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Analysis of various models of chronic osteomyelitis in experimental animals

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Abstract

Introduction: Chronic osteomyelitis occurs in 3-25% of cases after open fractures and in 1-8% of cases after surgical treatment of closed fractures. It accounts for 3 to 10% in the structure of purulent surgical diseases. Traditional treatment methods many times do not provide a complete cure for osteomyelitis. In modern medicine, many experimental models have been created on different types of laboratory animals in order to reproduce the model of chronic osteomyelitis and subsequently improve its therapy. Of all the experimental animals, we chose rabbits for the application of the developed method for the treatment of chronic osteomyelitis and for the selection of the optimal model of bone tissue suppuration.

The **objective** was to conduct a clinical, radiological and histological analysis of various models of chronic osteomyelitis in experimental animals.

Materials and methods: The experiment was carried out on 90 outbred rabbits. All rabbits were randomly divided into 5 groups. The rabbits were kept in a room with controlled temperature (16-21 °C) and relative humidity (45-65%). The rabbits were placed in special cages – 2-3 rabbits per each. Surgical intervention was performed under general anesthesia. Surgical access was made along the anterior surface of the distal metaepiphyseal area of the left femur. All animals were intraosseus injected with Staphylococcus aureus as an infectious agent. The development of chronic osteomyelitis was assessed by clinical, radiological, microbiological, histological and statistical methods.

Results: The study showed that X-ray and histological methods play an important role in the analysis of experimental models. The signs of osteomyelitis development can be seen on the 14th day after the operation with the help of the X-ray method, while wound healing, weight and temperature cannot be indicators. According to the results of the experiment, the signs of chronic osteomyelitis developed in all 5 groups, but its activity was different. Histological examination showed that osteomyelitis developed differently in different groups, fibrosis developed unevenly.

Key words: bone infection, Staphylococcus aureus, rabbits, osteomyelitis model, experimental model

Introduction

The share of chronic osteomyelitis (CO) in the structure of purulent surgical diseases is 3-10% of cases with the probability of recurrence after surgical treatment [1-3]. In recent decades, the incidence of surgical infection has remained unchanged against the background of the use of modern antiseptics, antibacterial agents and improved surgical techniques [1-5]. The percentage of complications caused by suppuration of the musculoskeletal system in mechanical trauma is observed in 5.3-75.4% of patients. This is due, among other things, to an increase in the occurrence of high-energy injuries, an increase in surgical activity, iatrogenic factors (operation technique violation, non-compliance

with the rules of asepsis and antisepsis by surgeons), a change in the species composition of microorganisms, and impaired activity of the body's immune system [6-8]. Osteomyelitis develops in 3-25% of cases as a result of open fractures and in 1-8% after surgical interventions in closed fractures. At the same time, relapses of osteomyelitis occur in 20-35% of cases, which requires the further use of radical methods of treatment, up to amputation [9-12].

Traditional methods of antibiotic treatment and alternative methods of therapy have not led to impressive results of complete osteomyelitis cure [13]. In research medicine, many experimental models of osteomyelitis have been created in different animal species in order to induce a disease state and subsequently treat it [14-17]. It was necessary to choose the optimal model for the development of osteomyelitis in rabbits. To this end, we analyzed 5 different models of osteomyelitis in experimental animals.

Objective: to conduct a clinical, radiological and histological analysis of various models of chronic osteomyelitis in experimental animals.

Research tasks:

1. To compare the most commonly used animal osteomyelitis models.

2. To conduct clinical, radiological, microbiological and histological analysis of the selected models.

3. To determine the optimal model of experimental osteomyelitis for further research.

Materials and methods

The experiment was carried out on 90 outbred rabbits. The average age of the rabbits at the beginning of the experiment was 2±1 months, and the average individual weight was 2±1 kg. All rabbits were randomly divided into 5 groups. The experiment was realized in the vivarium of Non-commercial joint-stock company «Karaganda Medical University». The conditions of animals keeping in the vivarium ensured the experiment purity. The rabbits were kept in a room with controlled temperature (16-21°C) and relative humidity (45-65%). The rabbits were placed in special cages – 2-3 rabbits per each. The experiment was fulfilled in accordance with international ethical standards, approved by the Ethical Committee of the university (protocol №4 (13) from 25.09.2017).

Surgical operation was performed under general anesthesia (xylazine 7 mg/kg+ ketamine 35 mg/kg intramuscularly). To prepare the surgical field, the hairline in the surgical area was removed with scissors, treated with antiseptic solutions for three times and covered with sterile linen. 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 retractors parted the edges of the wound. The femur was skeletonized with a raptor over an area of 2.0x1.0 cm, defects in the periosteum, cortical layer, and spongy substance were formed by a drill with a diameter of 2.0 mm in all groups.

Staphylococcus aureus (ATCC 43300) was used as an infectious agent in all animals, as it was the most frequently detected representative of the wound flora in patients with chronic osteomyelitis. The animals were infected with a daily culture mixture in sterile saline at a dose of 106 CFU/ml.

In group I, 0.2 ml of Staphylococcus aureus (SA) was injected into the bone defect after perforation. In group II, a cotton swab pre-soaked in a solution with SA was input into the bone defect, followed by closing the defect with Prime-Dent filling material. In group III, Ethoxysclerol (a sclerosing drug) and a cotton swab moistened with SA solution were added to the created bone tissue defect. In group IV, a cotton swab pre-soaked in solution with SA without additional sealing of the hole was added to the perforated hole. In group V, Ethoxysclerol was injected after perforation of the distal femur, and it was also filled with cotton swab soaked in SA solution. However, then the hole was closed with Prime-Dent filling material. Wounds in all groups were sutured in layers.

The development of chronic osteomyelitis was assessed by clinical, radiological, microbiological and histological data. Clinical evaluation was performed based on the activity of the rabbit, measurements of temperature and body weight, as well as the condition of the postoperative wound. A visual assessment of the severity of the inflammatory process was carried out in the area of the postoperative wound and adjacent soft tissues. The assessment was carried out in points: 0 points – no inflammation, 1 point – inflammation of soft tissues in the projection of the postoperative wound, 2 points – suppuration of the postoperative wound.

X-ray examination was performed for all rabbits in 2, 4, 6 and 8 weeks after the defect formation. Radiography was performed in frontal and lateral projections using the device «Arman 1.8L3» in dose 75 kV, 25 mAc, 18 mA on digital cassettes. Features of the shape and structure of the bone tissue were visualized in the area of the focus, their exact location and configuration were determined. Digital X-ray images are archived on the PC of the Department of Surgical Diseases. Radiography was performed 1 time in 2 weeks for 8 weeks. X-ray assessment was carried out according to a scoring system, where the following indicators were evaluated: periosteal reaction: no – 0 points, yes – 1 point; osteolysis, foci of destruction: no – 0 points; yes – 1 point; involvement into the process of the diaphysis: no – 0 points, yes – 1 point; involvement of the bone marrow canal in the process: no – 0 points, yes – 1 point. The highest score of 16 points showed the severity of the osteomyelitic process throughout the entire observation period.

Microbiological studies were carried out at the Department of Clinical Immunology, Allergology and Microbiology of Non-commercial joint stock company « Karaganda Medical University». Biosubstrate sampling for bacteriological analysis was performed at 2 weeks after the bone defect creating. The material for the study was the metaepiphyseal zone of the femur. The sampling of material from the lesion with a total weight of up to 1 gm were placed in a sterile container. The material was taken by the postoperative wound dissection after the re-introduction of the rabbit into anesthesia. Microorganisms were identified by the classical cultural method on an analyzer [18].

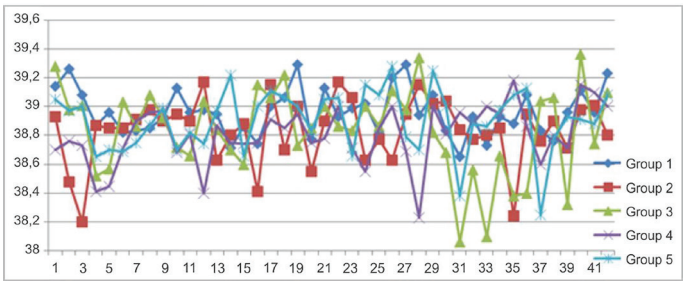
Histological examination was carried out after 2, 4, 6 and 8 weeks. All rabbits were euthanized from the experiment. Animals were euthanized by an overdose of anesthesia, then the test material was taken. The exarticulation of the femur was carried out after dissection of the implantation zone and the adjacent muscle mass, followed by osteotomy within healthy tissues at the level of the middle third of the thigh. The obtained material was sent to the Pathomorphological laboratory of the Department of Pathology of Non-commercial joint stock company «Karaganda Medical University».

Statistical data processing was carried out using a Microsoft Excel 2016 spreadsheet and STATISTICA 8.0 software (StatSoft, USA). The mean (M) and standard deviation (SD) were calculated for quantitative data with a normal distribution, in the case of a non-normal distribution – the median (Me) and quartiles (Q25; Q75). Qualitative values were presented as a percentage (in %) and its 95% confidence interval.

Results

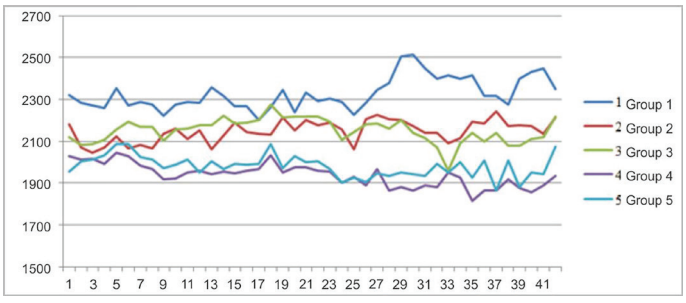
The body temperature of all experimental animals was measured with a non-contact thermometer during the entire observation period – 8 weeks. The body temperature curve in all five groups was similar: it gradually increased from the 1st to the 3rd day with a gradual decrease from the 3rd day to the 14th and was maintained at a stable level from the 14th to the 42nd day. The body temperature of rabbits in groups III, IV was higher than in groups I, II, V on the 3rd day ($p<0.05$) and there were no differences between five groups from the 3rd day to the 42nd ($p>0.05$) (Figure 1).

Figure 1 - Body temperature of rabbits in dynamics



The body weight of the rabbits was measured using standard scales during the entire observation period at the same time every day. The body weight of rabbits was lowest on the 3rd day. Rabbits of groups I, II, V lost less weight than in groups III, IV on the 3rd day ($p<0.05$). Body weight gradually increased from the 3rd day to the 42nd day and there was no difference between the five groups ($p>0.05$). On average, the total weight loss was 200±20 g (Figure 2).

Figure 2 - Body weight of rabbits in dynamics



Clinical evaluation of the data showed that in all groups, except group I, the animals showed exhaustion, decreased appetite, general condition depression, weak motor activity, and hair loss. After anesthesia termination, the operated animals on the first day after the operation did not lean on the operated limb, squeezing it, pulled the paw during examination, which was regarded as a sign of pain. Data on the state of the postoperative wound are shown in Table 1.

Table 1 Visual assessment of the postoperative wound

Group	2 weeks	4 weeks	6 weeks	8 weeks
Group I	0	0	1	0
Group II	0	0	1	0
Group III	0	1	2	2
Group IV	0	1	2	2
Group V	0	1	2	0

As can be seen from the Table 1, hyperemia, local temperature increase, fluctuation were observed from the 4th to the 8th week of observation in groups III and IV. The total score in these groups was 5, which indicates the severity of the clinical course. In group V, there was a moderate release of purulent exudate of a creamy consistency from the wound at the 6th week of observation, which healed on its own. In groups I and II, 1 point was registered – inflammation of soft tissues around the postoperative wound was noted at the 6th week and spontaneously disappeared during the next week.

The support ability of the limb improved from the second day after the operation in all groups, despite changes in the wound. The rabbits were actively moving. The general condition of the animals returned to normal.

Staphylococcus aureus was determined in the wound discharge in all groups in all animals during microbiological study after 2 weeks.

X-ray changes in the experimental groups are presented in Table 2.

As can be seen from Table 1, the most expressed periosteal reaction was observed in group III in all radiographs (15 points for the entire observation period). The least expressed reaction in terms of duration was observed in group I (1 point for the entire observation period). Osteolysis with destruction foci in groups III and IV was manifested in 3 out of 4 presented radiographs. The reaction from the diaphyseal part of the bone was the longest in group III throughout the entire observation period, while in group I it was completely absent. The reaction from the bone marrow canal was also observed throughout the observation period in group III and in group I there were no changes at all. Thus, the X-ray picture was most expressed in group III (15 points for all assessment indicators for the entire observation period), and the least expressed in group I (1 point for the entire observation period for all assessment indicators).

As can be seen from the presented series of X-ray images, X-ray changes in group I were noted only in the form of a periosteal reaction and persisted for 4 weeks. Changes regressed by 6-8 weeks of the study (Figure 3). Signs of osteolysis, destruction foci involving the medullary canal were noted in groups II and V from the 4th week and began to decrease by the 8th week (Figure 4 and 7). In groups III, IV, an extensive defect of the bone tissue in the metaepiphyseal area with alternating foci of clarification and darkening was revealed, while in group III the joint was involved (Figure 5, 6).

Table 2 X-ray control in dynamics

Group/ week / Parameter	Group I				Group II				Group III				Group IV				Group V			
	2	4	6	8	2	4	6	8	2	4	6	8	2	4	6	8	2	4	6	8
Periosteal reaction	0	1	0	0	0	1	1	0	1	1	1	1	0	1	1	1	0	1	1	0
Osteolysis, destruction foci	0	0	0	0	0	1	1	0	0	1	1	1	0	1	1	1	0	1	1	0
Involvement into the diaphysis process	0	0	0	0	0	1	1	0	1	1	1	1	0	1	1	1	0	1	1	0
Marrowy canal	0	0	0	0	0	1	1	0	1	1	1	1	0	1	1	1	0	1	1	0

Figure 3 - Radiography of group I in dynamics (A – 2 weeks, B – 4 weeks, C – 6 weeks, D – 8 weeks)

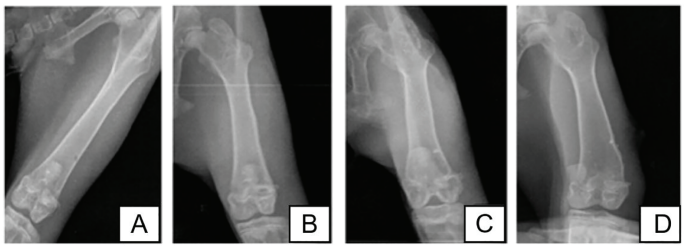


Figure 4 - Radiography of group II in dynamics (A – 2 weeks, B – 4 weeks, C – 6 weeks, D – 8 weeks)

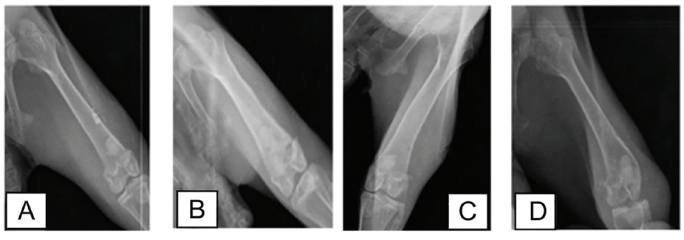


Figure 5 - Radiography of group III in dynamics (A – 2 weeks, B – 4 weeks, C – 6 weeks, D – 8 weeks)

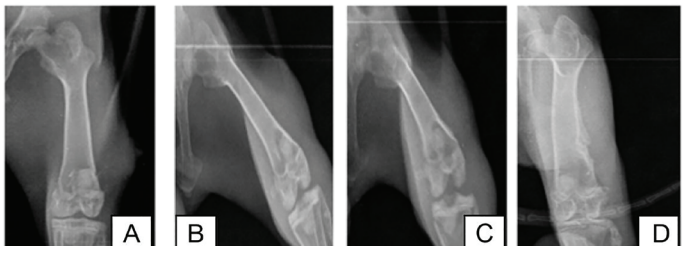


Figure 6 - Radiography of group IV in dynamics (A – 2 weeks, B – 4 weeks, C – 6 weeks, D – 8 weeks)

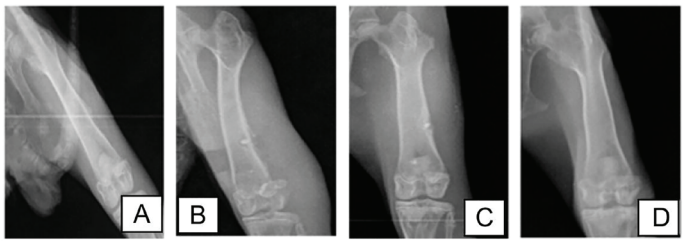
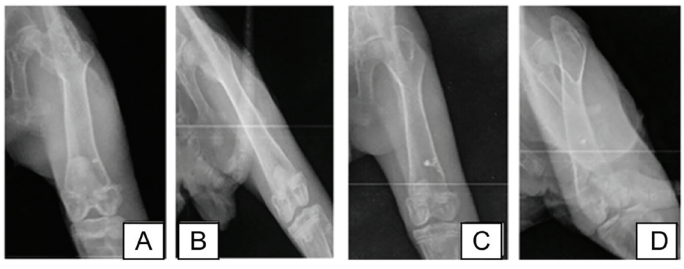


Figure 7 - Radiography of group V in dynamics (A – 2 weeks, B – 4 weeks, C – 6 weeks, D – 8 weeks)



Histological examination was performed once in 2 weeks after surgery. The following indicators were evaluated: the presence of periosteal reactions, changes in the medullary canal, the presence of osteonecrosis, soft tissue necrosis, granulocytic, lymphocytic, plasma, macrophage infiltrate. Osteomyelitis was considered to be actively proceeding in the presence of all these factors.

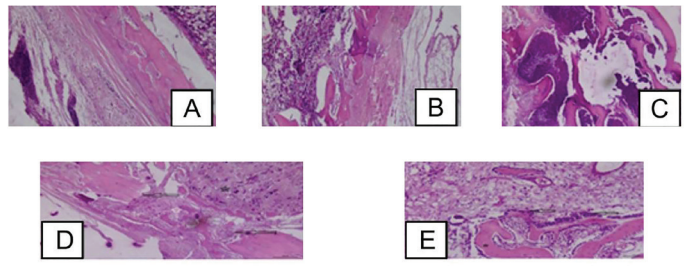
Histological examination revealed the following changes: chronic inactive osteomyelitis was formed in group I. The formed defect of the cortical plate was partially replaced by fibrous tissue with activated osteoblasts. Chronic inflammation was noted in the adjacent soft tissues and periosteum. The intramedullary area was represented by bone marrow with mature adipose tissue (Figure 8A).

Chronic inactive osteomyelitis was also registered in group II. For microscopy, the part of the formed cortical defect was presented, where the bone plate was partially replaced by fibrous tissue. The intramedullary area was with mild fibrosis, predominantly in the subcortical region. The integrity of the cortical plate was preserved. Osteocytes in the bone matrix were located in lacunae. Inactive osteoblasts were determined on the surface of bone trabeculae. The bone marrow area was filled with mature fat (Figure 8B).

Chronic active osteomyelitis of the 3rd stage and severe degree was detected in group III after 2 weeks of the study. Cortical and intramedullary cavities were filled with necrotic detritus and leukocytes merging with each other. The bone beams were of indefinite shape, thinned, in a state of necrosis and necrobiosis. The adjacent intramedullary area was presented by fibrous tissue infiltrated by lymphocytes, plasma cells, and granulocytes (Figure 8C).

Chronic active osteomyelitis developed gradually in group IV. The histological picture showed mild osteomyelitis after 2 weeks of the study, which gradually progressed to stage 3 at the 4th and the 6th weeks of the experiment. For the study, a place for modeling a defect in the bone was selected. The cortical bone defect was replaced by fibrous tissue with newly formed bone trabeculae surrounded by osteoblasts. Lymphoplasmacytic infiltration and fragments of foreign material with single giant multinucleated cells of foreign bodies were noted in the thickness of the fibrous tissue (Figure 8D).

Figure 8 - Histological examination of chronic osteomyelitis (A – group I, B – group II, C – group III, D – group IV, E – group V



Chronic active osteomyelitis of the 1st stage and mild degree was formed in group V. Severe fibrosis with mild lymphoplasmacytic infiltration and fragments of foreign material with single giant multinucleated cells of foreign bodies were noted. Newly formed subperiosteal bone tissue with active osteoblasts was recorded. The bone tissue lacked the plates or lines present in mature bone. Fibrous tissue with mild lymphohistiocytic infiltration was present in the intramedullary space. The intramedullary space was replaced by fibrous tissue with scattered lymphoplasmacytic infiltration. Bone beams were thin, single, with active osteoclasts (Figure 8E). A total of 90 rabbits were used, 5 groups of 18 rabbits per group.

Discussion

5 variants of osteomyelitis models were selected to achieve the objective and tasks of the study. Animals were subjected to clinical observation after the formation of all 5 models of osteomyelitis. Radiography and histological analysis were performed. According to the obtained results, the optimal model was chosen for the creation of chronic osteomyelitis

in order to conduct further research aimed at treating this pathology. Methods for the formation of models of experimental osteomyelitis are described in detail in the materials and methods chapter.

The earliest authors of experimental models of osteomyelitis in rabbits were Sheman in 1941 [19] and Norden in 1970 [20]. Both researchers developed osteomyelitis on the tibia of rabbits.

Norden used a sclerosing agent in the metaphysis of the tibia, injected with a suspension of S. aureus to produce progressive chronic osteomyelitis. The infection rate was 70 or 100%, depending on the bacterial load. In addition, the degree of infection ranged from mild to severe local bone changes in the analyzed studies, especially when the time to develop osteomyelitis varied from 2 to 4 weeks and according to differences in the bacterial strain used. The sclerosing material has mainly been used to evaluate new antibiotic therapies or antimicrobial drug delivery systems [21, 22, 23-31].

The models proposed by Scheman [19] and Norden [20] resembled hematogenous osteomyelitis. It usually occurs in children and becomes chronic if left untreated and diffuses through the tibial metaphysis.

Early publications include studies by Rodeheaver et al. in 1983 [32]. They inoculated S. aureus and E. coli into the intramedullary canal of the femur through an opening in the medial femoral condyle. Bone cement was injected through the same opening into the canal to promote the formation of osteomyelitis. Jacob et al. [33] reported their model of osteomyelitis on the femur in 1985 according to the method of Norden and Kennedy. Osteomyelitis was caused by intramedullary injection of a sclerosing agent (3% sodium tetradecyl sulfate) and S. aureus (5×106 CFU). X-ray showed osteomyelitic changes in 10 of 13 animals that survived over the 10-week time period. The diagnosis was confirmed histopathologically in 8 out of 10 cases. Later, Schulz et al. (2001) [34] reported a similar femoral model: 1.0 ml of bone marrow was taken through a biopsy needle inserted into the interpuncture fossa. Next, 0.1 ml of sodium morruate, S. aureus, and saline were injected. The results showed that the incidence of chronic progressive osteomyelitis was increased up to 100% using this method.

Rutledge et al. (2003) [35] created the same model by percutaneous injection. Models from other publications are similar – sclerosing agents and pathogens are injected through holes made in the proximal femur [36-37].

Despite the presence of studies indicating that the development of osteomyelitis with the addition of just SA or with the use of a filling preparation allows the use of these methods for an experimental model, in our case they did not confirm themselves. In groups where SA is simply added to the perforation hole and when using a filling preparation, the clinical picture of chronic osteomyelitis rarely develops, self-curability does not allow evaluating the possibilities of treatment. The use of a sclerosing drug allows the development of rapidly flowing chronic osteomyelitis with a high prevalence. The use of medical cotton wool (hygroscopic material as a carrier) allows the development of long-term chronic osteomyelitis, which cannot be cured on its own.

Conclusions

1. Clinical assessment showed an expressed severity of the course of the disease in group III, where the score was 15 points according to the scale. In group I, 0.2 ml of Staphylococcus aureus (SA) was injected into the bone defect after perforation. In group II, a cotton swab pre-soaked in a solution with SA was input into the bone defect, followed by closing the defect with Prime-Dent filling material. In group III, Ethoxysclerol (a sclerosing drug) and a cotton swab moistened with SA solution were added to the created bone tissue defect. In group IV, a cotton swab pre-soaked in solution with SA without additional sealing of the hole was added to the perforated hole. In group V, Ethoxysclerol was injected after perforation of the distal femur, and it was also filled with cotton swab soaked in SA solution. However, then the hole was closed with Prime-Dent filling material. Wounds in all groups were sutured in layers.

2. Temperature changes in the groups showed a gradual increase from the 1st day to 3nd with a gradual decrease from the 3nd day to the 14th. The body temperature of rabbits in groups III, IV was higher than in groups I, II, V on the 3rd day ($p<0.05$). There was no difference between 5 groups from the 3rd day to 42nd ($p>0.05$).

3. Changes in the body weight of rabbits showed that on the 3rd day there was a maximum weight loss of up to 220 g ($p<0.05$). The average total weight loss was 200±20 g.

4. X-ray assessment showed that the X-ray picture was most expressed in group III (15 points for all evaluation indicators for the entire observation period), and the least Expressed in group I (1 point for the entire observation period for all evaluation indicators).

Clinical assessment does not give a clear picture of the development of chronic osteomyelitis. The clinical picture of experimental animals is blurred, so X-ray and histological studies are required, which show local changes after 2 weeks from the start of the study. The X-ray and histological picture showed an actively flowing chronic osteomyelitis

It was revealed that the use of a sclerosing agent and a hygroscopic material gives the greatest effect. The introduction of a sclerosing drug leads to a rapid course of chronic osteomyelitis. Thus, the study showed that the modeling of osteomyelitis with hygrocosmic material (medical cotton wool) in 100% of cases leads to the development of classical active chronic osteomyelitis, which develops gradually. But the use of a sclerosing drug (Ethoxysclerol) led to a rapid course of chronic osteomyelitis.

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