10%

formalin (Biovitrum, Italy) at +4°C for 24 hours. Tissue trimming was performed according to a standard protocol developed at the Veterinary Diagnostic Laboratory of Washington University to ensure uniformity of sections for all animals [17].

B) a histomorphometric assessment of regeneration in a model of chronic femoral osteomyelitis using NCB with gentamicin was performed on days 14, 28, 42. Microscopic assessment was performed according to the certificate of entry of information in the State Register of Rights to Objects Protected by Copyright No. 9335 dated 20.04.2020 "Algorithm for morphological assessment of osteomyelitis in an animal model" [16]. Staining was performed with hematoxylin and eosin and Masson's trichrome.

6. Statistical processing of the results was performed using the Microsoft Excel spreadsheet editor (Microsoft Office 2010) and Statistica 6 specialized software intended for statistical data analysis. Quantitative indicators are presented as median and interquartile range — Me (Q1; Q3). The Mann–Whitney U test was used to compare two independent samples. Fisher's test was used for qualitative data. Differences were considered statistically significant at $p < 0.05$.

Results

General toxicity study. With intramedullary implantation, the NCB, being a non-toxic substance, does not have a statistically significant effect on locomotor activity, exploratory behavior, anxiety indicators, or all types of metabolism; histological and morphometric analysis in all organs is dominated by minimal, potentially reversible changes without reaching thresholds of acute and chronic toxic damage (mild dystrophic and vascular changes after intramedullary administration of the NCB are consistent with active phagocytic clearance and moderate immunometabolic stress in the highly vascularized bone marrow compartment).

Microbiological purity. Three repeated direct inoculations of tenfold serial dilutions of the test substances showed that the presented samples were microbiologically pure. According to the State Pharmacopoeia of the Republic of Kazakhstan, implants are subject to requirements as medicinal substances for parenteral use.

Results of the antimicrobial activity study: the biocomposite impregnated with gentamicin demonstrated antibacterial activity against S. aureus, E. coli and B. subtilis: the growth inhibition zone diameters were more than 23 mm in all cases (S). In the monovariant, the biocomposite does not possess bactericidal activity (R).

Bacteriological examination of the postoperative wound on days 3 and 7 revealed no pathogenic microflora in rats of either group; only saprophytic normal flora of the skin and hair coat of the animals was isolated: *S. xylosus, S. nepalenisis, S. gallinarum.*

According to HPLC-MS results, on day 14 after treatment of chronic osteomyelitis, the gentamicin concentration in animal plasma remained low in both study groups. In the autologous blood clot group, the median concentration was 0.193 [0.158; 0.202] μg/ml, whereas in the nanocellulose biocomposite group it was 0.178 [0.171; 0.253] μg/ml. No significant differences between the groups were found, indicating the absence of significant systemic accumulation of the antibiotic and confirming the predominantly local nature of its release from the implanted materials.

The obtained data suggest that by day 14 after treatment, systemic entry of gentamicin from the implantation area is minimal, and the use of the nanocellulose biocomposite is not accompanied by an increase in antibiotic levels in the bloodstream compared with the traditional carrier. This indirectly indicates localized drug release in the area of the bone defect and a low risk of systemic toxic effects of gentamicin when the biocomposite is used.

Histological assessment of healing of the osteomyelitic defect showed that the use of NFCB compared with the traditional method:

1. Promotes faster resolution of the inflammatory-necrotic phase (on day 14, osteonecrosis, soft tissue necrosis and inflammatory infiltration were reduced 2-fold compared with the control group);
2. Prevents prolonged fibrous replacement: on day 14, the volume of fibrous tissue in the bone plate area in the NFC group was higher (57.5[51;71]) than in the control (27[23.8;27.5]), whereas by day 42 it was, conversely, significantly lower (12.8[9.3;16] versus 34 [28;39.8]). At the early stages after debridement of the defect, the temporary connective tissue matrix acts as an organizing framework that provides filling of the cavity, neoangiogenesis and cell migration. With a favorable course of regeneration, this early fibrous component subsequently undergoes remodeling and is replaced by bone tissue. This is exactly the dynamic observed: the early increase in fibrous tissue in the NFC group is followed by a sharp decrease in its volume and a simultaneous increase in bone tissue.
3. Accelerates the formation and maturation of bone regenerate (a significantly greater volume of newly formed bone tissue in the bone plate repair area in the NFC group at all observation periods, especially on day 28 (56[49;63] versus 28.5[20.5;36.7]) and day 42 (80.8[78;87] versus 50[42.5;60.5]), indicating that the effect of NFC is not limited to anti-inflammatory action but is also associated with activation of reparative osteogenesis);

Conclusions:

1. According to the results of clinical, hematological and histological studies, nanocellulose-based biocomposite does not have a general acute and chronic toxic effect in vivo: there were no statistically significant differences in the studied parameters between the experimental and control groups ($p>0.05$).
2. The histological picture of regeneration in the model of chronic osteomyelitis showed that the use of BNC reduces leukocyte infiltration by 2.0 times ($p = 0.0008288$), and active restoration of the bone plate is also noted by 2.0 times compared with the control group ($p = 0.002271$).
3. The biocomposite based on HC with an antibiotic showed antibacterial activity in vitro, exceeding the standard by 5 mm, and provided prolonged release of gentamicin ivo; its plasma concentration on day 14 was 7.8% lower than in the control group without statistically significant differences, indicating a predominantly local nature of its release in the area of the bone defect.

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