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Original article

Reprint

Evaluating biocompatibility of vaterite-mineralized polycaprolactone matrices in subcutaneous implantation tests on white rats

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Abstract:
Objective: to estimate biocompatibility of matrices manufactured from polycaprolactone (PCL) and mineralized by vaterite ($CaCO_3$) via studying local and systemic manifestations of inflammatory reaction in subcutaneous implantation tests on white rats.
Material and Methods. The experiment was conducted on 40 rats divided into four groups of equal sizes: control, comparison (rats with imitation of implantation), negative control (rats with implanted non-biocompatible matrices) and experimental group, comprised of animals with implanted PCL/$CaCO_3$-matrices. Local inflammatory manifestations were analyzed by morphological investigation of implantation area tissues. Systemic inflammatory manifestations were estimated via TNF-α and interleukin-1β (IL-1) concentrations in blood serum by ELISA.
Results. The changes in cellular population content demonstrated that, on day 21 after the implantation, the PCL/$CaCO_3$-matrice was evenly colonized by fibroblast cells and afterwards vascularized. Such matrices did not cause intense inflammatory reaction observed in negative control animals. It was accompanied by systemic manifestations, such as statistically significant increase in TNF-α and IL-1 concentrations.
Conclusion. Our data confirmed the biocompatibility of PLC/$CaCO_3$-scaffolds, thus experimentally substantiating the potential for their use in tissue engineering.

Keywords: regeneration, scaffolds, vaterite, biocompatibility, polycaprolactone.

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Introduction

Currently, one of the most important areas of tissue engineering is the development of scaffolds designed to stimulate regeneration processes. Scaffolds are three-dimensional porous structures that, when implanted into a defect zone, function as an extracellular skeleton. On the periphery and in the depth of these structures, regeneration occurs due to the matrix colonized by cellular elements [1]. A wide range of materials of both natural and artificial origins is used to manufacture scaffolds [2]. To ensure the possibility of chemical modification and high multifunctionality, the preference in the development of scaffolds is given to artificial materials, one of which is polycaprolactone (PCL) [3]. This polymer is capable of biodegradation in vivo with formation of the products without toxic effect on surrounding tissues and the whole body [4].

To improve osteoinductive and osteoconductive properties of the matrices, scaffolds include inorganic substances stimulating such processes. One of these compounds is vaterite ($CaCO_3$) capable of activating proliferation of bone tissue cells and, upon transformation to calcite, participating in the target delivery of the substances and releasing pre-absorbed biologically active molecules in defect areas [5, 6].

One of the major properties required in scaffolds is their biocompatibility. Matrices should have an ability to vascularize and to be colonized by cellular elements. Also, they should not lead to the negative changes in the body. Subcutaneous implantation tests are among important and mandatory items in scaffold biocompatibility assessment [7].

The objective of our study was to assess the biocompatibility of matrices from vaterite-mineralized polycaprolactone via identifying local and systemic

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manifestations of inflammatory reaction in subcutaneous implantation tests conducted on white laboratory rats.

Material and methods

The experiment was performed on 40 white non-pedigree male rats weighing 200-250 g, divided into four equal groups of ten: the control group consisting of intact rats; comparison group, including sham-operated animals, which underwent a full-scale surgery without implantation of the matrices; negative control group (animals implanted with PCL-based non-biocompatible matrices with absorbed foreign protein), and experimental group with subcutaneously implanted PCL/CaCO3 scaffolds.

Experimental work was conducted along the guidelines of several official documents: European Convention for Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (adopted by the Council of Europe in 1986), Declaration of Helsinki by the World Medical Association (1989), International Guiding Principles for Biomedical Research Involving Animals (1985), and the Order No. 267 of the Russian Federation Ministry of Health from June 19, 2003 "On approval of laboratory practice rules". The study was carried out in accordance with the recommendations of the Ethics Committee of V.I. Razumovsky Saratov State Medical University (protocol No. 6 of February 6, 2018). A combination of telazol (Zoetis Inc., USA) at a dose of 0.1 ml/kg and xylazine (Interchemie, Netherlands) at a dose of 1 mg/kg was administered intramuscularly to anesthetize animals, while performing all manipulations on them.

Matrices based on PCL, made by electroforming at the Institute of Nanostructures and Biosystems, Saratov State University, were used for implantation. Animals of the negative control group were implanted with non-mineralized matrices with adsorbed foreign protein (native ovalbumin) on the surface. Scaffolds mineralized with vaterite sensu the method, described by M.S. Savelyeva, A.N. Ivanov, M.O. Kurtukova et al. [5], were used for implantation into animals of the experimental group.

To implant matrices, an incision on depilated and antiseptic-treated interscapular region, was performed. Then in the wound under the skin, a pocket sized 15x15 mm was formed, using the ramus of the forceps, into which the scaffold shaped as a 10 mm disk was placed. After placing the scaffolds in the subcutaneous pocket, the wound was sutured tightly using a non-absorbable monofilament yarn – Resorpen 3-0 USP (RESORBA MedicalGmbH, Germany), and treated with 70% ethyl alcohol.

On the 21st day after the true or false implantation of the matrices, blood samples were taken by the puncture of the venous heart under anesthesia. Five-milliliter blood samples were collected in Vacuette tubes with clot activator and barrier gel. Blood serum was obtained by centrifuging at 3000 rpm. Aliquots of serum were frozen and stored at -20oC. The concentrations of TNF (tumor necrosis factor) and IL-1 (interleukin-1) were determined in the serum of experimental animals by ELISA (enzyme-linked immunosorbent assay) using the "IL-1β rat" and "TNF-α rat" reagent kits by eBioscience (BenderMedSystems, Austria) and a microplate spectrophotometer Anthos-2020 (Biochrom, the United Kingdom).

After the blood sampling, the animals were removed from the experiment by an overdose of narcosis drugs. For histological examination, a scaffold with surrounding tissues was removed as a single unit. The material for the study was fixed in a 10% neutral formalin solution (OOO "Biovitrum", Russia), dehydrated in ethyl alcohols, and then poured into paraffin. Slices 5–7 μm thick were stained with Mayer's hematoxylin (OOO "Biovitrum", Russia) and eosin (OOO "Biovitrum", Russia). Bio-Monht (BioOptica, Italy) was used to cover the slices.

The specimens were studied with a microscope AxioImager Z2 (CarlZeiss, Germany) and a microvisor of transmitted light, μVizo-103 series (OOO "Lomo Photonica", Russia). Histological specimens were tested for local signs of inflammatory reaction: oedema, hyperemia, or infiltration of scaffold and surrounding tissues with immunocompetent cells. A morphometric count of the cell number in each cell population was performed at 63x magnification: fibroblasts, fibrocytes, neutrophils, eosinophils, lymphocytes, plasma cells and macrophages in five fields of view of the scaffold and its perifocal area.

Statistical data processing was performed using the software package Statistica 10.0. We tested the hypotheses regarding the type of variation series distribution (Shapiro – Wilk criterion). Most of our data did not comply with normal distribution; hence, to compare the values, we used the Mann-Whitney U-test, on the basis of which Z-criterion values and the confidence values of the difference (p) were calculated. Differences were considered significant at $p < 0.05$.

Table 1. Cell populations of perifocal area of PCL/CaCO3-scaffolds and matrices with absorbed foreign protein in comparison with connective tissue of the imitated implantation zone of sham-operated animals

Mean number of cells in five fields of view	Comparison group (n=10)	Negative control group (n=10)	Experimental group (n=10)
Fibroblasts	15 (10; 18)	30 (15; 34) $p_1<0,05$	13 (10; 15) $p_1>0,05$ $p_2<0,05$
Fibrocytes	9 (5; 12)	9 (6; 13) $p_1>0,05$	11 (7; 16) $p_1>0,05$ $p_2>0,05$
Polymorphonuclear leukocytes	0 (0; 0)	3 (2; 5) $p_1<0,001$	0 (0; 0) $p_1>0,05$ $p_2<0,001$
Lymphocytes	2 (0; 3)	7(3; 11) $p_1<0,05$	0 (0; 1) $p_1>0,05$ $p_2<0,001$
Macrophages	2 (2; 5)	6 (4; 7) $p_1<0,05$	1(1; 2) $p_1>0,05$ $p_2<0,001$

Median, upper and lower quartiles are presented in each case; p_1, p_2 – as compared with sham-operated animals and with animals of negative control group correspondingly

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Results

During the morphological study of imitated matrix implantation areas in the comparison group on the 21st day of the experiment, local signs of inflammation, including oedema, vascular congestion and leukocyte infiltration, were not detected. Fibroblastic cells prevailed in the connective tissue of the implantation area.

Thus, the proportion of fibroblasts was 54% of the total cell number. Fibrocytes were detected at 32%. Single leukocytes, mainly lymphocytes and macrophages (*Table 1*), were also found. Concentrations of pro-inflammatory cytokines in the group of sham-operated animals on the 21st day of the experiment did not have statistically significant differences from the levels of TNF and IL-1 of intact rats (*Table 3*).

On the 21st day after the implantation of a matrix with foreign protein in negative control group, a connective tissue barrier infiltrated with leukocytes, tissue oedema of the perifocal region, as well as a vascular congestion of arterial and venous beds in this zone were detected around the scaffold. Morphometric analysis showed that, in the perifocal area of a non-biocompatible scaffold, the number of fibroblasts was two times higher than that in the specimens of sham-operated animals. At the same time, the numbers of fibrocytes among the specimens of these groups did not have statistically significant differences (*Table 1*). Thirteen per cent of lymphocytes and 11% of macrophages dominated the total blood cell counts in the leukocyte infiltration of the perifocal area of scaffolds in the negative control group. In addition, polymorphonuclear leukocytes, predominantly neutrophils, in significantly higher numbers than in sham-operated animals, were found in the cell populations of the perifocal area of the scaffold in animals of this group (*Table 1*).

Table 2. Cell populations of PCL/CaCO3-scaffolds and matrices with absorbed foreign protein in comparison with connective tissue of the imitated implantation zone of sham-operated animals

Mean number of cells in five fields of view	Comparison group (n=10)	Negative control group (n=10)	Experimental group (n=10)
Fibroblasts	15 (10; 18)	5 (1; 12) $p_1<0,001$	38 (21; 47) $p_1<0,001$ $p_2<0,001$
Fibrocytes	9 (5; 12)	2 (0; 6) $p_1<0,001$	22 (13; 32) $p_1<0,001$ $p_2<0,001$
Polymorpho-nuclear leukocytes	0 (0;0)	13 (5;16) $p_1<0,001$	0 (0;1) $p_1>0,05$ $p_2<0,001$
Lymphocytes	2 (0; 3)	3 (2; 3) $p_1>0,05$	2 (0; 4) $p_1>0,05$ $p_2>0,05$
Macrophages	2 (2; 5)	4 (2; 6) $p_1>0,05$	2 (0; 4) $p_1>0,05$ $p_2<0,05$

Median, upper and lower quartiles are presented in each case; p_1, p_2 – as compared with sham-operated animals and with animals of negative control group correspondingly

Table 3. Concentrations of pro-inflammatory cytokines in blood plasma of experimental animals

Groups	TNF , pg/ml	IL-1, pg/ml
Control (n=8)	9,5 (5,6; 11,6)	16,1 (13,9; 16,6)
Comparison group (n=6)	7,2 (6,8; 8,4) $p_1>0,05$	14,9 (14,2; 15,6) $p_1>0,05$
Negative control (n=9)	15,7 (12,4; 16,5) $p_1<0,05$ $p_2<0,05$	27,5 (23,6; 31,5) $p_1<0,001$ $p_2<0,05$
Experimental group (n=7)	7,6 (3,5; 8,4) $p_1>0,05$ $p_2>0,05$ $p_3<0,05$	16,6 (12,2; 18,1) $p_1>0,05$ $p_2>0,05$ $p_3<0,05$

TNF – tumor necrosis factor α; IL -1 – interleukin-1 β; median, upper and lower quartiles are presented in each case. p_1 – as compared with animals of control group; p_2 – as compared with sham-operated animals; p_3 – as compared with animals of negative control group

For animals of the negative control group, a weak colonization of the matrix with fibroblastic elements was observed, while no signs of vascularization were detected. The numbers of fibroblasts and fibrocytes in non-biocompatible matrix were 3 and 4.5 times, respectively, lower than in the connective tissue of the imitated implantation zone of sham-operated rats (*Table 2*). In PCL-scaffold with adsorbed foreign protein, we observed the prevalence of polymorphic leukocytes, which accounted for about 48% of the total cell count (see *Table 2*). Lymphocytes and macrophages averaged 11 and 15%, respectively, of the total blood cell count (see *Table 2*).

For negative control animals, on the 21st day of the experiment, a statistically significant increase in TNF concentration was detected compared with control and sham-operated rats: by a factor of 1.7 or 2, respectively (see *Table 3*). An increase in the concentrations of IL-1 was also revealed: by 1.7 and 1.9 times compared with the control group and sham-operated animals, respectively (*Table 3*).

On the 21st day of the experiment, moderate blood filling of the microvasculature was indicated in the perifocal zone of the PCL/CaCO3 scaffold for the experimental group animals. However, oedemas, leukocyte infiltration, signs of the formation of a boundary barrier around the PCL/CaCO3 scaffold for the animals of this group were not detected. The predominant cell type in the perifocal area was fibroblasts. Thus, after morphometry, it was established that the number of fibroblasts and fibrocytes reached 52% and 44% of the total cell counts (*Table 1*). Simultaneously, the number of fibroblasts in the perifocal area of the animals in this group was 2.3 times lower than around non-biocompatible matrices in the negative control group (*Table 1*). The numbers of leukocytes, macrophages and neutrophils in the perifocal area of the PCL/CaCO3 scaffold did not differ significantly from the cellular composition of the tissues in the imitated implantation area of sham-operated animals but was significantly lower than in the perifocal area of non-biocompatible matrices in the negative control group (*Table 1*).

On the 21st day after implantation, PCL/CaCO3 scaffold was colonized by fibroblastic elements and then vascularized. The number of fibroblasts and fibrocytes in the PCL/CaCO3 scaffold was 2.5 times higher than in non-biocompatible matrices (*Table 2*). These cell populations amounted to 34% and 60% of the total cell count, respectively. Single

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leukocytes were found in the structure of PCL/CaCO3 scaffold but their numbers did not differ significantly from the cellular composition of the connective tissue in the imitated implantation area of sham-operated animals, (*Table 2*). The number of leukocytes in PCL/CaCO3 scaffolds was 1,5 – 2 times lower than in non-biocompatible scaffolds for rats in the negative control group (*Table 2*).

The concentrations of TNF and IL-1 in the blood of animals from the experimental group did not differ from those of rats of the comparison and control groups. The concentrations of TNF and IL-1 in the serum of rats on the 21st day after the implantation of PCL/CaCO3 scaffolds were 2.1 and 1.7 times lower, respectively, than in the animals of the negative control group (*Table 3*).

Discussion

The results of our study suggested that on the 21st day of the experiment, sham-operated animals had neither evidence of inflammation (in the area of imitated matrix implantation) nor systemic manifestations of inflammatory response. Therefore, by the 21st day of the experiment, the inflammatory changes due to post-surgery tissue injury were completely disappearing.

For negative control animals in contrast to comparison group rats, distinct inflammatory changes were noted in the implantation zone of the matrix with a foreign protein. Previously published studies demonstrated that, after subcutaneous implantation of matrices with adsorbed foreign protein, local inflammatory vascular changes were persistently recorded from the 7th through the 21st day of the experiment [7-9]. Other published studies noted that non-biocompatible scaffolds were not colonized by connective tissue elements and did not vascularize [10]. Our data implied that negative control animals had a significant increase in the concentrations of pro-inflammatory cytokines TNF and IL-1 in their blood, which represented systemic manifestations of inflammatory response. Elevated levels of TNF and IL-1 in rats with subcutaneous implantation of non-biocompatible scaffolds explained the mechanism of vascular changes. Consequently, after the subcutaneous implantation of non-biocompatible scaffolds, white rats experienced a significant inflammatory reaction, characterized by typical local and systemic manifestations on the 21st day.

Our results indicated that subcutaneous implantation of PCL/CaCO3 scaffolds in white rats of experimental group, in contrast to the negative control group, did not result in local signs of inflammation in the perifocal area. The composition of cell populations of the perifocal zone of PCL/CaCO3 scaffolds did not differ significantly from the connective tissue of the implantation imitation zone in comparison group rats. The concentration of cytokines in blood on the 21st day after PCL/CaCO3 scaffold implantation was similar to cytokine concentration in intact rats, which confirmed an absence of inflammation manifestations. However, on the 21st day of the experiment, an active colonization of PCL/CaCO3 scaffolds with connective tissue elements and their vascularization were observed, which, along with the absence of inflammatory changes in the perifocal zone, suggested biocompatibility of this type of matrices.

At present, the most promising concept of developing composite scaffolds for stimulating bone tissue regeneration involves a combination of synthetic polymers, such as PCL, and mineral compounds in the structure of matrices [11-14]. The results of numerous studies conducted abroad implied a high degree of biocompatibility of PCL matrices containing calcium phosphate compounds as the basis of the mineral component for the bone tissue [11, 12, and 14]. These studies demonstrated the biocompatibility of PCL scaffolds mineralized with hydroxyapatite [14], calcium beta-triphosphate [11, 12], and two-phase calcium phosphate, which is essentially a mixture of the first two compounds [13]. Our earlier research was consistent with results of international authors and confirmed biocompatibility of both non-mineralized PCL matrices and PCL matrices mineralized by hydroxyapatite [10].

Vaterite is the least stable form of calcium carbonate [15]. In vitro experiments demonstrated that vaterite nanoparticles in hydrogel matrices do not possess cytotoxicity and, under the influence of intercellular fluid or phosphate buffer, are transformed into hydroxyapatite [15]. However, the PubMed database up to date has only two publications [5, 16] devoted to PCL matrices mineralized with vaterite. It was shown that PCL / CaCO3 scaffolds had mechanical parameters matching human cancellous bone tissue, while osteoblasts had the ability for conglutination on these matrices in vitro [16]. Our data on the composition of cell populations of the matrices and on the absence of systemic manifestations in the course of their implantation confirmed previously published studies [5, 9] and permitted us to conclude that PCL/CaCO3 scaffolds were not inferior to polycaprolactone scaffolds judging from their biocompatibility properties.

Conclusion

Lack of scaffold biocompatibility showed itself as inflammation in the area of its implantation, which was accompanied by a characteristic change in the composition of cellular populations of connective tissue in the perifocal area. Consequently, scaffold cell populations were represented mainly by leukocytes dominated by neutrophils. Local tissue reactions were associated with systemic manifestations of the inflammatory response, characterized by an increase in the concentration of pro-inflammatory cytokines: TNF and IL-1. When PCL/CaCO3 scaffolds were implanted, neither local nor systemic signs of inflammation in the surrounding scaffold tissues were observed. The composition of cell populations of scaffolds was characterized by prevalence of fibroblastic cells. The combination of biochemical and morphometric data indicated a high degree of PCL/CaCO3 scaffolds biocompatibility, thus experimentally substantiating the possibility of their use for tissue engineering.

Conflict of interest

The work was performed as part of the Federal assignment to V.I. Razumovsky Saratov State Medical University by the Russian Ministry of Healthcare “Development of technology for assessing the regenerative potential of matrices for replacing bone tissue defects based on their vascularization parameters”. Registration number: AAAA-A18-118020290178-3.

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