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HISTOLOGICAL AND MICROBIOLOGICAL RATIONALE FOR THE USE OF MATRIX MATERIALS IMPREGNATED WITH ANTIBIOTICS FOR THE RECONSTRUCTION OF BONE TISSUE DEFECTS

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Studying the combination of fibrous scaffold materials with therapeutic agents as a drug delivery system is important for regenerative medicine. The results of the antibiotic-absorbing capacity of the fibrous non-woven polycaprolactone matrices created by us, as well as their influence on the regeneration of bone tissue, were analyzed in the study. The results of microbiological studies indicated the pronounced hydrophilic properties of the matrices we've created; it was confirmed by a decrease in the activity of the antibiotic only at 16.4 % after 7 days ($p<0.05$). Instead, in an experiment on laboratory animals, the specified frame effect of fibers was confirmed by the beginning of the formation of an organized bone structure, namely, by an increase in osteoid up to 34.38 % ($p<0.05$), at an early stage so far.
Key words: matrix materials, polycaprolactone, antibiotic impregnation, cefazolin, lincomycin, histological analysis, bone tissue.

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ГІСТОЛОГІЧНЕ ТА МІКРОБІОЛОГІЧНЕ ОБГРУНТУВАННЯ ВИКОРИСТАННЯ МАТРИКСНИХ МАТЕРІАЛІВ, ІМПРЕГНОВАНИХ АНТИБІОТИКАМИ, ДЛЯ РЕКОНСТРУКЦІЇ ДЕФЕКТІВ КІСТКОВОЇ ТКАНИНИ

Вивчення поєднання волокнистих каркасних матеріалів із лікувальними засобами, як системи доставки ліків має важливе значення для регенеративної медицини. У дослідженні проаналізовано результати антибіотик-сорбуючої здатності створених нами волокнистих нетканих полікапролактонових матриксів, а також їхній вплив на регенерацію кісткової тканини. Результати мікробіологічних досліджень свідчили про виражені гідрофільні властивості створених нами матриксів, що підтверджувалось зниженням після 7 діб активності антибіотика всього на 16,4 % ($p<0,05$). Натомість, в експерименті на лабораторних тваринах вказаний каркасний ефект волокон підтверджувався початком формування організованої структури кістки, а саме, збільшенням остеоїду до 34,38 % ($p<0,05$) вже на ранніх термінах.
Ключові слова: матриксні матеріали, полікапролактон, імпрегнація антибіотиками, цефазолін, лінкоміцин, гістологічний аналіз, кісткова тканина.

The study is a fragment of the research project "Comprehensive morphofunctional research and rationale for the use of modern technologies for the treatment and prevention of dental diseases", state registration No. 0121U109242.

To date, a new direction is being pursued in medicine, including the combination of fibrous materials with therapeutic agents, as a delivery system for medicines and living cells. This approach promotes a purposeful management of the structural-functional condition of cells involved in regenerative processes [1, 11].

Natural polymers (hyaluronic acid, collagen, gelatin, fibrinogen, chitosan, pectins, agarose, alginates, cellulose) and synthetic materials (polycaprolactone, polylactide) are considered to be the most promising tools for the controlled reconstructive tissue repair [5, 6].

An existing method of forming porous non-woven matrices is electrospinning. The three-dimensional frame of the implant due to its architecture and the presence of active functional groups (which is determined by the type of polymer material) promotes the adhesion and migration of cells to the area of the tissue defect, provides complex cascades of intercellular signaling interactions underlying angiogenesis, trophicity and repair [2].

In the reconstructive surgery of necrotizing infectious processes of soft tissues, the tissue implants are used at the same time as local delivery systems of antimicrobial medicines (antibiotics, silver sulfadiazine, metal nanooxides) into the damaged area [9, 10, 12]. In surgical practice, in particular in surgical dentistry, such microfiber materials as scaffolds for bone tissue reconstruction have not yet been widely used. Electrospinning itself as a method, is an expensive and energy-intensive one. In addition, in the process of synthesis of micro- and nanofibers according to this method, solvents for polymers that are toxic to living cells are used, and the resulting matrix structure has very small pores for the growth of tissues and capillaries. Currently, a cheaper and safer method of synthesis of fibrous matrices and the use of such matrix implants impregnated with antibiotics in reconstructive surgery, remains relevant.

The purpose of the study was to investigate the antibiotic-absorbing and framework capacity of the fibrous non-woven polycaprolactone matrices created by us, intended for the reconstruction of bone tissue defects.

Materials and methods. Samples of microfibrous non-woven matrices made by our method from polycaprolactone PCL (invention patent of Ukraine No. 119958), were used in the work. Sterilization of microfibrous matrices by γ-radiation was performed using the "Elektronika ELU-4" linear accelerator. Collagen fragments were used as a control for microbiological studies. Impregnation of matrix samples was carried out in aseptic conditions by applying antibiotic solutions (cefazolin in a final dose of 30 μg and lincomycin – 10 μg) with a micropipette, followed by drying in a dry-air sterilizer at a temperature of no more than 30°C. Cefazolin (Borshchahivskiy CPP, Ukraine) and Lincomycin hydrochloride (Pharmaceutical company "Darnytsia", Ukraine) were used in the study.

All samples were divided into 3 series, which were stored for 3 weeks in different conditions: at a room temperature, at a room temperature in the dark, and in the dark in a refrigerator at a temperature of +4°C. During the 1st, 3rd, 5th, 7th, 14th, 18th and 21st days of the experiment, samples for microbiological studies were taken from each series.

To assess the preservation of antibiotics in samples of matrix materials and the possibility of their release in an active condition into the environment, the most accessible and sufficiently sensitive biological test was used [8]. As a biosensor, a culture of a clinical strain of *S. aureus* sensitive to the specified antibiotics and identified on the basis of a complex of morphological and cultural properties in accordance with the recommendations of the 9th edition of "Bergey's Manual of Bacteria" [7] and biochemical microtests "STAPHYtest 16" (Lachema, Czech Republic), was used. The test-strain used in the study, was checked for sensitivity to cefazolin and lincomycin by the disc diffusion method (HiMedia discs, India) [4].

The samples selected at the appropriate terms were placed on the surface of nutrient agar pre-inoculated with the S. aureus test culture (standardized according to the optical turbidity standard of 5×10^5 CFU/ml). After cultivation in a thermostat at a temperature of 37°C for 18 hours, the diameters of the growth retardation zones of the test-cultures were determined. Digital images of cultures on plates were obtained; they were processed using the UTHSCSA ImageTool 2.0 computer program (The University of Texas Health Science Center in San Antonio, ©1995-1996) [13].

The experimental part of the research in laboratory animals was performed using adult, sexually mature male rabbits weighing 1100–1400 g, kept in a vivarium on a regular diet. Animals were kept and manipulated in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1985).

Experimental animals were divided into 2 groups. The first main group included 30 animals with a polymeric fibrous non-woven matrix made of polycaprolactone PCL implanted into the bone tissue. The second comparison group consisted of 30 animals with surgically formed defect in the bone tissue followed by sutures' overcasting. Material was collected from bone tissue in both groups during the 1st, 2nd, 3rd, 4th and 5th months of the experiment. When the material was taken, the experiment was completed by an overdose of 2 % sodium thiopental solution 1.5 ml intravenously.

To perform a general histological examination, special histological examinations, fragments of bone tissue were fixed in a 10 % solution of neutral formalin (Ph-7.0). Histological sections of bone tissue were stained with hematoxylin and eosin and according to Masson.

The results of the experiments were processed using the methods of variational statistics and one- and two-factor analysis of variance (ANOVA).

Results of the study and their discussion. With the help of the initial control microbiological test on standard disks, it was determined that the values of the diameters of the growth inhibition zones of the test culture were for cefazolin (CZ 30 μg) – 29.96±0.14 mm, lincomycin (L 10 μg) – 29.60±0.17 mm.

Taking into account the results of the sensitivity of the S. aureus test strain, the final doses of antibiotics for impregnation into the material samples were determined. Experimentally, it was found that the sorption properties of the matrix materials are able to retain a volume of water without loss that corresponds to their mass (1:1, m/v). Samples of matrix materials were divided into fragments weighing 15.0 mg, which in terms of surface area corresponded to standard paper discs for antibiotic susceptibility testing. Antibiotics (cefazolin and lincomycin) were previously diluted with a sterile isotonic solution to the required working concentrations. Cefazolin was applied in a final dose of 30 µg per sample in the form of a solution with a volume of 6 µl. Lincomycin was applied in a final dose of 10 µg per sample in the form of a solution with a volume of 6 µl.

During the 1st day of the experiment, the content of antibiotics in samples of matrix materials was investigated immediately after applying solutions and after drying the samples for 60 minutes. The drying procedure of the samples (both collagenous and polycapron) had absolutely no effect on the activity of cefazolin (Table 1).

Table 1

The content of antibiotics in samples of matrix materials before and after the drying procedure (diameters of growth retardation zones of S. aureus test-culture, mm)

Antibiotics	Collagen		PCL	
	Before drying	After drying	Before drying	After drying
Cefazolin 30 µg	28.91±0.25	29.02±0.44	27.23±0.41	27.78±0.46
Lincomycin 10 µg	29.64±0.43	29.29±0.47	30.11±0.54	27.31±0.75*

Note: * – p<0.05 when comparing samples before and after drying.

The activity of lincomycin in the drying process did not change only in the case of impregnation of the antibiotic into the collagenous matrix. A slight decrease in the activity of lincomycin was observed on the polycaprolactone matrix after drying.

Antibiotic retention in samples of matrix materials was evaluated during the 1st, 3rd, 5th, 7th, 14th, 18th and 21st days of the experiment. The obtained experimental data indicate that both used medicines (both cefazolin and lincomycin) were stored in significant quantities in both collagenous and polycaprolactone matrices throughout the observation period. This is evidenced by the formation of distinct, comparable in size zones of growth inhibition of the test-culture of Staphylococcus aureus.

Antimicrobial activity of polycaprolactone matrix samples impregnated with cefazolin, remained at the initial level for 3 days, collagenous matrix – for 5 days. After 7 days of storage at a room temperature, the activity of impregnated cefazolin decreased at 9.0 % (p<0.05) on the collagenous matrix, on polycaprolactone – at 16.4 % (p<0.05). The decrease in the activity of cefazolin impregnated into the collagenous matrix continued until the 14th day of observation (it reached 20.7 %, p<0.01), but it had already stopped at longer storage periods of the samples (during the 18th–21st day). During the same period, a progressive decrease in the activity of cefazolin impregnated into the polycaprolactone matrix, was observed. At the end of the observation period (after the 21st day of storage), the diameter of the growth retardation zone of the test-culture decreased at 35.2 % (p<0.01).

Antimicrobial activity of matrix samples impregnated with lincomycin decreased during their storage at a faster rate. During the 7th day of observation, the diameters of the growth inhibition zones of the S. aureus test-culture around the samples of collagenous matrix decreased at 21.4 %, polycaprolactone – at 39.8 % (p<0.01). The progressive decrease in the activity of lincomycin impregnated into the collagenous matrix, continued until the end of the observation period (the 21st day) and reached 37.6 % (p<0.01).

The influence of the storage time of samples of matrix materials on the antimicrobial activity of antibiotics impregnated into them, was confirmed during the statistical processing of the obtained experimental data by the method of unifactor analysis of variance (ANOVA) (Table 2). For all studied samples of matrix materials, the decrease in the activity of impregnated antibiotics during storage for 3 weeks, was statistically significant.

Table 2

Unifactor analysis of variance (ANOVA) of the influence of storage terms of matrix materials samples on the antimicrobial activity of antibiotics impregnated into them

Samples under study	Fisher's criterion F	Value P	F critical
Collagen + Cefazolin	26.57417	0.000239	4.747225
PCL + Cefazolin	18.75035	0.000978	4.747225
Collagen + Lincomycin	19.66946	0.000814	4.747225
PCL + Lincomycin	12.81067	0.003788	4.747225

Thus, it can be concluded that antibiotics impregnated into the collagenous matrix, are preserved somewhat better than when impregnated into polycaprolactone, which is associated with a smaller diameter of pores in collagen and, accordingly, a more pronounced capillary effect. However, the concentration of the antibiotic retained on the polycaprolactone matrix, is quite sufficient for a pronounced antimicrobial effect at the initial stages of tissue regeneration.

Histological examination of the bone tissue 1 month after the implantation of the polymer matrix showed the growth of connective tissue with a loose arrangement of connective tissue fibers mainly in the central and peripheral parts of the defect. Multiple osteoid foci were also noted – 18.96 % per 1 μm^2 in close contact with the fibrous non-woven polymer matrix, which indicated the beginning of bone mineralization and regeneration processes in the area of the defect (Fig. 1).

In 2 months after implantation, the process of osseointegration of the matrix and bone tissue increased, which is confirmed by the increased growth of circularly located densely adjacent collagenous fibers to the polymer matrix and the increase of osteoid up to 34.38 % ($p<0.05$), which was significantly different from the previous term.

At the end of 3–4 months, mineralized lamellar bone tissue has already been noted in the area of the bone defect. The presence of a large number of osteocytes, in our opinion, indicated the completion of the osteogenesis process and the presence of the already formed bone. The share of nonmineralized bone plates was only 8.91 % ($p<0.05$), which is significantly lower than the corresponding index at the end of the 2nd month of the experiment and reflected the process of active mineralization and compaction of bone tissue (Fig. 2).

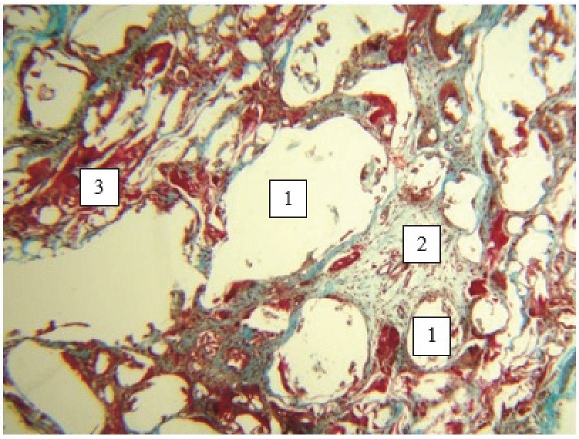

Fig. 1. Bone defect within 1 month after implantation of the polymer matrix. Staining: according to Masson. Magnification: ocular lens 10, field lens 20. 1 – location of polymer implant fibers, 2 – connective tissue fibers, stained blue, 3 – osteoid, stained red.

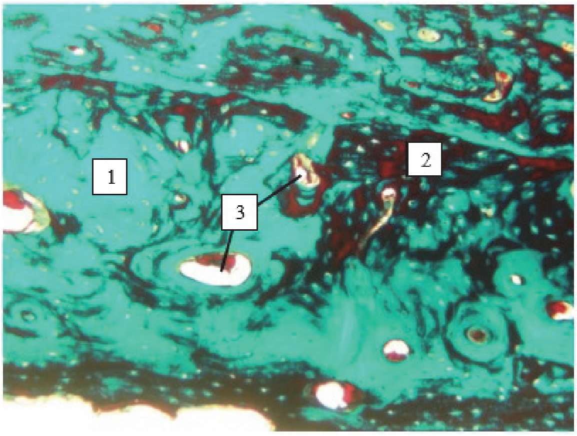

Fig. 2. Bone defect within 3 months after implantation of the polymer matrix. Staining: according to Masson. Magnification: ocular lens 10, field lens 20. 1 – mineralized bone matrix, 2 – osteoid, 3 – central canals of osteons.

During the 5th month of the experiment, the presence of fully mineralized bone tissue with lysis of the fibers of the non-woven polymer matrix and the presence of microosteoid foci, was noted in the defect zone, the proportion of which was 0.13 % ($p<0.05$), which is almost 25-fold lower than in the previous experimental group and at 86 % less than the index of the control group (the share of osteoid of the control group is 0.94 %).

As shown by the results of the histological studies, the active formation of the connective tissue matrix in the area of the bone defect was noted already in the early stages of the experiment and was replaced by osteoid with subsequent formation of a formed and organized bone structure in three mutually perpendicular directions. The percentage share of osteoid (34.38 % ($p<0.05$)) in the early stages in the experimental group, in comparison with the control indices, indicated the pronounced frame effect of the implanted microfiber polymer matrix. The specified matrix effect was also noted in the compact and circular arrangement of the collagenous fibers around groups of polymer microfibers. That is, a group of polymer fibers created a kind of scaffold for building bone tissue on it.

In our opinion, the release of the antibiotic impregnated into the sample in the gel medium, was evaluated due to the transition of the antibiotic to the soluble phase under the conditions of sufficient humidity and subsequent diffusion into the agarous gel, as indicated by the diameter of the zone of 27.23±0.41 growth retardation of the test-culture. This process simulates the events occurring after the matrix is implanted into the tissue and saturated with tissue fluid. According to the laws of diffusion, the impregnated medicine enters the surrounding tissues. But the elution of the antibiotic stops quite quickly due to a rapid decrease in the concentration gradient at 16.4 % ($p<0.05$) after 7 days of observation. Its duration depends on the dose of the antibiotic impregnated into the matrix material.

The matrix materials developed by us, are the tools of one-time local delivery of the medicine to the tissues in the area of damage. Taking into account the above considerations, it is possible to predict their greatest effectiveness in terms of prevention of postoperative infectious complications [3, 10]. This is especially relevant in surgical dentistry, since even strict adherence to the rules of asepsis cannot protect against the ingress of single microbial cells from the surface of the mucous membrane of the oral cavity and from saliva into the area of surgical intervention. Immediate contact of microbial cells with an antibiotic eluted from the implanted matrix, causes their rapid death and prevents the realization of their invasive potential. Pathogenic and conditionally pathogenic oral microflora is represented mainly by streptococci, staphylococci and actinomycetes, which are mostly characterized by high sensitivity to cephalosporins and lincosamides (which led to the choice of antimicrobial medicines for this development).

Further research may be directed at examining the clinical efficacy of implants made of worked out antibiotic-impregnated matrix materials. The duration of elution of impregnated antibiotics from different matrix materials needs clarification. A long-term issue may be the development and study of new matrix materials capable of providing prolonged release of not only antibiotics into tissues, but also various biologically active substances and growth factors to accelerate regeneration.

Conclusions

1. Lincomycin impregnated into polymer matrices is characterized by less stability during storage, than cefazolin.

2. Antibiotics impregnated into the collagenous matrix are preserved somewhat better than when impregnated into polycaprolactone.

3. Antibiotic concentrations of impregnated test samples of matrix materials at the level of control values are actively maintained for a period of 5 days.

4. The results obtained of histological studies of bone tissue in the experiment with the implantation of a fibrous matrix, indicated an increase in reparative osteogenesis in the form of an increase in osteoid zones up to 34.38 %.

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