**ANNOTATION**

dissertation work for the degree of Doctor of Philosophy

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| Topic: "Development and experimental morphological substantiation of antibiotic impregnation of a bone allograft harvested according to the Marburg system on a model of osteomyelitis" |
| Specialty: 6 D 110100 "Medicine"Performed by: doctoral student Koshanova Amina Supervisor: Doctor of Medical Sciences, Head of the Department of Surgical Diseases Tuleubaev B.E.Scientific consultant: Doctor of Medical Sciences, Professor of the Department of Pathology Tussupbekova M.M.Foreign scientific consultant: Ph.D., Researcher at the Research Center of the Military Medical Academy named after S.M. Kirov Ministry of Defense of Russia, St. Petersburg Vorobyov K.A.  2022 |

**Relevance.**

 The relevance of the problem of treating chronic osteomyelitis is determined by the significant prevalence of the disease among all age groups, which is determined by the steady increase in injuries, the severity and duration of the course of the pathological process, the activity of introducing surgical methods of treatment, as well as the difficulties in the prevention and treatment of this pathology ( Bozhkova S.A., Novokshonova A.A., Konev V.A. Modern possibilities of local antibiotic therapy of periprosthetic infection and osteomyelitis (literature review)// Traumatology and Orthopedics of Russia, 2015; 3(77): P. 93-103; Derkachev V.S., Alekseev S.A. , Bordakov V.N., Elin I.A., Derkachev D.V. On the issue of complex treatment of chronic post-traumatic osteomyelitis // Traumatology is not orthopedics. - 3-4 (33-34) / 2015. - P.43 -44; Dyachkova G.V., Klyushin N.M., Migalkin N.S., Larionova T.A., Leonchuk D.S., Dyachkov K.A., Begimbetova N.B. X-ray and histological parallels of the stages of chronic osteomyelitis // Bulletin of the Russian military medicine nsk academy, 2017; 4 (60): pp. 17 – 22). Despite the widespread use of minimally invasive treatment methods and active surgical tactics, chronic osteomyelitis remains an unresolved problem in the world. It has been established that after the treatment of chronic osteomyelitis, there are still high rates of poor outcomes and disability , reaching 50-90% ( Dyachkova G.V., Klyushin N.M., Migalkin N.S., Larionova T.A., Leonchuk D. S., Dyachkov K.A., Begimbetova N.B. X-ray and histological parallels of the stages of chronic osteomyelitis // Bulletin of the Russian Military Medical Academy, 2017; 4 (60): pp. 17 - 22 ). Adequate treatment of chronic osteomyelitis is: the elimination of infection, through sanitation and the creation of high concentrations of the antibiotic in the lesion, the simultaneous filling of a bone defect. The traditional methods of treating osteomyelitis are systemic antibiotic therapy and surgical debridement of the focus of infections. After any necrosequestrectomy , defects remain in the bone that do not regenerate on their own and constantly maintain inflammation ( Lewis CS , Supronowicz PR , Zhukauskas RM , Gill E , Cobb RR . Local antibiotic delivery with demineralized bone matrix. Cell Tissue Bank. 2012 Mar;13 (1):119-27. doi: 10.1007/s10561-010-9236-y. Epub 2011 Jan 1; Shah M. RukeshRP , \_ Randhirsinh V.S. \_ Shailendra HG Estimation of drug absorption in antibiotic soaked bone grafts. Indian J Orthop . 2016 Nov - Dec ; 50(6): 669–676. doi : 10.4103/0019-5413.193486 ). The space created by debridement must be filled to prevent transmission and recurrence of the infection. The basis of the pathogenetic mechanisms of chronic osteomyelitis is a complex of ischemic, infectious-inflammatory and reparative changes in the bone and surrounding soft tissues. Structural and functional changes are determined by the characteristics of the pathogens of the infectious process, the nature and severity of inflammatory and reparative processes in the affected area. Histological examination is the "gold standard" in relation to any imaging techniques in combination with radiation and microbiological methods. Pathological changes in osteomyelitis are currently well studied, showing the differences between acute and chronic types of inflammation in the bone (Uskokovi ć V , Desai T.A. \_ In vitro analysis of nanoparticulate hydroxyapatite/chitosan composites as potential drug delivery platforms for the sustained release of antibiotics in the treatment of osteomyelitis. J Pharm Sci . Feb 2014 ;103 (2):567-79. doi : 10.1002/ jps.23824 . Epub 2013 Dec 30 ). Antibiotic-impregnated various fillers can act as a local anti- infective drug release system that not only fills the resulting bone defect after surgical debridement, but also provides high concentrations of antibiotics in the focus, without increasing the level of antibiotic in the blood serum. Currently, in traumatology and orthopedics, there is a significant number of both biodegradable and non-degradable materials involved in the mechanisms of the bone tissue reparative process. Non-degradable materials require repeated surgical interventions, which reduces their scope. The negative aspects of the use of bone autografts are trauma during harvesting, limited resources of donor zones, the risk of fractures and chronic pain syndromes at the site of donor material sampling ( Bozhkova S.A., Novokshonova A.A., Konev V.A. Modern possibilities of local antibiotic therapy for periprosthetic infection and osteomyelitis (literature review)// Traumatology and Orthopedics of Russia, 2015; 3(77): pp. 93-103). Bone autografts , which are similar in structure, are especially popular today . However, when harvesting them, the negative aspects are the difficulty of obtaining them, the need for a special production base, where they will be processed, sterilized, degreased, decellularized. Existing synthetic biodegradable materials are expensive, they are not produced and are not registered on the territory of the Republic of Kazakhstan.

Given the above, there is a need to find ways to create a bone allograft impregnated with an antibiotic of our own production. The solution of this problem will favorably affect the treatment of this category of patients, as well as affect the economic component of this problem.

**The working hypothesis** is the development of a bone allograft impregnated with an antimicrobial agent and harvested according to the Marburg system with testing on a model of chronic osteomyelitis will prove its ability to effectively transport the antibiotic with the suppression of the main strains in the osteomyelitis focus.

**The aim of the study** is to develop a bone allograft impregnated with an antibiotic, harvested according to the Marburg system with a study of its effectiveness on a model of chronic osteomyelitis with an assessment of the clinical and morphological features of the bone tissue reparative process in the experiment.

**Research objectives**.

1. To develop a method for antibiotic impregnation of a bone allograft prepared according to the Marburg system.
2. Create a model of chronic osteomyelitis in rabbits for testing the proposed method of bone allograft impregnation with an antibiotic.
3. To give a histological characterization of the bone tissue using a bone allograft impregnated with an antibiotic according to the proposed method "Impregnation of a bone allograft with an antibiotic" and according to the standard (conventional) technology.
4. To evaluate the antibacterial efficacy of the bone allograft impregnated with an antibiotic according to the developed technology on the main strain of *osteomyelitis S. aureus*.

**Scientific novelty**

- For the first time, a histological characterization of the reparative process of bone tissue was carried out using a bone allograft impregnated antibiotic harvested according to the Marburg system.

- For the first time, an antibacterial evaluation of the effectiveness of using a bone allograft prepared according to the Marburg system and impregnated with an antibiotic according to the developed technology on the main strain of osteomyelitis was given.

**The main provisions for defense:**

- the proposed method of antibiotic impregnation of a bone allograft harvested according to the Marburg system allows uniform impregnation solutions of medicinal substances spongy tissue of the bone allograft throughout its entire thickness;

- the created model of chronic osteomyelitis on rabbits allows you to repeatedly reproduce the model of chronic osteomyelitis in different periods of its development with the same end result;

- an "Algorithm for the morphological assessment of osteomyelitis in an animal model" was created , which allows for a macroscopic and histological assessment of the activity of osteomyelitis, as well as to evaluate the restructuring of the bone tissue reparative process and compare the effectiveness of surgical treatment at different stages based on the data obtained ;

- evaluation of antibiotic therapy showed a positive effect of the use of a bone allograft impregnated with an antibiotic according to *the* original method in relation to *S. aureus* in osteomyelitis.

**Practical significance**

The research carried out in the dissertation work expands the existing ideas about the use of bone tissue substitutes for plastic surgery of extensive bone defects in chronic osteomyelitis using the presented new biodegradable bone allograft impregnated with an antibiotic according to the original technology, which is confirmed by the results of clinical and morphological studies.

The accumulated scientific base substantiates the potential possibility of using a bone allograft impregnated with an antibiotic according to the original technology in clinical practice, which can be used as an alternative material for filling bone defects in chronic osteomyelitis and has a morphological substantiation of the stages of the reparative process.

The product obtained by us will, after clinical trials, cover the need for a biological implant with a targeted antibacterial effect for the treatment of patients with chronic osteomyelitis.

 **Relationship of the thesis with other research papers**

dissertation work was carried out within the framework of grant funding of the Ministry of Education and Science of the Republic of Kazakhstan No. AP05133674 “Development and application of an antibiotic-impregnated allograft prepared according to the Marburg bone bank system for the treatment of osteomyelitis” .

**Author's personal contribution**

The dissertation student, together with the supervisor and the research team, developed a device for perforating bone allografts for the purpose of their further impregnation with an antibiotic. An experimental study was independently implemented on 144 laboratory animals to study structural changes in the defect zone and the efficiency of filling with a bone allograft using the original technology impregnated with an antibiotic with a histological examination, and an assessment of the adhesive properties of the allograft . The author carried out modeling of a femoral defect in experimental animals, followed by sampling of material for histological examination, removal of animals from the experiment. All operations on animals were performed by the author personally in a team with colleagues. The received material is systematized, documented and designed in the form of a dissertation work personally by the applicant.

**Approbation of work**

The main provisions of the dissertation were reported and discussed:

- at the international foreign congress "26 th EORS Annual Meeting " ( Galway , Ireland , 2018);

- at The III International scientific and educational conference “The Internationalization of continuing medical education. Prospection”, Aktobe , Kazakhstan , 2019 ;

- on international foreign 20th EFORT Congress ( Portugal ) 2019 ; \_ \_

- at the "Russian Science in the Modern World" XXVII International Scientific and Practical Conference. Digest of articles. Part 1. - Moscow: Research and Publishing Center " Actuality.RF ", 2020;

- on the European Congress of Trauma and Emergency surgery . - Oslo , Norway , 2020,

- at the 28th EORS Annual Meeting. - Izmir, Turkey, 2020;

- on 1st Virtual EFORT Congress, 2020;

- at an expanded meeting of the Department of Surgical Disciplines and the Department of Pathology of NJSC "Medical University of Karaganda";

- at the regional society of traumatologists and orthopedists.

**Publications**

Based on the materials of the dissertation, 18 scientific papers were published, of which:

- 3 in scientific publications recommended by the Committee for Control in Education and Science of the Ministry of Education and Science of the Republic of Kazakhstan;

- 3 publications in international scientific journals included at the time of publication of articles in the Scopus information base

- Received a utility model patent; 2 certificate of entering information into the state register of rights to objects protected.

**The structure and scope of the dissertation**

The dissertation is presented on 85 pages of computer typing of the Microsoft text editor Word, consists of an introduction, 3 sections of the main part, a conclusion and a list of sources used. The dissertation is illustrated with 6 tables and 49 figures. The list of references includes 211 sources in Russian and English.

**Materials and research methods.**

Research on the topic of the dissertation work was carried out in the period from 2018 to 2021 at the Department of Surgical Diseases, Vivarium, Department of Microbiology, Vivarium, Pathological Anatomical Laboratory of the Clinic of the Medical University NAO "MUK".

In the course of the experimental work, clinical, histological and microbiological changes were studied in the treatment of chronic osteomyelitis using a bone allograft prepared according to the Marburg system of a bone bank impregnated with an antibiotic.

The study as a whole was conducted on 144 outbred rabbits of both sexes of comparable age and weight.

Study design:

Formation of a model of chronic osteomyelitis

Group 2 - 18 rabbits (a cotton ball was introduced into the bone defect, previously soaked in a solution with SA, followed by closing the defect with Prime-Dent filling material)

Group 1 - 18 rabbits (0.2 ml of Staphylococcus aureus (SA) strain was introduced into the bone tissue defect)

Group 3 - 18 rabbits (a sclerosing drug (Ethoxysclerol) was added to the bone defect, then a cotton ball pre-moistened in SA solution)

group 4 - 18 rabbits (a cotton ball was introduced into the defect of the bone tissue, previously soaked in SA without additional filling of the hole)

Group 5 - 18 rabbits (a sclerosing drug (Ethoxysclerol) was introduced into the bone tissue defect, it was filled with a cotton ball pre-moistened in SA, the hole was closed with a filling preparation "Prime-Dent")

14 day

(n - 6)

28 day

(n - 6)

42 day

(n - 6)

Histological examination

Microbiological research

14 day

(n - 6)

7 day

(n - 6)

Study in rabbits with osteomyelitis model

Group 1 - 18 rabbits (biodegradable drug "PerOssal" impregnated with an antibiotic)

group 3 - 18 rabbits (perforated bone allograft impregnated with an antibiotic)

group 2 - 18 rabbits (one-piece bone allograft impregnated with an antibiotic)

14 day

(n - 6)

28 day

(n - 6)

42 day

(n - 6)

Histological examination

Microbiological research

7 day

(n - 6)

14 day

(n - 6)

The bone allograft used in the study was harvested from salvaged femoral heads from living donors after hip arthroplasty . All patients received a notarized consent to the removal of donor waste material. Permission was received from the Bioethics Committee (No. 4 of September 25, 2017) to conduct the study. Sampling was carried out at the clinical base of the NAO MUK in the trauma center of the Multidisciplinary Hospital named after Professor Kh.Zh. Makazhanova ". Criteria for inclusion of the femoral head in the study :

- diameter of the femoral head from 40 mm to 54 mm;

- the absence in the anamnesis of the patient - the donor of such diseases as: Hepatitis B or C, HIV;

Criteria for exclusion of femoral heads from the study were :

- the presence of cystic formations in the head of the femur

- the presence of severe osteoporosis of the femoral head

- the presence of aseptic necrosis.

All selected material was randomly divided into 2 groups depending on the method of application. The experiment included perforated and non-perforated femoral heads according to the described criteria. Perforation was carried out according to the developed technique using the original device for perforation . The device has through holes that fix the direction of the drill, which eliminates the "cleaver effect" with the risk of wedging and cracking of the bone. The presence of a given wall thickness in the oriented holes of the device of 10 mm allows you to avoid the smallest deviations of the drill during the formation of channels. The head of the femur is firmly fixed in the device, which prevents displacement and intersection of the channels. The use in the graft fixation device makes it possible to neglect the unevenness of the bone surface. The presence of the guiding holes of the device ensures uniformity in the distribution of channels, their parallelism, maintaining a given distance, as well as preventing the intersection of channels drawn from two sides, which has a positive effect on the strength of the allograft and on the impossibility of forming a cavity, in the place of which a hollow cavity can form during the formation of bone tissue . defect . A perforated bone allograft , unlike a solid one, makes it possible to evenly impregnate the bone tissue throughout its entire thickness, which was previously studied *in vitro .* After preparation and perforation, all bone allografts were placed in special containers with subsequent heat treatment in the Lobator sd-2 apparatus (according to the Marburg system) (Fig. 4) at an average temperature of 82.5 0 to 140 0 in the center of the femoral head . bones within 94 min. After heat treatment, bone allografts were soaked in an antibiotic. Gentamicin was chosen as an antibacterial drug for impregnation, which met the requirements for thermal stability and confirmed its characteristics at the preliminary stages of the study *in vitro by a* team of authors. Antibiotic impregnation of bone allografts in both groups was carried out by soaking them in a 4% solution of gentamicin for 90 minutes at room temperature.

*Staphylococcus* was used as an infectious agent in all animals. *aureus* (ATCC 43300) as the most frequently detected representative of the wound flora in patients with chronic osteomyelitis. Inoculum prepared from the third passage of a test culture of *Staphylococcus aureus* on soy broth ( tryptone soy broth) and casein digested soy agar ( tryptone soy agar ). The suspension density was adjusted to 5\*10 9 CFU/ml. A buffered saline solution with the addition of 15% gelatin was used as a diluent in order to increase the viscosity. This form of inoculum ensured the stability of the bacterial content in the experimental focus for a sufficiently long period. The medium was stored at 25°C in a sterile ­sealed container. Pre-suitability of *Staphylococcus strain aureus was* assessed by determining the minimum inhibitory concentration (MIC) with gentamicin (Protocol No. 14 of 10.04. 2018 - MIC = 0.5 µg/ml) by dilution method. The plates were incubated at 37°C for 24 hours. After 24 hours, the *Staphylococcus strain aureus* was used to form chronic osteomyelitis.

An experiment to create a model of chronic osteomyelitis was carried out on 90 outbred rabbits. The average age of the rabbits at the beginning of the experiment was 2±0.56 months, and the average individual weight was 2.4±0.49 kg. All rabbits were randomly divided into 5 groups . The experiment was carried out in the vivarium conditions of NJSC " Karaganda Medical University" in accordance with international ethical standards, approved by the ethical committee of the university (protocol No. 4 (13) dated September 25, 2017). The rabbits were kept in a room with controlled temperature (16-21°C) and relative humidity (45-65%). They were placed in special cages, 2-3 rabbits per cage, of the same age and the same weight. Animals of all groups were kept under the same conditions. They received a balanced diet consisting of mixed fodder, oats, forb meadow hay, carrots and apples.

During the simulation, daily clinical observation of experimental animals was carried out. Animals were weighed and thermometered throughout the entire experimental period.

A microbiological study of necrotic areas of soft tissues was performed on the 7th and 14th days of the experiment in order to determine the microbial agent.

An X-ray examination to assess the state of the bone tissue was carried out three times: on the 14th, 28th and 42nd day from the start of the experimental modeling.

A histological study of bones and bone tissue affected by osteomyelitis was performed on days 14, 28 and 42 of experimental modeling after euthanasia of rabbits by an overdose of an anesthetic drug.

Surgical procedures :

Surgery was performed under general anesthesia ( xylazine 7 mg/kg + ketamine 35 mg/kg im). The rabbit was fixed to the table in a lateral position. After removal of wool, the region of the lower third of the diaphysis of the left femur, the limb was treated with antiseptic solutions three times . The access was carried out along the anterior surface of the distal metaepiphyseal region of the left femur, a longitudinal incision with a layered dissection of the skin, subcutaneous tissue, fascia 3.0 cm long. Sharp hooks parted the edges of the wound. Raspator skeletonized femur on an area of 2.0 x 1.0 cm. A drill with a diameter of 2.0 mm in all groups formed defects in the periosteum, cortical layer and spongy substance . Depending on the method of modeling osteomyelitis in all rabbits, they were randomly divided into 5 groups . In group I , after perforation, 0.2 ml of *Staphylococcus aureus* ( SA ) strain was injected into the formed bone defect . In the second group , a cotton ball was introduced into the formed bone defect , previously soaked in a solution with SA, followed by closing the defect with the filling material " Prime - Dent ". In the III g of the group , a sclerosing agent was added to the formed bone defect ( Ethoxysclerol ), then a cotton ball previously soaked in SA solution. In group IV , a cotton ball pre-soaked in SA without additional filling of the hole was injected into the formed bone defect. In group V , a sclerosing preparation ( Ethoxysclerol ) was injected into the formed bone tissue defect , it was filled with a cotton ball previously moistened with SA, the hole was closed with a filling preparation " Prime - Dent " . After the surgical manipulations, the wounds in all groups were sutured in layers, and then treated with antiseptic solutions. The duration of the model development was 1.5 months. In the postoperative period, all rabbits received anesthetic Ketorol 0.1 ml per kg of body weight for 3 days. The presence of chronic osteomyelitis was assessed using clinical, microbiological, radiological and histological methods.

*of* rabbit infection with *St. aureus* was chosen and modeled chronic osteomyelitis, which worked out the surgical method of treatment. Experimental animals were randomly divided into 3 groups depending on the method of filling the bone defect. There were 18 rabbits in each group. Under general anesthesia (35 mg/kg of ketamine + 7 mg/kg of xylazine , i.m.), after 4-fold treatment of the surgical field with a solution of betadine , in the projection of the postoperative suture, excision of scars, sequesters, and non-viable tissues was performed. Excision was carried out within healthy tissues. The wound is abundantly washed with antiseptic solutions (hydrogen peroxide, chlorhexidine ). In group I , the defect was filled with a biodegradable drug " Perossal " impregnated with an antibiotic. Before using Perossal , according to the instructions, it was immersed in an antibiotic solution for 10 minutes. The recommended dose of the antibiotic was calculated from the calculation of 6 granules per therapeutic dose of the antibiotic. In group II , the defect was filled with a solid bone allograft prepared according to the Marburg system, impregnated with an antibiotic according to the previously described method. In group I - II , the defect was filled with a perforated bone allograft prepared according to the original method and soaked in an antibiotic solution according to the previously described method. After filling the defect, layer-by-layer suturing of the postoperative wound was performed. Assessment of the clinical condition of rabbits after treatment was carried out by measuring the temperature, weight, general motor activity of animals and the degree of wound healing. For this purpose, daily examination of animals, weighing and measuring body temperature was carried out. Determination of wound healing in operated rabbits was carried out by the presence of signs of inflammation, discharge of pus from the wound, marginal necrosis, consistency of sutures. Wound healing was considered satisfactory in rabbits without signs of inflammation, the absence of marginal necrosis, with full consistency of the sutures. Wound healing was considered unsatisfactory in the event of marginal necrosis, suture failure, and discharge. A visual assessment of the severity of the inflammatory process in the area of the postoperative wound and soft tissues was carried out, which was assessed in points: 0 points - no inflammation, 1 point - soft tissue infiltration in the projection of the postoperative wound, 2 points - suppuration of the postoperative wound.

In order to conduct a microbiological assessment, material was taken on the 7th and 14th day after the operation. The wound discharge and nearby tissues were placed in a sterile container directly from the focus of the treatment. Immediately after sampling, all the material was delivered to the Department of Biomedicine, where it was sown on a nutrient medium. The plates were incubated at a temperature of 37°C. The evaluation of the results was carried out by measuring the growth zone with a ruler with an accuracy of 0.1 mm of microorganisms from the edge of the seeding point after 24 hours. After the termination of the vital activity of the experimental animal, by means of an overdose of the anesthetic preparation, histological material was taken on the 14th, 28th and 42nd days. A representative fragment was a sample of bone tissue of a longitudinal section of the central part of the bone in the area of the formed defect, limited by the distal and proximal edges with the capture of visually normal bone tissue in an area of at least 10 mm. Morphometric evaluation was carried out on an area of 1 cm 2 at the site of surgical intervention (formed defect). Microscopic examination assessed the presence and / or duration of acute, chronic inflammation, the degree of regeneration / dysregeneration , the reaction of the bone plate to the manipulations. Acute inflammation is considered to be the presence of tissue necrosis (outside the allograft ) and granulocytic cells at the site of injury. Histological findings were described according to the nature of the morphological picture. Healing in all groups was assessed on a 5-point scale, which also included estimates of bone defect closure, lysis, and allograft restructuring.

The processing of the obtained results by statistical methods was carried out by the program STATISTICA v8.0. (StatSoft) with the calculation of the average value, standard deviation. For independent groups, testing of statistical hypotheses for quantitative indicators was carried out using non-parametric Mann-Whitney tests (when comparing 2 groups) and Kruskal-Wallis (for multiple comparisons). Results were considered statistically significant at p<0.05.

**CONCLUSIONS:**

1. The developed original method of antibiotic impregnation of a perforated bone allograft prepared according to the Marburg system allows all layers of bone tissue to be impregnated with an antibiotic.

2. The created model of chronic osteomyelitis in the experiment allows you to reproduce chronic osteomyelitis. The advantage of which is the absence of an acute course, the stable condition of laboratory animals, the development of a chronic purulent process.

3. The use of a perforated bone allograft impregnated with an antibiotic shows a 2-fold decrease in leukocytes on day 42 compared with the use of a whole bone allograft with an antibiotic, which indicates a decrease in the inflammatory process. At the same time, an active reparative process of bone tissue in the lesion was noted, as evidenced by active neoplasm of bone tissue 9 times more on day 14, and 2 times more on day 42 than with the use of a whole bone allograft with an antibiotic. Perforated bone allograft impregnated with an antibiotic by the formation of newly formed bone tissue in the lesion dominates over the biodegradable drug " PerOssal " by 1.2 times.

4. Microbiological evaluation showed that in the group with the use of a perforated bone allograft CFU after 2 weeks compared with the group where the biodegradable drug " PerOssal " was used decreased by 0.1 times (p>0.05), and after 4 weeks CFU decreased 34 times (p<0.05).