Clinical and therapeutic efficacy of biodegradable nanostructures in experimental infections

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Abstract. One of the main promising directions in the development of pharmacology is the development of selective drugs and effective approaches to their production using nanoscale drug delivery vehicles. Providing the necessary therapeutic concentration of drugs in target cells is not an easy task. To solve it, a specific drug carrier is required. The most promising is the use of biodegradable delivery systems, due to the lack of the need to use expensive equipment, and it is also possible to synthesize them in production volumes. The need to develop systems for the controlled delivery of drugs, especially antibacterial drugs, is due to their clear advantage over antibiotics in standardized dosage forms. Increasingly, in poultry industry, high mortality rates of chickens began to be observed in the acute form of enterotoxemia due to Clostridium perfringens and its associations with another microflora. The aim of this paper is to study the therapeutic efficacy of a free and included in biodegradable system antibacterial drug (cefotaxime) for bacterial infections in birds. There were formed 3 experimental and 1 control group of broiler chickens of the cross Cobb-500 daily age of 15 animals each. At the age of 10 days, the birds of group 1-3 were orally infected with virulent diurnal cultures of microorganisms: Clostridium perfringens. During the experiment, daily monitoring of the general condition and behavior of the birds, consumption of feed and water was carried out, the clinical status of the sick birds, the time of onset of positive dynamics and the time of recovery were evaluated. After completing the course of therapy, monitoring of the state of the birds continued for 7 days. After completion of the experiment, bacteriological examination showed that the use of cefotaxime at a dose of 10 mg/kg for 7 days does not eradicate Clostridium perfringens, while the use of cefotaxime based on chitosan and cefotaxime based on exosomes at a dose of 10 mg/kg provides 100% therapeutic efficiency.

1. Introduction
One of the main promising directions in the development of pharmacology is the development of selective drugs and effective approaches to their production using nanoscale drug delivery vehicles. Providing the necessary therapeutic concentration of drugs in target cells is not an easy task to solve which requires a specific drug carrier. In our opinion, the most promising is the use of biodegradable delivery systems, due to the lack of the need to use expensive equipment, and it is also possible to synthesize them in production volumes.

The need to develop systems for the controlled delivery of drugs, especially antibacterial drugs, is due to their clear advantage over antibiotics in standardized dosage forms. Thus, the use of nanocapsulation of the active substance of the drug will ensure the constancy of the concentration and...
pharmacokinetics of the drug at a low course dose of the drug, which prevents negative side reactions of antibiotic therapy, which are to reduce the toxic effect (1).

The use of nanotechnology in biomedicine will change and achieve controlled pharmacokinetic and toxicological properties. Nanostructured drug delivery systems can improve biopharmaceutical properties such as drug absorption, distribution, metabolism, elimination. The inclusion of antibacterial substances in microcontainers ensures their high efficiency, accurate dosed and prolonged action. The new technology makes it possible to efficiently obtain nanostructured forms of various forms of substances, both hydrophobic and amphiphilic antibiotics, and hydrophilic antibiotics, which differ in their location in the carrier. Chitosan and exosomal biopolymer nanosystems from the whole variety of alternatives have the most suitable characteristics that allow them to be used as a nanoscale drug delivery vehicle. These carriers are well tolerated by the body without causing toxic and allergic effects and are completely biodegradable by the body without the formation of toxic metabolites (3). A promising material for creating micro- and nanoscale drug delivery systems is chitosan, a deacetylated derivative of the natural chitin polysaccharide. The main advantages of chitosan include low toxicity and high biocompatibility (1, 3, 10).

As a rule, exosomes include a fraction of membrane vesicles with a diameter of 40-100 nm, which are secreted into the intercellular space by various types of cells of tissues and organs. It is known that the structure of the exosomal capsule is a double lipid membrane with an integrated layer of external transmembrane proteins. Microvesicles are found in various biological fluids (bronchoalveolar lavage, serum, urine, breast milk, cerebrospinal fluid, saliva, malignant pleural effusions, etc.), both during normal functioning of the body and in the event of various diseases. They provide transportation of proteins, lipids and nucleic acids in organs and systems, bypassing the plasma membrane, which allows them to be used as containers for targeted delivery of drugs to target cells (2,7,8). The most widely used among researchers was the exosome isolation protocol, which includes ultracentrifugation in a sucrose density gradient. The lack of a unified standard approach for producing exosomal dispersions is actively discussed in the literature; attempts are made to standardize protocols. An additional difficulty is the fact that neither the qualitative nor the quantitative composition of micro-vesicles can be strictly specific. It should be noted that the use of methods such as differential centrifugation of dispersions of micro-vesicles increase the risk of their fragmentation into smaller fragments [1]. To eliminate this problem, we recommend the use of ultrafiltration through membranes. In addition, in any physiological fluid and in the intercellular space, obviously, at the same time, populations of subcellular formations with a size in the range of 40-100 nm can be present at the same time. Therefore, the size of exosomes is not an absolute criterion for their difference, since both viruses and protein complexes can be detected in this range.

Cefotaxime is a semi-synthetic antibiotic of the third generation cephalosporins group, mainly for parenteral use. Of particular importance is the use of cefotaxime for the treatment of infections of the respiratory tract, skin and soft tissues, bacterial meningitis and other infectious diseases. Studies have shown that the development of a nanocapsulated form of cefotaxime can reduce the toxic effects of its use, reduce the effective dose of the drug, and also provide a prolonged effect of the drug substance (4,5,9).

Increasingly, in poultry industry, high mortality rates of chickens began to be observed in the acute form of enterotoxemia due to *Clostridium perfringens* and its associations with another microflora.

The aim of this study is to study the therapeutic efficacy of a free and included in biodegradable system antibacterial drug (cefotaxime) for bacterial infections in birds.

2. Materials and methods

During the work, reagents of the “Sigma-Aldrich” company, USA were used: chitosan (190 - 310 kDa), sodium tripolyphosphate, cefotaxime, acetic acid, acetonitrile, ammonium acetate > 98%, ultrapure water (type I according to ASTM, Millipore system, USA).

Chitosan nanoparticles with cefotaxime incorporated were prepared by ion cross-linking according to a modified method of Calvo et al. [3]. Chitosan was dissolved in 1% acetic acid to a concentration of
3 mg/ml and filtered through a filter with a pore diameter of 0.2 μm (Millipore, USA). A solution of cefotaxime and sodium tripolyphosphate was added to the chitosan solution with stirring on an Ultra-Turrax dispersant (IKA, Germany) at 2500 rpm for 30 minutes.

Then the suspension of particles was centrifuged at 10,000 rpm for 5 min at 4 °C, washed twice with water. The resulting preparation was stored at a temperature of (2–8) °C. Two methods have been developed to extract exosomes from the blood, by ultracentrifugation and ultrafiltration. The source of exosomes was the blood serum of outbred males of the vivarium of the Department of Therapy and Pharmacology of Stavropol State Agrarian University. The manipulations were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Scientific Purposes. Blood was taken from the saphenous vein of the forearm, after preliminary processing the place of sampling (cut hair along the veins, skin disinfection), into vacutainers with coagulation activator. To thoroughly mix the blood with the coagulation activator, the tube was gently turned upside down several times, then centrifuged at 3000 rpm for 10 minutes.

Aliquots of 1.8 ml of blood serum were taken into 2 ml tubes, then centrifuged using a MiniSpin microcentrifuge (Eppendorf, Germany) at 4000 x g rpm for 30 min. 1 ml of the obtained supernatant was taken with a medical syringe and filtered simultaneously through the following membranes: Millex-GV (Durapore) with a membrane diameter of 25 mm and a pore size of 0.22 μm; LCR (hydrophilic PTFE, Millipore), with a membrane diameter of 25 mm and a pore diameter of 0.45 μm, and a Millipore installation (USA) with a vacuum pump, using UPN-50 filters (50 kDa, “Vladipor”, Russia). Exosomes isolated from blood served as a container for the active antibacterial substance.

The average size and morphology of the nanoparticles was studied by scanning probe microscopy in an electron microscope for biological studies EVO LS 10 (Carl Zeiss, NTS Germany). The homogeneity of the preparations was evaluated based on flow cytometry data (Attune, Applied Biosystems, USA).

To study the in vitro release profile of cefotaxime from nanoparticles, 1 ml of the freshly prepared dispersion was placed in a dialysis bag (12-14 kDa, 10 mm wide), which, in turn, was transferred to a glass containing 100 ml of 0.02 M phosphate-buffered saline (pH 2.12, 4.76 and 7.21). The solution with an immersed dialysis bag was stirred on a magnetic stirrer at 50 rpm and a temperature of (37 ± 1) °C. Based on the data on the antibiotic content in the samples, a dependency chart of the percentage of the released antibiotic versus time was built.

The cefotaxime concentration in nanoparticle dispersions was determined by high performance liquid chromatography on an Ultimate 3000 chromatograph (Dionex Corp., United States); Reprosil-Pur 300 ODS-3 column (250 × 3 mm, particle size 5 μm) was used in all experiments. Elution was carried out in isocratic mode using two mobile phases: 20 mM ammonium acetate solution, pH 4.70 and acetonitrile, phase ratio 90:10, flow rate 0.4 ml / min. Column thermostat temperature 25 °C. The volume of the injected sample was 10 μl; detection was carried out at 252 nm.

Encapsulation efficiency was calculated using the following formula:

\[ A = \frac{B - C}{B} \times 100\% \]

where A – inclusion efficiency, %; B – initial concentration of cefotaxime in the reaction medium, μg / ml; C - the concentration of cefotaxime in the supernatant, μg / ml.

To study the clinical and therapeutic effectiveness of biodegradable nanostructures in experimental infections, 3 experimental and 1 control groups of Cobb-500 cross of 24-hour-old broiler chickens were formed with 15 animals each. At the age of 10 days, poultry of group 1-3 was orally infected with virulent diurnal cultures of microorganisms: Clostridium perfringens (name according to the passport - type C “BT”). Group 4 of broilers were not infected and served as the control one. One day after infection, chickens were slaughtered selectively (3 heads from each group) for bacteriological studies (intestines). The treatment was carried out according to the scheme described in table 1.

During the experiment, daily monitoring of the general condition and behavior of the poultry, consumption of feed and water was carried out, the clinical status of the sick poultry, the time of onset...
of positive dynamics and the time of recovery were evaluated. After completing the course of therapy, monitoring of the state of the poultry continued for 7 days.

Table 1. Scheme of the use of antibacterial drugs.

| No. | Group     | Drug                        | Method of administration | Dose  | Therapy duration   |
|-----|-----------|-----------------------------|--------------------------|-------|-------------------|
| 1   | 1st       | cefotaxime                  | Intramuscular            |       |                   |
| 2   | 2nd       | cefotaxime based on chitosan| Intramuscular            | 10 mg/kg | Every 12 hours, for 7 days |
| 3   | 3rd       | cefotaxime based on exosomes| Intramuscular            |       |                   |
| 4   | 4th       | control                     | -                        |       |                   |

3. Results and discussion

During optimization of the conditions for the formation of nanoparticles, it was found that ionic crosslinking occurs in a narrow range of concentrations of chitosan and sodium tripolyphosphate in the reaction medium (0.5–2.0 and 0.25–1.25 mg/ml, respectively). Large concentrations of reagents contribute to the formation of particulates, and low concentrations contribute to a sharp decrease in the yield of nanoparticles. The ratio of chitosan/tripolyphosphate also has a significant effect on the quality of dispersion. It was shown that an increase in the fraction of tripolyphosphate in the reaction mixture leads to an increase in particle size.

The finished preparations of chitosan microparticles obtained by ionic crosslinking were colorless solutions with a slight odorless opalescence. The average particle size in the preparations was 100 ± 50 nm. An analysis of the results of electron microscopy allows us to conclude that the main number of particles in concentrated preparations is part of temporary associates with other particles that do not affect the stability of the quality indicators of the drug. The size of such complexes ranges from 400 nm to 2 μm. Flow cytometry data of experimental samples of preparations containing chitosan-based particles with an antibiotic on indicate a relative homogeneity of the obtained dispersions.

The effectiveness of incorporating cefotaxime into chitosan nanoparticles depends on the concentration of the particle components and is proportional to the amount of chitosan in the reaction mixture. The highest efficiency of cefotaxime inclusion (up to 46.6%) was obtained with a 1.5/1 ratio of the amount of chitosan/tripolyphosphate. The relatively low values of the efficiency of incorporation of cefotaxime into nanoparticles can be explained by the high solubility of the compound in water, as well as by its small molecular weight (455 g/mol) and the antibiotic charge.

Figure 1. Dependence of the formation of chitosan nanoparticles with cefotaxime incorporated on the concentration of components.
The nature and rate of release of a substance encapsulated in nanoparticles is one of the most important parameters of the drug. Most of the free cefotaxime is released from the dialysis bag within the first 6 hours. According to the data obtained, the release of the antibiotic from the particles occurs unevenly. The most intense process occurs in low pH mediums. So in acidic solutions, 50% of the drug is released in the first 24 hours, while during dialysis in a neutral solution, 30% of the drug is released.

![Figure 2. Transition to cefotaxime solution during dialysis.](image)

The release of a drug substance from nanoparticles of chitosan (NPC) proceeds according to several mechanisms: polymer swelling [4], drug diffusion through the polymer, erosion or degradation of the polymer matrix, and a combination of erosion and degradation [5]. The initial release of the encapsulated substance from chitosan nanoparticles proceeds according to the polymer swelling mechanism, pore formation, or drug diffusion from the polymer surface [6]. In addition, chitosan nanoparticles exhibit pH-dependent drug release due to the solubility of chitosan in acidic solutions [7,8]. The observed release of cefotaxime in vitro, apparently, also occurs through the initial stage of particle swelling, followed by pH-dependent erosion of the nanoparticles. In vivo antibiotic release occurs faster than in vitro, since the destruction of the chitosan nanoparticle is additionally accelerated by enzymatic destruction of the polymer chain [9].

In the study of therapeutic efficacy, it was found that during the first days after infection, the poultry was lethargic, drowsy and thirsty, which is probably associated with stress. On day 3 after infection, broiler chickens of groups 1 and 2 showed lethargy, crowding, thirst, these signs after 2 days were observed in single individuals, then disappeared. On day 6 after infection in group 1, broiler droppings had viscous white filamentary inclusions, which indirectly indicates persistence of Clostridium perfringens in the gastrointestinal tract.

As a result of daily observation, it was found that feed and water intake by chickens did not statistically significantly differ and corresponded to standards according to the Cobb-500 cross (table 2).

| Table 2. Feed and water intake per head |
|----------------------------------------|
| Age, days | Feed, g | Water, ml |
|-----------|---------|-----------|
| 1-5       | 12      | 33        |
During the experiment in group No. 1, the mortality of the poultry was 3 individuals, the mortality of the poultry in groups 2-4 was not observed. In the pathoanatomical autopsy of a dead poultry, that the main lesions are localized in the gastrointestinal tract - acute catarrhal hemorrhagic enteritis, the contents of the intestines are foamy, fluid, with blood, sometimes with gas bubbles, on the intestinal mucosa - multiple banded and petechial hemorrhages. The kidneys are hyperemic, softened with extensive hemorrhages. Hyperplasia of the spleen. Acute heart enlargement, myocardial dystrophy.

### Table 3. The results of the control weighing of poultry.

| Group number | Average weight, g |
|--------------|-------------------|
| 10th day     | 24th day          |
| 1-st group   | 296±41            | 1015±123*        |
| 2-nd group   | 319±24            | 1301±99         |
| 3-rd group   | 289±22            | 1312±97         |
| 4-th group   | 317±27            | 1325±111        |

Note: the significance of differences is indicated * - p<0.05.

The live weight indices of group 1 broiler chickens are significantly lower than in groups 2-4.

The excrements for bacteriological examination of the droppings were collected immediately after defection using a sterile glass rod. The study was conducted in accordance with GOST 26503-85 Agricultura animals. Methods of laboratory diagnosis of clostridiosis. The results of bacteriological studies of poultry droppings are presented in table 4.

### Table 4. The results of bacteriological studies of poultry droppings.

| Before infection | 1st day after infection | 3rd day after infection | 5th day after infection |
|------------------|-------------------------|-------------------------|-------------------------|
| 1-st group       | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi, clostridia |
| 2-nd group       | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi, clostridia |
| 3-rd group       | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi, clostridia |
| 4-th group       | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi | streptococci, micrococci, gram-positive bacteria, yeast-like fungi |

The data in the table show that at the bacteriological examination of the droppings on the 3rd day after infection, the presence of Clostridium perfringens in groups 1-3 was established. On the 5th day after
infection in groups 2-3, Clostridium perfringens is absent in the samples, however, E. coli is presented in the droppings of both groups, which indicates a weakening of the poultry organism against the background of infection. Normal microflora was found in the droppings of the control group.

Selected intestinal samples were examined under sterile conditions in a bacteriological research laboratory. Crops on the Kitta-Tarozzi medium were carried out in a laminar cabinet, followed by cultivation in thermostat at a temperature of 37 °C for 24 hours. Cultures were examined in accordance with GOST 26503-85 Agricultural animals. Methods of laboratory diagnosis of clostridiosis. The research results are shown in table 5.

Table 5. The results of bacteriological examination of the intestines.

| Group number | One day after infection | At the end of the experiment |
|--------------|-------------------------|------------------------------|
| 1            | Clostridium perfringens | Clostridium perfringens      |
| 2            | Clostridium perfringens | not found                    |
| 3            | Clostridium perfringens | not found                    |
| 4            | not found               | not found                    |

As a result of bacteriological examination of the intestine one day after infection, the presence of Clostridium perfringens in the experimental groups was confirmed. After completion of the experiment, bacteriological examination showed that the use of cefotaxime at a dose of 10 mg/kg for 7 days does not eradicate Clostridium perfringens, while the use of cefotaxime based on chitosan and cefotaxime based on exosomes at a dose of 10 mg/kg provides 100% therapeutic efficiency.

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