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In vivo effectiveness of polymer hydrogels impregnated with an antibacterial drug in chronic osteomyelitis

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Abstract

Introduction Polymethyl methacrylate (PMMA) is a common depot system in the treatment of chronic osteomyelitis. However, a lot of its shortcomings do not allow us to consider it ideal. **Purpose** of the work was to study *in vivo* the effectiveness of a polymer hydrogel containing an antibiotic for chronic osteomyelitis of the tibia in a rabbit model and compare it with PMMA **Materials and methods** The study was performed on the lower leg of 25 mature Chinchilla rabbits. A model of chronic osteomyelitis of the tibia was created. A methicillin-sensitive strain of *Staphylococcus aureus* (MSSA), highly active against cefazolin, was chosen as an infectious agent. Surgical debridement started 21 days after the clinical, laboratory, radiological and microbiological confirmation of the diagnosis, the technique for all animals was the same. The experimental group (n = 11) was treated by implantation of a polymer hydrogel, the comparison group (n = 11) with PMMA, and the control group (n = 3) had no implanted substance. In the postoperative period, monitoring of the local status, weight and body temperature of the animals, microbiological and radiological studies were carried out. Animals were taken out of the experiment by stages. Biopsies were sent for bacteriological and histomorphometric studies. Statistical comparison of the groups was performed using the Mann - Whitney, Kruskell - Wallis and Tukey criteria, descriptive statistics were used for the control group. **Results** In the experimental group, in all cases, postoperative wounds healed in a timely manner, the levels of WBC and CRP significantly ($p = 0.040$) decreased from 14 and 21 days, respectively. Microbiologically, the growth of microflora from the wound discharge and biopsy specimens was not detected; radiographic progression of chronic osteomyelitis was not observed; histomorphometry revealed a significant ($p = 0.002$) effective relief of the inflammatory process. In the comparison group, systemic antibiotic therapy was required from postoperative day 7. Levels of inflammatory markers decreased less effectively than in the experimental group. MSSA was verified from wound discharge and biopsy specimens at almost every follow-up time-point. X-rays and histomorphometry ($p = 0.001$), on average, detected exacerbation of osteomyelitis. In the control group, systemic therapy did not give positive dynamics. **Discussion** A comparative analysis showed that the hydrogel impregnated with an antibacterial agent, unlike PMMA, reliably arrests chronic osteomyelitis without auxiliary systemic antibiotic therapy and does not cause material-associated bone resorption. The clinical and laboratory picture is fully consistent with the data of microbiology, radiology and histomorphometry. **Conclusion** Hydrogel impregnated with an antibiotic reliably and effectively stops chronic osteomyelitis compared to PMMA.

Keywords: chronic osteomyelitis, polymer hydrogel, bone cement, polymethyl methacrylate, orthopedic infection, PMMA, *in vivo* study, experimental model

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INTRODUCTION

Chronic osteomyelitis is a challenging problem in modern traumatology and orthopaedics since it is considered one of the most severe infectious complications [1] and accounts for 3-25 % of all diseases of the musculoskeletal system [2]. As is known, chronic osteomyelitis triggers a whole cascade of successive reactions, resulting in inflammation, microbial biofilms, sequesters and destruction of bone tissue [1, 3].

The main method of combating microbial biofilms is the removal of an infected implant, if present, radical surgical debridement, and a combination of local and systemic antibiotic therapy. The combination of local and systemic therapy has shown better eradication of infection in animal models compared to the use of systemic antibiotics alone, since the bacterial biofilm hinders their penetration and ultimately contributes to a change in the metabolic state of microbial colonies and the acquisition of resistance to the drug used [4]. Depot systems are used as local therapy. The most common system in clinical practice for delivering antibiotics to the site of infection is bone cement based on polymethyl methacrylate (PMMA). However, despite the many ways to improve the methods of treatment and the structure of PMMA, recurrence of chronic osteomyelitis is observed in 20-30 % of cases, and in 16.75 % the treatment ends with amputation of the limb [1]. Moreover, such limitations as the elution of only 10 % of the impregnated drug, hydrophobicity and bioinertness of the material, requiring repeated surgical intervention to remove it [4] and contributing

to an increase in the duration of treatment and additional stress on the patient's body [1], as well as high temperature polymerization (up to 120 ºC), which causes bone tissue necrosis and limits the range of drugs used, does not allow us to consider the PMMA system as ideal. Therefore, a search for new local transport systems has been recently carried out that would be devoid of the shortcomings of bone cement. Preferences are mainly given to biodegradable depot matrices, one of which is hydrogel [5].

Purpose: to study *in vivo* the effectiveness of chronic tibial osteomyelitis on a rabbit model with an antibiotic-impregnated polymer hydrogel and compare it with PMMA.

MATERIALS AND METHODS

In vivo study was performed on 25 adult Chinchilla rabbits aged 5-6 months with an average weight of 2608 ± 112 g. All animals were males to exclude the effect of sex differences on the results of the study. Before inclusion in the study, the rabbits were subjected to examination by a veterinarian and were found to be healthy. The animals were allowed to acclimatize for 10 days prior to the start of the experiment. Feeding was carried out 3 times a day, water supply was constant. Sawdust was used as bedding. Much care was paid to a daily cleaning of the space and cages in order to prevent exogenous infection.

In all cases, the study segment was the lower leg. Modeling of chronic osteomyelitis was performed in the proximal tibia according to the previously described method [6]. The surgical technique was the same for all rabbits. Animals were anesthetized by intramuscular administration of Zoletil 100 and Meditin 0.1 % at a dosage of 30 mg/kg and 0.5 ml / 5 kg, respectively. Methicillin-susceptible *Staphylococcus aureus* (MSSA) [7, 8, 9], which is highly active against cefazolin and was isolated from intraoperative biopsies of patients treated by us, was used as an infectious agent as the leading etiological structure of orthopedic infections. After being infected, the rabbits developed chronic osteomyelitis for 21 days [6, 10]. Once the diagnosis was confirmed, they underwent surgical debridement of the focus of infection. Surgical debridement was performed according to the same technique. The distribution of rabbits into groups (experimental, comparison and control) was carried out randomly. After being infected and until the end of the study, all animals were kept in individual cages under vivarium conditions with 12-hour cycles of light and darkness at a temperature of 20 ± 1 °C and air humidity of 50-70 %.

In the postoperative period, like at the stage of infection and debridement, the wound area was treated with a 0.05 % chlorhexidine solution until it was completely healed. Monitoring of the local status, weight and body temperature of animals and blood parameters (hemoglobin (HB), leukocytes (WBC), C-reactive protein (CRP) and ESR) was performed on days 3, 7 and then with an interval of one week up to 21 days at the stage of infection and up to 42 days after the debridement. A microbiological study of wound discharge (if any) and of biopsy material taken at the stage of withdrawal of the animals from the experiment was conducted. Radiographic study was performed the next day after the operation, on the 21st day of the infection stage and on the 3rd, 15th, 30th and 45th days of the sanitation stage.

The duration of the sanitation phase was 45 days. The animals were taken out of the experiment on the 15th, 30th and 45th day. The rabbits were euthanized under anesthesia by air embolization through the ear vein. After harvesting bone and soft tissue biopsy material, including fragments of the compared depot systems, for microbiological examination, the bone was placed in a 10 % formalin solution and sent to the pathoanatomical laboratory for further histomorphometric examination. Micropreparations were examined with an AxioLab A1 light-optical microscope at a magnification from 100× to 400×.

Technique of surgical debridement of the infection focus In a fixed supine position under general anesthesia, having previously taken the discharge material if there was a fistula or wound, the hair was shaved and the surgical area was disinfected. After treating the surgical field three times with antiseptic solutions, a 3-cm long skin incision was made along the old postoperative scar of the tibia. Purulent streaks/ pockets were opened to evacuate the contents, if any. After excision of the altered soft tissues, the area of the defect was exposed and expanded with a utter to a size of 0.6 × 0.6 cm and a depth of 4 mm. Further, an additional hole was drilled somewhat distally from the defect to the same depth and size of 0.4 × 0.4 cm in order to take a bone biopsy for histological confirmation of infection. Next, the bone marrow canal was debrided with spoons for removal of pus, small sequesters and granulation tissue. Intra-operative biopsies were sent to the microbiological laboratory. The canal was abundantly washed with 20 ml of an antiseptic solution and hemostasis was performed.

After changing gloves, changing and additional processing of the surgical field, we proceeded to the stage of implantation of intra-operatively manufactured depot systems based on polymer hydrogel and PMMA, saturated with 300 mg of cefazolin, according to the previously described method [5]. The experimental group (n = 11) was injected with 2 ml of polymer hydrogel into the medullary canal using a syringe through a catheter. The comparison group (n = 11) received solitary bone cement prior to the start

of the polymerization reaction. In the control group (n = 3), no material was implanted. The operation was completed by layer-by-layer suturing of the wound.

The morphometric assessment of the infection process on histological preparations was carried out using the HOES scale (Histopathological Osteomyelitis Evaluation Score) [11], which is a graduated semi-quantitative and additive form for assessing the criteria for acute (A1-A3) and chronic (C1-C2) osteomyelitis. The following criteria were assessed: A1 – osteonecrosis, A2 – soft tissue necrosis, A3 – granulocyte infiltrate, C1 – bone neogenesis/fibrosis, and C2 – lymphocyte/macrophage infiltrate. Depending on the severity, determined by the number of formed elements per unit area of the infiltrate, each criterion was assigned a score from 0 to 3, where 0 is the absence of inflammation sign; 3 is severe inflammation. The sum of points A1-A3 $\geq$ 4 of the histopathological picture corresponded to acute osteomyelitis; A1-A3 and C1-C2 $\geq$ 6 to exacerbation of chronic osteomyelitis; C1-C2 $\geq$ 4 to chronic osteomyelitis; C1-C2 $\leq$ 4 points to subsidence of chronic osteomyelitis; C1-C2 $\leq$ 1 point to no signs of osteomyelitis. The area was measured using the MegaMorph12 morphometric program.

The obtained data were statistically processed using the IBM SPSS Statistics 22 and SigmaPlot 11.0 software package. The weight of animals and laboratory blood tests for the experimental and comparison groups of rabbits on the check days of the study are presented as the mean ± standard deviation (μ ± Sd). In order to identify intergroup significant differences, the Mann – Whitney test was used. Values were considered significant at $p < 0.05$. Descriptive statistics were used for the control group.

The significance of histomorphometric results was determined by the nonparametric Mann – Whitney test and the Kruskal – Wallis multiple comparison test. When intergroup differences were found, one-way analysis of variance with Tukey's post hoc comparisons was used. Values were considered significant at $p < 0.05$. Statistical data are presented as median and interquartile range (Me (25 %; 75 %)) as a range chart.

The experimental work was approved by the ethics committee of the scientific council of the Federal State Budgetary Institution Priorov National Medical Research Centre for TO of the Ministry of Health of the Russian Federation and was carried out in accordance with the ethical standards for the treatment of animals in compliance with the recommendations and requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 2006). All manipulations on animals were carried out in accordance with the Declaration of Helsinki on the Humane Treatment of Animals (2000) and with the order of the Ministry of Health and Social Development of the Russian Federation No. 708n dated August 23, 2010 "On approval of the rules of laboratory practice". Animals were kept under the conditions specified in the "Guide for Care and Use of Laboratory Animals" (1996).

RESULTS

By the 21st day of the infection stage, the development of a fistula in the area of the postoperative scar was noted in 60 % of rabbits (n = 15), an abscess in 12 % (n = 3), wounds with thick purulent discharge in 16 % (n = 4), hyperemia and /or hyperthermia of the skin in 3 (12 %) cases. In 44 % of the animals (n = 11), the above symptoms were accompanied by an increase in body temperature, which averaged 41.7 ± 0.9 °C by the end of the infection stage.

All animals during the observation period had decreased appetite, were less active, more than half of the cases acquired a forced, limb-sparing position, which caused hypotrophy of the thigh and lower leg muscles. Lameness on the involved limb by walking was noted in 22 (88 %) rabbits. The body weight of the animals during the infection stage decreased on average by 564 ± 113 g. The levels of inflammatory markers in blood tests were higher than normal. The data of the results of laboratory blood parameters for each group are presented in Table 1.

By the 21st day after infection, there were radiographic signs of chronic osteomyelitis: heterogeneous structure of bone tissue with alternating foci of sclerosis and areas of enlightenment (Fig. 1 a), osteoporosis, periostitis of varying severity (Fig. 1 b, c), blurred contours of post-trepanation defect and bone sequestration detected in 7 (28 %) cases. Bacteriological study of the fistula discharge (n = 15) and intra-operative biopsies taken from all rabbits (n = 25) during surgical debridement verified the growth of MSSA.

Table 1

Laboratory blood tests versus the reference values on day 21 after infection of the rabbit's tibia

Tests	Reference values	Day 21 upon contamination (μ ± Sd)		
		Experimental group (n = 11)	Comparison group (n = 11)	Control group (n = 3)
Hb	110-136	104.6 ± 6.5	107.4 ± 8.8	108.3 ± 10.6
WBC	2.5-6.9	11.1 ± 1.8	10.5 ± 2.2	9.6 ± 1.9
ESR	1-4	8.8 ± 3.4	8.5 ± 3.1	6.3 ± 2.1
CRP	0-1	41 ± 15.5	43 ± 16.3	36 ± 22.6

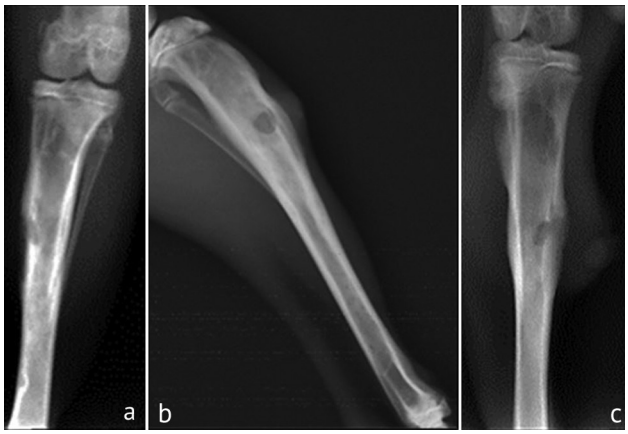

Fig. 1 Radiographs of the rabbit's tibia on the 21st day after infection: *a* heterogeneous structure of the bone tissue; *b* periosteal reaction and osteosclerosis; *c* fistula at the top of the abscess

Histological study showed that there were moderate and significant amounts of lipocytes in the reticular stroma of the bone marrow and the Haversian canals of the cortical plate (Fig. 2 a) and the septa between which were thickened and rich in fibroblast-like cells, gentle basophilic and oxyphilic fibers. Between the latter, cells of the lymphoplasmacytic series were detected in a moderate and significant amounts (Fig. 2 a). The fields of the lamellar bone in the proper bone marrow and some bars of the cancellous bone did not have osteocytes, and the lacunae were empty over large and small areas. Dilated Haversian canals showed signs of resorption and osteoreparative regeneration with layers of new bone substance with intact osteocytes and unusual bending in the lines of bone lamellar binding (Fig. 2 b). At the same time, the resorbable bone matrix at the border with the inflammatory infiltrate had a sharply oxyphilic edge, which indirectly indicated local acidification of the interstitial substance and, accordingly, the ongoing infectious inflammatory process.

In general, the results of clinical laboratory, radiological, microbiological and histological studies confirmed the development of chronic osteomyelitis of the tibia in rabbits by day 21 of the infection stage.

After surgical debridement of the infection focus in the experimental group (n = 11), wound healing proceeded without complications in all animals. The body temperature of the rabbits with the implanted hydrogel fluctuated within the normal range (N = 37-39.5 ºC). In the comparison group (n = 11), by the end of the first week, six (54.5 %) animals required systemic antibiotic therapy by intramuscular injection of cefazolin at a dosage of 30 mg/kg 3 times a day due to the absence of regression of clinical symptoms of chronic osteomyelitis. By week 3, the dosage of the drug was increased to 40 mg/kg in three (27.3 %) rabbits without positive dynamics. However, despite intensive therapy, we failed to achieve complete arrest of the infection in two (18.2 %) cases. In the control group, systemic antibiotic therapy turned out to be ineffective, since at each control period the animals retained certain signs of inflammation. Body temperature by the end of the study in that group was 40.7 ºC, which is above the upper limit of the norm.

Intergroup statistical data on the body weight of animals of the studied groups in dynamics are presented in Table 2.

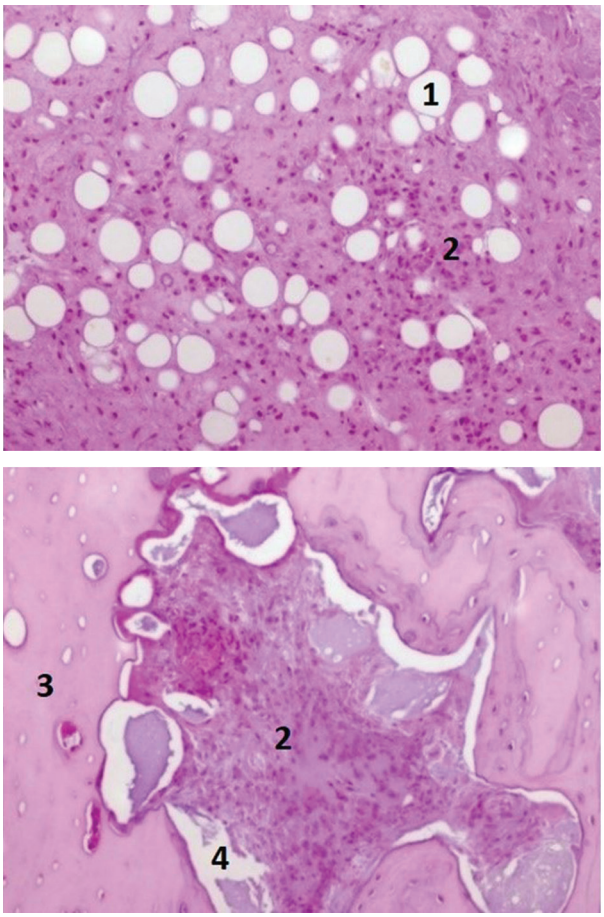

Fig. 2 *a* reticular stroma of the bone marrow with lipocytes (1), infiltration by lymphocytes and plasma cells (2); *b* bone matrix fields devoid of osteocytes (3). Bone resorption (4). Bone marrow infiltration of Haversian canals with lymphocytes and plasma cells (2). Stained with hematoxylin and eosin. ×200

Table 2

Weight of rabbits in each study group after surgical debridement on the control days of the study

Check day	Experimental group (μ ± d)	Comparison group (μ ± Sd)	Control group (μ)	p value (Mann – Whitney test)*
3	2418 ± 110.7	2435 ± 126.3	2294	p = 0.870
7	2989 ± 139.9	2909 ± 140.9	2764	p = 0.279
14	3639 ± 213.5	3512 ± 200.7	3323	p = 0.324
21	4223 ± 263.5	4012 ± 274.8	3945	p = 0.123
28	4901 ± 280.8	4544 ± 306.7	4751	p = 0.006
35	5578 ± 263.1	5175 ± 324.2	5393	p = 0.005
42	6308 ± 152.3	5948 ± 283.3	6116	p = 0.000

Note: * statistical difference for experimental and comparison groups

The dynamics of laboratory blood tests for the experimental and comparison groups presented as arithmetic mean values with a standard deviation, and for the control group as arithmetic mean values are given in Table 3. The statistical data of blood tests in the control periods of the study for the experimental and comparison groups are shown in Table 4.

Table 3

Dynamics of blood tests in the control study days in the experimental and comparison groups

Day	Blood tests			
	HB	WBC	ESR	CRP
	N = 110-136	N = 2,5-6,9	N = 1-4	N = 0-1
Experimental group (μ ± Sd)				
3	115.2 ± 9.7	9.4 ± 1.1	4.6 ± 2.1	35.1 ± 5.7
7	117.1 ± 7.3	8.6 ± 1	3.6 ± 1.6	23.5 ± 10.1
14	122.2 ± 6.2	7.4 ± 1.5	3.5 ± 2.7	15.1 ± 8.7
21	124.2 ± 8.2	6.9 ± 2.8	2.5 ± 0.7	12.5 ± 12.6
28	129.6 ± 7.4	5.4 ± 2.3	3 ± 2.5	8.2 ± 7.7
35	128 ± 5.8	5.2 ± 3.2	1.8 ± 1	4.7 ± 4.1
42	129.7 ± 4.5	3.9 ± 1.5	1.7 ± 1.1	2.7 ± 4.1
Comparison group (μ ± Sd)				
3	115 ± 12.1	10.1 ± 1.9	5 ± 1.8	42 ± 11.6
7	116.1 ± 5.9	9.6 ± 1.8	4.1 ± 1.7	29 ± 13.1
14	117.2 ± 11.7	8.7 ± 1.7	3.8 ± 1.8	19.8 ± 15.5
21	119. ± 12.6	8.8 ± 2.4	3.3 ± 1.5	18.3 ± 8.1
28	121.8 ± 17.9	8.1 ± 3.5	3.6 ± 2.1	14.3 ± 5.5
35	120 ± 19.1	8.1 ± 3.6	3.4 ± 1.9	14.7 ± 10.1
42	119 ± 14.3	7.7 ± 4.5	3.1 ± 2.4	11.8 ± 9.4
Control group (μ)				
3	121	9.03	4.7	37
7	116.3	8.6	4	20.7
14	121.7	8.1	4	17.7
21	118.5	8.2	2	11.5
28	126.5	9.2	2	11
35	132	6.8	2	19
42	128	7.9	4	24

Table 4

Statistical differences in inflammation markers in rabbits of the experimental and comparison groups (Mann – Whitney test)

Day	Blood tests			
	HB	WBC	ESR	CRP
3	0.478	0.478	0.748	0.116
7	0.478	0.151	0.652	0.519
14	0.606	**0.040***	0.332	0.562
21	0.297	**0.024**	0.340	**0.040**
28	0.436	**0.040**	0.258	*0.063*
35	0.209	*0.053*	*0.097*	**0.011**
42	*0.053***	**0.011**	0.383	**0.011**

Note: * statistically significant differences ($p < 0.05$) for polymer hydrogel are in bold; ** – statistical values, where $0.05 < p < 1$, are in italics

There were no obvious signs of progression of chronic osteomyelitis in the experimental group studied by X-rays; post-trepanation defects were replaced evenly, by the end of the study, the holes were almost completely closed (Fig. 3). In the comparison group, in all checking periods, on average, there were radiographic signs of ongoing infection of the tibia such as periostitis of varying severity, subperiosteal cystic cavities, heterogeneous structure of bone tissue, sequestration, and delayed osteoreparation (Fig. 4). In a number of cases, the spread of the osteosclerosis zone to the middle third of the bone diaphysis was observed. Resorption at the "bone-cement" border was traced on the images from the 15th day of the study. In the control group, the same situation was observed but the difference was in a pronounced degree of periosteal response and osteosclerosis of the entire thickness of the upper third of the tibia with spread to its lower parts (Fig. 5).

Microbiological analysis of wound discharge in the experimental group showed no growth of microflora. Negative results were also obtained in the bacteriological study of biopsy specimens of gradually withdrawn rabbits. The obtained single positive growth of MSSA from the soft tissue biopsy of the animal removed from the experiment on the 30th day after the implantation of the hydrogel was regarded by us as a contamination due to a violation of the material sampling technique and its transfer into a test tube. The strain was detected neither on the hydrogel itself, taken from the bone marrow canal and from the surface of this soft tissue biopsy, nor in the bone tissue and other parts of the soft tissue structures, nor in the smear from the area of the entire surgical intervention. In the comparison group, the growth of MSSA was verified at each checking point both from the wound discharge (up to45.5 %) and from the samples of animal materials harvested on the 15th day (9.1 %) and 45th day (27.3 %). It is worth noting that in one (9.1 %) of 2 cases by the end of the study, MSSA on bone cement was detected in a rabbit with a stable remission of the infection.

In the control group, the positive growth of the strain was detected in all rabbits while there was wound discharge, and from biopsy specimens of the animal withdrawn from the experiment on the 15th day of the study. In rabbits withdrawn on the 30th and 45th days, it was not possible to obtain a biopsy material from the medullary canal of the tibia due to the overlapping of the area of the holes with massive periosteal layers and pronounced osteosclerosis, and the inoculation of purulent detritus, taken by the end of the study from the area of the corresponding projection of the infiltrate, did not detect growth of microflora.

Histologically, the reticular stroma of the bone marrow was with weak signs of lymphocytic infiltration and with a large number of macrophage cells on the 15th day in the experimental group. Active osteoreparative signs were found such as an endosteal and periosteal reaction with the formation of reticulofibrous bone tissue featuring trabecularization on the cortical wall where the post-trepanation holes were located. The hydrogel itself occupied a vast area surrounded by giant cells of foreign bodies resorbing it (GCFB) (Fig. 6 a).

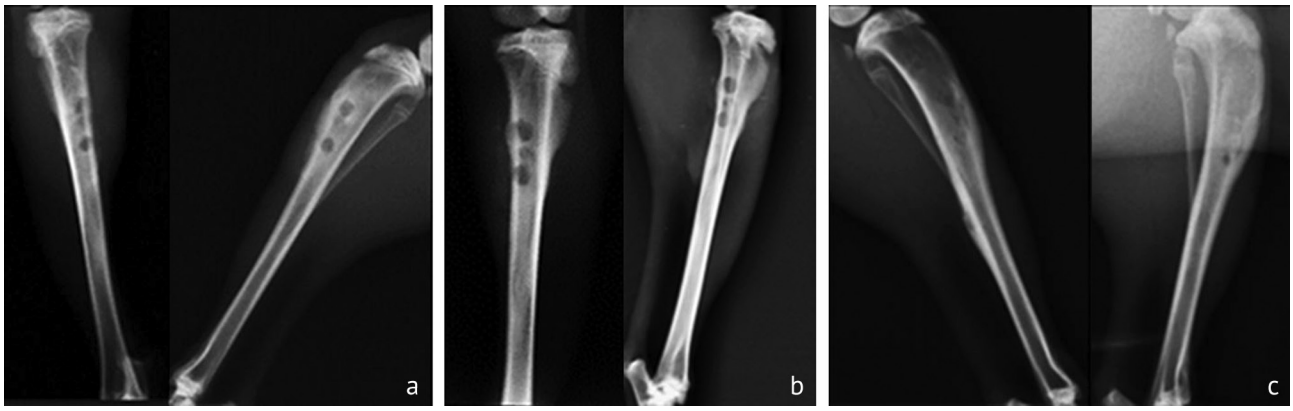

Fig. 3 X-ray images of the lower leg bones of the animals in the experimental group: *a* on the 15th day of withdrawal from the experiment; *b* on the 30th day of withdrawal from the experiment; *c* on the 45th day of withdrawal from the experiment

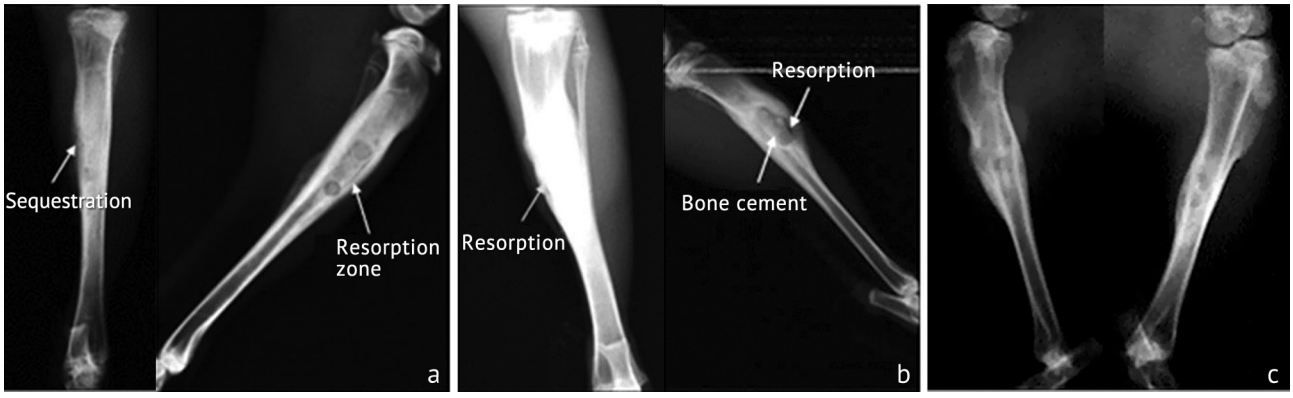

Fig. 4 X-ray images of the lower leg bones of the animals in the comparison group: *a* on the 15th day of withdrawal from the experiment; *b* on the 30th day of withdrawal from the experiment; *c* on the 45th day of withdrawal from the experiment

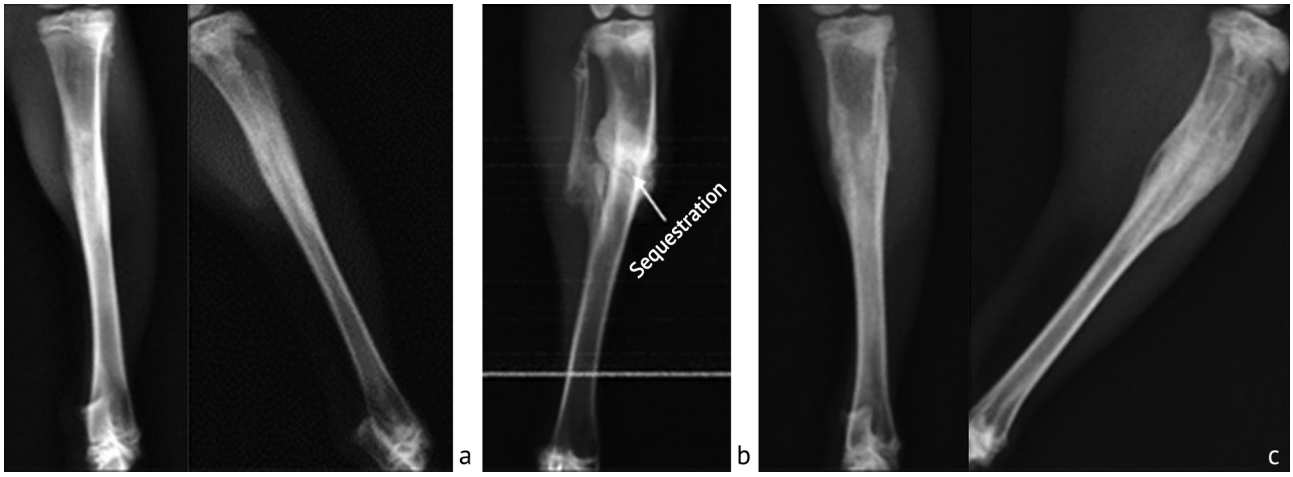

Fig. 5 X-ray images of the lower leg bones of the animals in the control group: *a* on the 15th day of withdrawal from the experiment; *b* on the 30th day of withdrawal from the experiment; *c* on the 45th day of withdrawal from the experiment

In the comparison group, the inflammatory infiltrate, represented by a significant number of lymphocytes and plasmocytes, occupied a large space, including between the exfoliated parts/granules of cement (Fig. 7 a). Proper reparative osteogenesis in the comparison group was less pronounced and contained a smaller volume of reticulofibrous bone tissue.

On the 30th day, a significant ($p = 0.002$) decrease in inflammation was noted in the experimental group. Cells of the lymphoplasmacytic series were determined in single quantities. The hydrogel volume decreased by that day, its fragments still continued resorption by GCFB (Fig. 6 b). No negative reaction such as material-associated bone resorption to the presence of the polymer hydrogel was found. At the same time, in the comparison group, despite the stable clinical and laboratory arrest of chronic osteomyelitis, histologically there was no positive dynamics in reducing the degree of inflammation (Fig. 7 b). Moreover, material-associated resorption of the newly formed bone tissue was traced at the "bone-cement" interface.

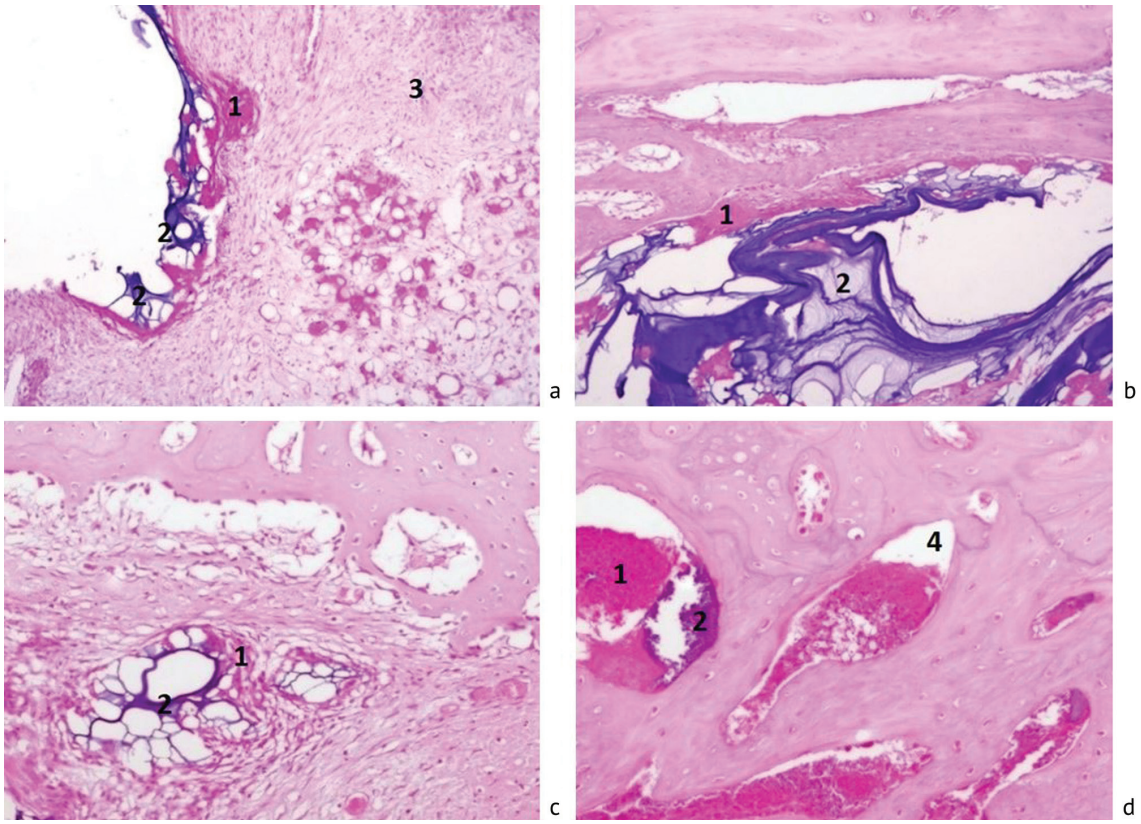

Fig. 6 Experimental group: *a, b* micropreparation on the 15th day after hydrogel implantation. GCFB (1) resorb the hydrogel (2). Weak infiltration by cells of the lymphocytic series (3) of the reticular stroma of the bone marrow. Stained with hematoxylin and eosin. ×200; *c* on the 30th day. GCFB (1) resorb hydrogel fragments (2); *d* on the 45th day. GCFB (1) resorb hydrogel residues (2) inside the lumen of the Haversian canals (4). Stained with hematoxylin and eosin. ×200

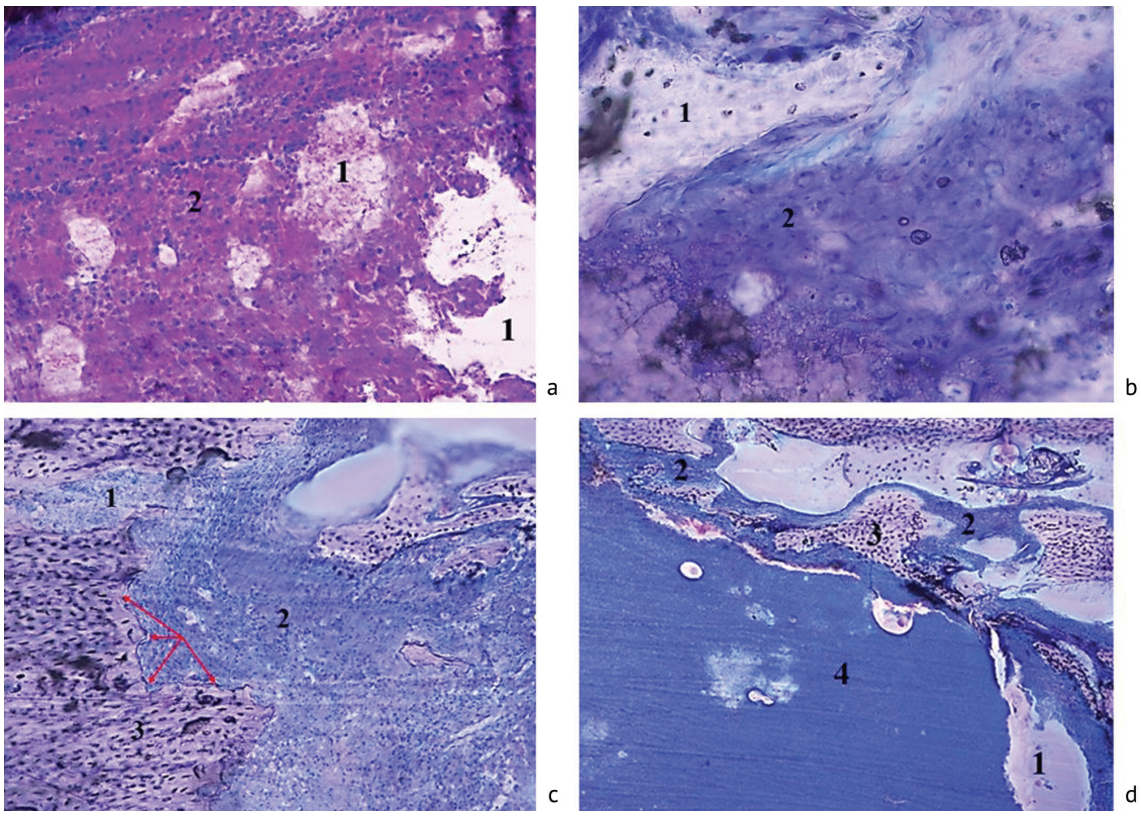

Fig. 7 Comparison group: *a* micropreparation on the 15th day after sanitation (inflammatory infiltrate of polymorphonuclear leukocytes (1) around PMMA granules (2), ×200); *b* on the 30th day (a granule of bone cement (1) of a stratified structure with signs of fragmentation surrounded by an inflammatory infiltrate (2); ×200); *c* on day 45 (resorption of bone substance (3) at the border with inflammatory infiltrate (2); ×200); *d* on the 45th day (fields of inflammatory infiltrate (2) in the bone marrow space; microabscess (4), ×50; staining – celestial trichrome)

By the end of the study, the inflammatory process almost completely subsided in the experimental group; only single plasma cells and cells of the macrophage series were found. The newly formed bone tissue in the area of post-trepanation holes was at the stage of remodeling, and the reticulofibrous bone tissue was at the stage of differentiation into adipose tissue of the yellow bone marrow. Residual hydrogel fragments, found in the lumens of the formed Haversian canals, were resorbed by GCFB (Fig. 6 c). Significant ($p = 0.001$) signs of exacerbation of hronic osteomyelitis were noted in the comparison group manifested by an aggravation of resorption of the newly formed bone tissue, more pronounced at the border with the inflammatory infiltrate (Fig. 7 c). In the area of post-trepanation holes, no bone tissue remodeling was observed. The area of the inflammatory infiltrate, represented by a significant number of polymorphonuclear neutrophils, increased significantly ($p = 0.001$) to such an extent that it occupied the space not only around and between the cement fields, but also the entire lumen of the medullary canal. More than half of the field of view of the histological preparation was occupied by the fields of microabscesses (Fig. 7 d)

In the control group, no histologically positive dynamics in arresting the infectious and inflammatory process was observed in any of the checking points (Fig. 8 a, b). The histological picture before the sanitation of the focus of infection and after it was the same. The difference was a high degree of compaction of the bone substance of the cortical wall and ongoing periosteal reaction (Fig. 8 c) and the detection of multiple foci of hemorrhage by the end of the study not only in the lumen of the narrowed medullary canal but also in the cortical plate (Fig. 8 d) .

The results of the morphometric study are presented as range diagrams in Figure 9.

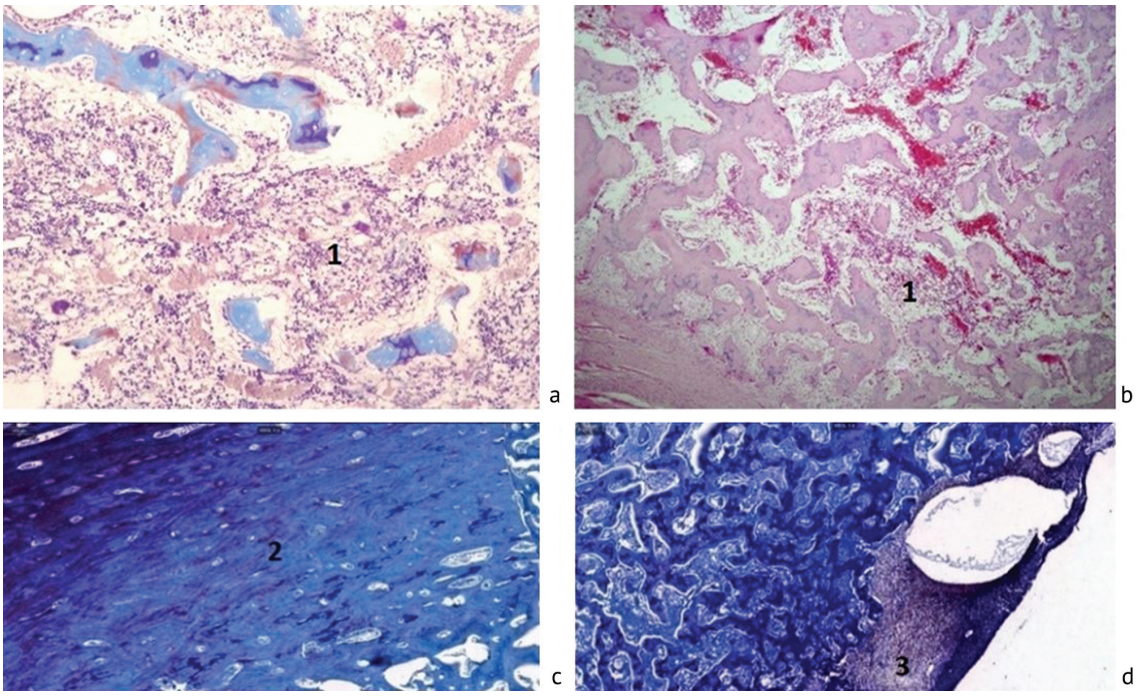

Fig. 8 Control group: *a* micropreparation of animal bone on the 15th day after sanitation (foci of lymphoplasmacytic infiltration (1) in the lumen of the medullary canal; staining with hematoxylin and eosin, ×100); *b* on the 30th day after sanitation (lymphoplasmacytic inflammatory infiltrate (1) in the reticular stroma of the bone marrow; staining with hematoxylin and eosin, ×50; *c,* d on the 45th day after sanitation; high degree of bone compaction (2) and focus of hemorrhage in the periosteal callus (3); Mallory stain)

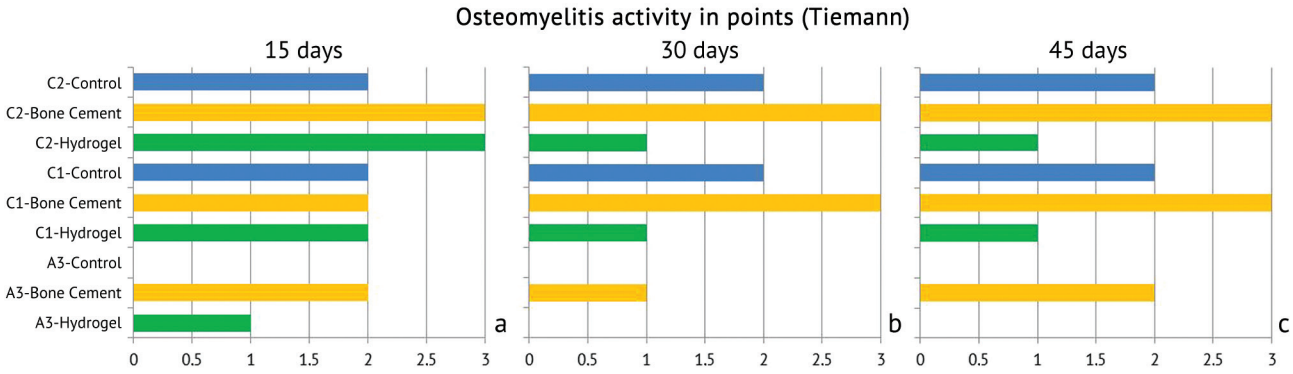

Fig. 9 Intergroup data on osteomyelitis activity following sanitation after: *a* 15 days; *b* 30 days; *c* 45 days

DISCUSSION

The analysis of the *in vivo* experiment showed that the local therapy provided by the polymer hydrogel in the experimental group contributed to the timely healing of the postoperative wound of the lower leg in all cases. In the comparison group, there was a need for auxiliary systemic antibiotic therapy in addition to local therapy from the 7th day until the end of the study due to the lack of regression of clinical symptoms of inflammation. However, despite enhanced systemic therapy, we failed to achieve remission of the infection in two cases. Treatment of chronic osteomyelitis with systemic antibiotic therapy alone is ineffective. Moreover, a significant ($p = 0.006$) increase in the body weight of animals from day 28 (Table 2) in the experimental group compared with the comparison group indirectly indicates a rapid postoperative recovery of their general condition.

Analysis of the inflammation markers showed a significant decrease in WBC ($p = 0.040$) and CRP ($p = 0.040$) in the experimental group from weeks 2 and 3 of the study, respectively (Table 4). In the control group, the blood test values obtained by the end of the study indicated a recurrence of the infection. For ESR and HB, there was no statistically significant difference between the groups (Tables 3 and 4). Despite the reliability we have established in WBC and CRP levels, it is not worth evaluating the effectiveness of treatment, focusing only on laboratory blood tests due to long-term fluctuations in their values without any definite dynamics. Foster et al. also observed that blood parameters in animals, and namely sheep, are not reliable criteria for the effectiveness of the infectious focus arrest [12]. Therefore, blood parameters in animals must be considered in conjunction with the data of clinical, microbiological, radiological and histological studies.

Despite a single positive case, which we regarded as contamination, no growth of microflora was found in the experimental group what we attribute to the high elution-antimicrobial and hydrophilic properties of the hydrogel [5]. In the comparison group, the MSSA strain was identified at almost every control period. The detection of growth of microflora on PMMA by the 45th day of observation in an animal with an arrested infection is associated with the hydrophobic surface of non-biodegradable PMMA predisposing to the adhesion of microorganisms, which subsequently contributes to the development of osteomyelitis recurrence and the acquisition of antibiotic resistance by microbes. The results obtained by us regarding bone cement, in general, do not contradict the conclusions of other authors. Ma et al. reported the presence of viable bacteria on the surface of spacers explanted from patients with managed periprosthetic infection (PPI). It was found that 30.8 % of cement samples had a high level of copies of bacterial 16S rRNA, which indicated the presence of viable microorganisms and was the cause of PJI recurrence in 10-20 % of the two-stage treatment [13]. Another study examined bacterial resistance before and after gentamicin spacer implantation in 33 patients with an infected hip joint. Before surgery, only 29 % of bacteria were resistant to gentamicin, and after treatment with bone cement, their proportion increased to 41 % [14]. George et al. compared the results of patients before and after implantation of PMMA saturated with vancomycin and found an increase in BMD in 36 % of cases [15]. Another *in vivo* experiment on rats showed a high percentage (78 %) of antibiotic-resistant *St. epidermidis* in the group where bone cement containing gentamicin was used as a prophylactic material [16].

As the analysis of the morphometric study showed (Fig. 9), there were significant differences in the histological picture of the groups. Thus, in the experimental group, as compared with the other groups, a significant ($p = 0.002$) effective arrest of the infectious and inflammatory process was observed. Thus, before the focus was sanated, the total result of C1 + C2 was 4 points, after implantation of the hydrogel, the amount significantly decreased to 2 points (Fig. 9 c). At the same time, only single cells of lymphocytes or plasmocytes were histologically noted, what may be due to a reaction to the presence of a foreign hydrogel material that was leveled after its complete resorption. The hydrogel itself did not affect the osteoreparative process and did not cause any toxic or negative reactions in the bone tissue. As seen in Figure 6 (a, d), the volume of the hydrogel matrix decreased with each control period. In contrast to the experimental group, implantation of PMMA causes a significant ($p = 0.001$) exacerbation of chronic osteomyelitis over time (A3 + C1 + C2 = 8 on the 45th day versus A3 + C1 + C2 = 4 before the sanation of the focus), and in the group without implantation depot system, but with systemic antibiotic therapy, there was no dynamics at all.

In general, the histomorphometric results obtained by us are consistent with the data of the radiographic study.

Comparison of our *in vivo* results with the experimental data of other studies on animal models is difficult, due to the long time passed on the scientific publications devoted to the study of PMMA, and many other circumstances (different conditions for performing the experiment, induction of chronic osteomyelitis, timing of observation, etc.). However, in general, the conclusions made by us regarding bone cement and the control group do not contradict the conclusions of other authors. Thus, Mendel et al. compared the *in vivo* results of the antimicrobial activity of PMMA and collagen and concluded that these matrices lead to a clear decrease in the number of bacteria, but are not capable of completely suppress

the infection [17]. Other authors compared the results of treatment of chronic osteomyelitis of the tibia in rabbits with spacer beads containing tazocin and surgical debridement without material implantation. Radiologically and histologically, the control group showed the worst result of treatment without the use of a local depot system. However, in the group of bone cement in 5 (41.7 %) out of 12 cases, a violation of reparative osteogenesis was found, of which in 25 % the onset of the reparative phase was noted by the 14th week after implantation, in 16.7 % there were no signs of reparative osteogenesis at all [18]. Tuzuner et al. showed that bone cement and calcium phosphate loaded with teicoplanin in the presence of MRSA were unable to prevent the development of femoral osteomyelitis in rats. Moreover, histological and radiological manifestations of osteomyelitis were significantly higher in the group of PMMA without antibiotic and PMMA with antibiotic without biodegradable material [19]. In another experiment on a rat model, no significant difference was found in the therapeutic effect of the treatment of chronic osteomyelitis upon implantation of a biodegradable matrix and PMMA impregnated with gentamicin [18]. Similar treatment results were obtained by Shirtliff et al. who compared the therapeutic effect of PMMA and hydroxyapatite impregnated with vancomycin [20].

The study revealed that PMMA implantation causes material-associated resorption over time. One of the reasons may be its bioinertness, which contributes to the formation of a fibrous layer at the "bone-cement" interface that prevents direct bone contact of the implant [21]. Another reason of loosening can be radiopaque agents added to the matrix (barium sulfate, zirconium dioxide), which initiate the release of pro-inflammatory cytokines and, consequently, the osteolytic reaction of tissues [22]. At the same time, barium sulfate has higher osteolytic properties compared to zirconium dioxide, characterized by more abrasive properties [23]. Implantation of the polymer hydrogel was not accompanied by such a reaction. It is one more merit of the many advantages compared to bone cement.

CONCLUSION

The results of the *in vivo* study have demonstrated the high efficacy of antibiotic-impregnated polymer hydrogel in the treatment of chronic osteomyelitis compared to PMMA. It was found that implantation of the hydrogel promotes timely healing of the postoperative wound of animals, significant ($p = 0.006$) rapid recovery of their general condition after surgery, does not cause progression of radiological signs of chronic osteomyelitis and significantly ($p = 0.002$) arrests the infectious and inflammatory process shown by histomorphometry without development any toxic effects in the bone tissue. And such properties of the material as hydrophilicity and biodegradability significantly reduce the risk of microbial adhesion and the formation of biofilms thus decreasing the likelihood of exacerbation of the infection.

Conflict of interests *Not declared.*

Funding *The authors did not have any grants for conducting the study.*

Ethical approval *The study was approved by the ethics board. The study was carried out in compliance with the principles of humane treatment of laboratory animals in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals (Strasbourg, 2006) used for experiments and other scientific purposes and with the order of the Ministry of Health and Social Development of the Russian Federation No. 708n dated August 23, 2010 "On approval of the rules of laboratory practice".*

Informed consent *Not required.*

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