Tissue response to the implantation of biodegradable polyhydroxyalkanoate sutures

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Polyhydroxyalkanoate (PHA) sutures were implanted to test animals intramuscularly, and tissue reaction was investigated and compared with the reaction to silk and catgut. Tested monofilament sutures made of PHAs of two types – polyhydroxybutyrate (PHB) and a copolymer of hydroxybutyrate and hydroxyvalerate (PHV) – featured the strength necessary for the healing of muscle-fascial wounds. The reaction of tissues to polymeric implants was similar to their reaction to silk and was less pronounced than the reaction to catgut; it was expressed in a transient post-traumatic inflammation (up to four weeks) and the formation of a fibrous capsule less than 200 μm thick, which became as thin as 40–60 μm after 16 weeks, in the course of reverse development. Macrophages and foreign-body giant cells with a high activity of acid phosphatase were actively involved in this process. PHB and PHB/PHV sutures implanted intramuscularly for an extended period (up to one year) did not cause any acute vascular reaction at the site of implantation or any adverse events, such as suppurative inflammation, necrosis, calcification of the fibrous capsule or malignant tumor formation. No statistically significant differences were revealed in the tissue response to polymer sutures of the two types. Capsules around silk and catgut sutures did not become significantly thinner.

1. Introduction

Polymers of hydroxy acids (polyhydroxyalkanoates, PHAs), biocompatible and biodegradable materials of microbial origin, have been actively investigated for medical applications [1, 2]. As PHAs are thermoplastic and not subject to chemical hydrolysis, they degrade in biological media gradually, retaining their mechanical strength for a long time. Due to the variable composition of PHAs, materials made of them can have different physicochemical properties [3]. It has been shown that PHAs can be used to manufacture non-woven materials, polymer films, sutures, and other products to be used in surgery, transplantology, tissue engineering, and pharmacology [4–7]. In one of the recent reviews on medical applications of PHAs Dr S. F. Williams and Dr D. P. Martin wrote: ''In fact, in the past 2 years PHAs have become one of the leading classes of biomaterials under investigation for the development of tissue-engineered cardiovascular products because they can offer properties not available in existing synthetic absorbable polymers'' [7].

Although the known PHAs are quite diverse [8], only few of them are being actively investigated for medical applications: polymers of β-hydroxybutyric acid (PHB) and heteropolymer PHAs: copolymers of hydroxyoctanoate and hydroxyhexanoate (PHO/PHHx), hydroxybutyrate and hydroxyhexanoate (PHB/PHHx), hydroxybutyrate and hydroxyvalerate (PHB/PHV) [4, 5, 7]. The functional properties of the last mentioned polymers are generally good [3, 6], but the results of investigating biocompatibility of PHB/PHV are contradictory. Some authors report the absence of adverse reactions to PHV [9], yet others present the evidence of possible inflammatory response of tissues to PHB/PHV implantation [10, 11].

The purpose of this research was to investigate tissue response to the implantation of PHB and PHB/PHV sutures in test animals compared with the response to traditional surgical materials (silk and catgut).

2. Materials and methods

2.1. Preparation of pure PHA polymers

The tested material was the PHA samples synthesized by the bacteria *Ralstonia eutropha*. The strain of *R. eutropha* B578 is registered in the Russian Collection of Industrial Microorganisms. The bacteria were grown under autotrophic conditions in batch cultures in a 10 l laboratory fermenter equipped with a turbine-type mixer at 1000 rpm; the culture contained the mineral salts

medium and carbon dioxide and hydrogen as sources of carbon and energy. A diaphragm-type compressor continuously pumped the gas mixture through the culture at a rate of 8–12 l/min, the volumetric efficiency being 0.3. The initial volume proportions of CO_2, O_2, and H_2 in the control were 1 : 2 : 7, respectively. To attain maximum accumulation of a PHA, we used a two-stage batch cultivation mode: in the first stage, the bacteria were grown in a nitrogen-deficient medium (for 25–30 h) and in the second, in nitrogen-free medium at pH 7.0 and temperature 30 °C (for 20–30 h). Carbon dioxide was the carbon source in the nutrient medium used to synthesize P3HB, and the medium used to synthesize the P3HB/PHV copolymers also contained sodium valerate at a concentration of 2 g/l.

The PHAs were extracted from bacterial biomass with chloroform and precipitated with ethanol. The extraction of PHAs from biomass was conducted in several stages. In the first stage, to partially destroy the cell wall and attain a fuller extraction of lipids, the bacterial biomass was centrifuged (for 15 min at 6000 rpm), collected, and covered with ethanol, pH 10.5–11.0 (0.5–0.7 g KOH/l ethanol). The sample was boiled using backflow condenser for 30 min. Then the alcohol was removed, the biomass was covered with 86% ethanol and separated from alcohol by centrifuging. In the next stage the partly destroyed and defatted biomass was covered with chloroform and boiled for 30–40 min using a water bath with a backflow condenser. The sample was cooled and placed into a funnel to separate the chloroform extract of the polymer from the biomass. After separation of the phases, the polymer was precipitated by adding ethanol as a reagent. The procedure of re-dissolution and further precipitation of polymers was repeated several times to prepare specimens that would not contain organic impurities of protein, carbohydrate, or lipid nature. All the organic solvents used in the procedure were preliminarily distilled to remove impurities.

The chemical purity of the resulting specimens was estimated by conventional biochemical methods. The presence of protein impurities was determined by the Kjeldal micro-method [12] and carbohydrates by the anthranone method [13].

The degree of purity of PHA samples and proportions of monomers in the copolymer PHA (PHB/PHV) were determined on a GCD plus chromatograph-mass-spectrometer (Hewlett Packard, USA). To determine the PHA composition, $\sim$ 4 mg of PHA were reacted in a small flask, using the backflow condenser, with a solution containing 1 ml chloroform, 0.85 ml methanol, and 0.15 ml sulfuric acid for 140 min at 100 °C in thermostatically regulated bath [14]. This method degrades PHA by methanolysis to its constituent β-hydroxycarboxylic acid methyl esters (FAME). After the reaction, 0.5 ml of distilled water was added and the tube was shaken for 1 min. After phase separation, the organic phase was removed, transferred into a vial and used for analysis. FAMEs were analyzed with gas chromatograph-mass spectrometer (GC/MS, model GCD Plus, Hewlett Packard, USA), equipped with a 30 m × 0.25 mm HP-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column. Chromatographic conditions were: carrier gas helium; flow rate 1 ml/min; sample input temperature 220 °C; initial temperature 70 °C, programmed to 230 °C at a rate of 8 °C/min; interface temperature 250 °C; ion source temperature = 175 °C; electron impact mode 70 eV; scanning from 45 to 450 amu at 0.5 s/scan.

Filaments were prepared from the PHA: polyhydroxybutyrate (PHB) (M_w 340 000 Da, crystallinity 70–78%) and a copolymer of β-hydroxybutyrate β-hydroxyvalerate poly(3HB-co-3HV) containing 15 mol % hydroxyvalerate (M_w 295 000–360 000 Da, crystallinity 55%).

2.2. Implants

Tested implants were monofilament samples obtained according to International Standard for Biological Evaluation of Medical Devices [15]. All the sutures used were of similar dimensions (size: 3–0 (2.0 metric)). The melt-spun experimental PHB and PHB/PHV fibers [16] had the following properties: force at fracture – 7.1 and 9.4 N; tensile strength – 205 and 274 MPa; tensile modulus 3.75 and 3.13 GPa. As positive control we used ''Black silk braided'' (HELM PHARMACEUTICALS GMBH, Hamburg, Germany/Allemagne); as a degradable reference suture – ''Catgut 0,41101'' (HELM PHARMACEUTICALS GMBH, Hamburg, Germany/Allemagne). The materials were sterile.

2.3. Animal model

A 24-week experiment was conducted according to International Standard for Biological Evaluation of Medical Devices [15] on sexually mature female Wistar rats (180–200 g). The total number of animals was 96, 18 in each group: Group I – negative control (intact); Group II – positive control (surgical silk); Group III – reference biodegradable suture (catgut); Group IV – test (PHB/PHV suture); Group V – test (PHB suture). The animals were ether-anesthetized under aseptic conditions. Longitudinal 2 cm skin and muscle incisions were made on the right femur. The muscle wound edges were closed with three sutures of the tested material (total length 3.0–3.5 cm), and the skin edges were closed with silk sutures. The wound edges in the animals of the positive control group were closed with silk. Three animals of each group were euthanized by an overdose of ether 1, 2, 4, 8, 16, and 24 weeks after the surgery and the implants were taken for analysis. Eight other animals with implanted PHA sutures were under observation for up to a year.

2.4. Sectioning

To compare the tissue reaction to the tested PHB and PHB/PHV implants and the silk and catgut ones, we estimated the tissue reaction at the site of surgery by conventional histological and histochemical methods. Fragments of tissues surrounding the implants were excised out of the femur muscle, fixed with 10% formalin, embedded in paraffin, and 5–10 μm-thick microscopic sections were prepared from the paraffin blocks. One fragment excised out one animal's femur was used to make 10–18 serial sections, which were divided into halves and stained by two methods. To

analyze the general tissue response and the processes of collagen fiber development, the sections were stained with hematoxylin–eosin, Van-Gieson, and with pyrofuchsin.

A Carl Zeiss Image Analysis System (Germany) was used for viewing microscopic images and analyzing morphometric characteristics of sections. The estimated parameters were the dynamics of formation and thickness of the fibrous capsule (FC) (the parameter shows the intensity of the connective tissue reaction in the area of implantation), its cellular composition; the number of fibroblast rows (FR) (an indication of the activity of production reaction at the site of implantation); the number of macrophages (M) in the capsule (the number of macrophages in 15 measuring points in different equidistant parts of the capsule) (an indication of the implant biodegradation rate); and the volume density of vessels (V) (an indication of blood supply to *de novo* tissue [17]).

The qualitatively estimated parameters were the duration and intensity of inflammation at the site of surgical incision and suture and the period of development and maturation of collagen fibers.

The starting quantitative data for morphometry were obtained as follows. The sample was placed into the substage of the microscope stage so that the examined portion of the capsule was in the field of vision when it was moved horizontally and vertically. The objective was moved along the capsule circumference and measurements were taken at equal distances. The eyepiece micrometer was used to measure the total thickness of a FC and to count the FR and the number of M in each measuring point. At the same time, using an electron ruler in the monitor, we recorded the capsule thickness and the number of fibroblasts in it. Based on the obtained numerical results, we calculated the parameters. We took at least 15 measurements in different parts of a capsule in each of the 3–5 serial sections of one animal. As there were three animals per each observation period, at least nine sections were analyzed for every experimental point. Thus, sampling for the determination of quantitative parameters (FC, FR, and M) was at least 135 in each experimental point. The means of FC, FR, and M were calculated by the formulae proposed in Pkhakadze *et al*. [17].

The mean capsule thickness was calculated as follows:

$$\mathrm{FC} = 1/n \sum_{i=1}^{n} \mathrm{FC}_i$$

where n is the number of measurements and FC_i is the capsule thickness in measuring points.

The number of FR rows was calculated as:

$$\mathrm{FR} = 1/n \sum_{i=1}^{n} \mathrm{FR}_i$$

where n is the number of measurements and FR_i is the number of FR rows in the capsule in measuring points.

The number of M was counted in sections in 15 measuring points.

Tissue phosphomonoesterases (acid and alkaline) were determined in the fibrous capsule and in the tissues adjacent to the implants histochemically by the method of Gomori [18].

2.5. Transmission electron microscopy (TEM)

For electron microscopy, tissue fragments were fixed in a 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and additionally fixed in a 1% solution of OsO_4 in the same buffer. The samples were dehydrated in alcohols of ascending concentrations and acetone; then they were embedded in the Epon–Araldit mixture of epoxy resins (''Serva''). Semi- and ultra-thin sections were cut with a Reichert Um-03 ultra-microtome (Austria). The semi-thin sections were stained with 1% toluidine blue for light microscopy (''Reichert'', ocular 8, objectives 20 and 40). The ultra-thin sections were additionally stained with a 3% solution of uranyl acetate in 30% ethanol and with a 0.05% aqueous solution of lead isocitrate. The resulting sections were studied using a JEM-100C electron microscope (Japan).

2.6. Statistical analysis

Results of the experiment were processed statistically using the standard Microsoft Excel software package. Statistical analysis of the results was made using the standard software package of Microsoft Excel. Arithmetic means and standard deviations were found. Data are presented as mean and standard error of the mean (SE). The arithmetic mean of the variants, the mean-square deviation, and the error in arithmetic mean were calculated. Significant differences between mean values in control and test groups were tested using Student's t test. A $p < 0.05$ was considered significant [19].

3. Results

Microscopic examination of the postoperative sutures demonstrated that PHB and PHB/PHV fibers, like silk and catgut, provided reliable holding of the suture of the muscular-fascial incision in the animals throughout the period of observation. Wound healing in all the animals of the test groups proceeded in the same way as in the positive control group (silk) and in the reference group (catgut), through primary stretching. None of the animals exhibited rejection of fibers, suture disjunction, or any other adverse reaction.

The tissue reaction to the surgical intervention and subsequent implantation of PHB and PHB/PHV fibers was generally similar to the reaction scheme typical of the wound process and foreign-body invasion. This scheme included the stages of traumatic inflammation, *de novo* formation of connective tissue, and formation and rearrangement of the cicatrix – like in the case of silk and catgut implants [20]. However, there were some differences in the tissue response to the tested polymer threads and the traditional sutures. These differences were recorded both at the stage of post-traumatic tissue inflammation and in the course of cicatrix formation and re-arrangement (Table I).

On day seven after the surgery, the microscopy of

TABLE I Morphometric parameters of tissue response to the implantation of suture material (M* ± m)

Suture material	Time, weeks	Thickness of capsule (TC), (μm)	Number of fibroblast rows (FR)	Number of macrophages (in the field of vision)
PHB	1	—	—	2.64 ± 0.31
	2	116.98 ± 6.46	9.07 ± 0.58	6.36 ± 0.42
	4	172.23 ± 13.64	10.64 ± 0.48	11.50 ± 0.85
	8	161.20 ± 5.93	9.50 ± 0.57	13.56 ± 0.87
	16	54.09 ± 3.28	4.64 ± 0.37	11.93 ± 0.98
	24	48.02 ± 5.25	3.50 ± 0.32	10.50 ± 0.85
PHB/PHV	1	—	—	3.14 ± 0.32
	2	130.86 ± 3.43	9.28 ± 0.76	5.50 ± 0.40
	4	169.67 ± 5.98	11.07 ± 0.65	10.79 ± 0.90
	8	158.08 ± 4.37	10.57 ± 0.67	12.79 ± 0.74
	16	43.71 ± 3.11	3.36 ± 0.37	11.93 ± 0.84
	24	33.73 ± 2.05	2.57 ± 0.27	12.64 ± 0.93
Silk	1	—	—	1.00 ± 0.19
	2	126.99 ± 2.70	13.29 ± 0.75	1.36 ± 0.14
	4	169.87 ± 13.45	10.64 ± 0.48	1.43 ± 0.19
	8	173.17 ± 5.46	11.43 ± 0.81	1.14 ± 0.19
	16	125.49 ± 2.63	10.79 ± 0.63	1.29 ± 0.21
	24	132.54 ± 3.84	9.71 ± 0.37	1.21 ± 0.23
Catgut	1	—	—	5.21 ± 0.66
	2	204.99 ± 17.53	21.00 ± 0.84	4.00 ± 0.65
	4	422.25 ± 6.51	38.79 ± 1.01	5.64 ± 0.70
	8	514.21 ± 12.01	46.29 ± 1.66	2.14 ± 0.45
	16	342.00 ± 9.68	21.76 ± 0.84	2.43 ± 0.45
	24	272.14 ± 4.11	20.86 ± 1.19	1.14 ± 0.22

* mean values ($n = 135$) (the mean of nine sections (three sections of each of three animals), with 15 measurements per section).

tissues around the PHB and PHB/PHV implants showed an insignificant edema. The sutures were surrounded mainly by monocytes (macrophages and lymphocytes), neutrophils, and fibroblasts. The onset of the formation of a FC around the sutures was also observed at this stage. The initial tissue reaction to the implantation of PHA sutures of both types (the character, intensity, and length of inflammation) was similar to the tissue reaction to silk and was much less pronounced than the reaction to catgut.

To analyze the biochemical restructuring that accompanies morphological changes in tissues after the surgery and implantation of sutures, we investigated the dynamics of activities of phosphomonoesterases, acid phosphatase (AcP), and alkaline phosphatase (AlP) (Figs. 1 and 2), whose response to tissue injury is the most conspicuous [21]. AlP, which is mostly present in neutrophils, is indicative of inflammation and neovascularization [22]. AcP of M and foreign-body giant cells (FBGC) can be a reliable indicator of the biodegradation rate of polymeric materials [23]. On day seven after the surgery the level of AlP in the tissues adjacent to the site of surgery and PHA implantation was significantly elevated (Fig. 1(a)). A similar response was recorded in the tissues surrounding silk and a much more pronounced one in the tissues around the catgut implant. In this period no significant activity of AcP was recorded histochemically in the tissues surrounding the implants of all types, including PHA ones (Fig. 2(a)). These data agree with the low number of M in the tissues in this period (2–3 in the field of vision) (Table I).

In two weeks after the surgery, the tissues surrounding PHA sutures were still inflamed and edematic (Fig. 3). The area of the edema, however, was significantly smaller around the tested PHA sutures and silk, but it was considerable around catgut. The number of mature secretory-phagocyte M in the field of vision around degrading PHB and PHB/PHV sutures increased in the capsule that formed around implants, to 6.36 ± 0.42 and 10.79 ± 0.90 in the field of vision, respectively.

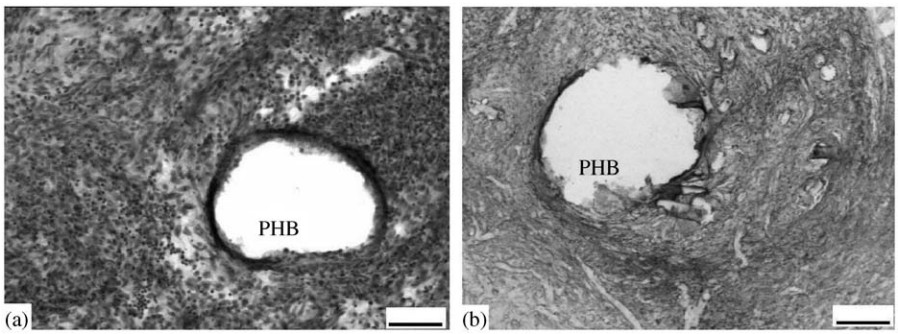

Figure 1 Histochemical tissue reaction to AlP by the method of Gomori: weeks one (a) and four (b) after the implantation of PHB suture. Labeling: p, polymer suture. Marker is 0.01 mm.

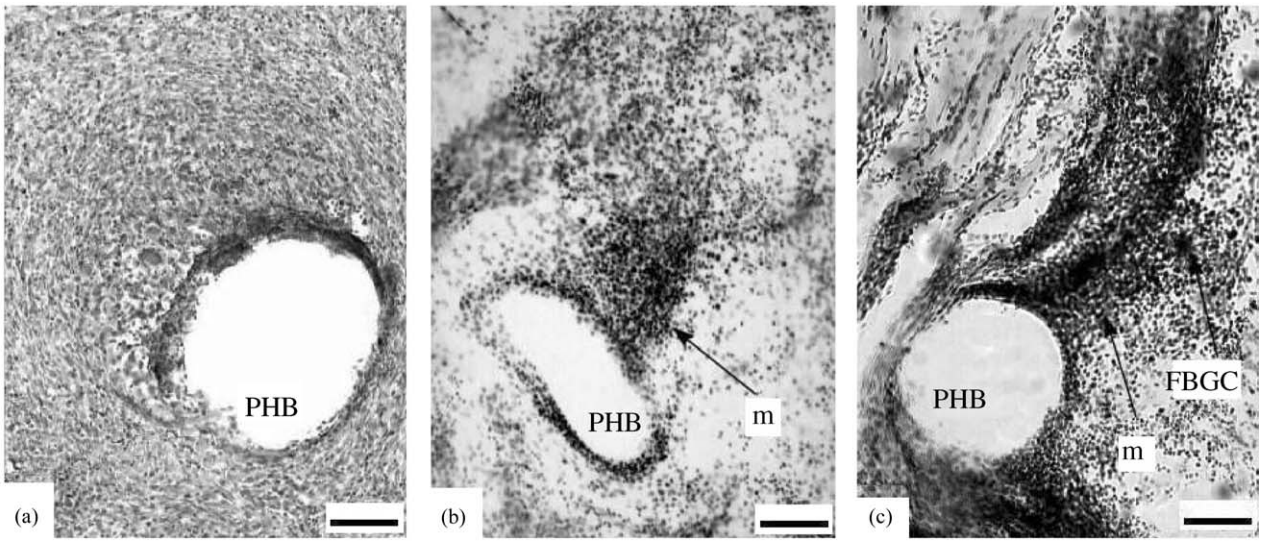

Figure 2 Histochemical tissue reaction to AcP by the method of Gomori: weeks one (a), two (b), and four (c) after the implantation of PHB suture. Labeling: m, macrophages; FBGC, foreign-body giant cells. Marker is 0.01 mm.

Histochemical investigations revealed an increase in the number of AcP granules in the M cytoplasm (Fig. 2(b)). Around catgut, the number of M was smaller, 5.64 ± 0.07, and in tissues surrounding the silk sutures, the number of M did not increase (Table I). The average thickness of the capsules (TC) around PHB and PHB/PHV was 116.99 ± 6.46 and 130.86 ± 3.43 μm and the number of FR 9.07 ± 0.58 and 9.28 ± 0.76, respectively (Table I). At that time the TC around silk was 126.99 ± 2.70 μm; no increase in the number of M in the capsule was observed. Leukocytes were still numerous in the surgery area around catgut; the TC was 204.99 ± 17.53 μm.

Four weeks after the surgery, the thickness of the FC around the PHB and PHB/PHV sutures increased to 172.23 ± 13.64 and 158.08 ± 4.37 μm (Fig. 4) and its thickness was similar to that of the FC around the silk implant (Fig. 5(b)). Vast inflammation was still observed around the catgut implant, which was also surrounded by a coarse FC (TC more than 400 μm) (Fig. 5(a)). Mature collagen fibers were dominant components of the capsules around silk and the tested PHA sutures (Fig. 3). The bundles of collagen fibers in the FC around catgut were much thicker and actually filled the capsule (Fig. 6(a)). At the sites of PHB and PHB/PHV implantation, the number of active M with numerous outgrowths and cell lysosomal structures kept increasing (to 11–12 in the field of vision) as did the number of FBGC and the activity of AcP in them (Fig. 2(c)). An increase in the activity of AcP is indicative of the activation of phagocytic reaction of M – active agents of PHA biodegradation [24, 25]. Some FBGC had 4–5 nuclei (Fig. 7(a)). Neovascularization was observed in the capsules around PHA implants (Fig. 8(a)) as well as around silk and catgut ones.

Eight weeks after the surgery, tissues surrounding the tested PHB and PHB/PHV implants, as well as silk and catgut ones, remained essentially unchanged. The capsules around PHA implants were well-vascularized. The densities of the vessels in the capsules around the tested PHA sutures and around the traditional suture materials were similar.

Sixteen weeks after the surgery, the capsules became structurally different. The capsules around the both PHAs became considerably (almost three times) thinner (Fig. 4): the average TC around the PHB and PHB/PHV implants decreased to 54.09 ± 3.28 and 43.71 ± 3.11 μm, and the number of FR in them to 4.64 ± 0.37 and 3.36 ± 0.37, respectively. However, the count of active M in tissues adjacent to the implants remained high (no statistically significant decrease relative to the previous stages was registered); there were M ''lying'' on the surface of polymer threads (Fig. 3); some FBGC had 10–12 nuclei (Fig. 7(b)). Mature cells prevailed among fibroblasts. Mature connective tissue was formed at the periphery of the capsule in the form of bundles of collagen fibers and fibrocytes adjacent to them (Fig. 3). Active PHA-phagocytizing M and FBGCs were also identified at the periphery of the capsule. The thickness of the FC around the silk implant did not change significantly: the TC was 125.49 ± 2.63 μm and the number of FR – 10.79 ± 0.63. The catgut implant, though actively degrading (by week 16 it was not determined in tissues), was still surrounded by a solid capsule, its thickness being 342.00 ± 9.68 μm in 16 weeks after the surgery. By that time, the number of M in the tissues adjacent to catgut decreased twice and amounted to 2.43 ± 0.45.

Twenty-four weeks after the surgery, further involution of the FC around PHB and PHB/PHV was observed (Fig. 4) and thickness of the FC reached 48.02 ± 5.25 and 33.73 ± 2.05 μm, respectively. Active PHA-phagocytizing M were still observed around PHA implants. The FC around silk and catgut implants did not become significantly thinner by the end of the experiment (Fig. 5(a) and (b)). The thickness of the FC around silk was 125–132 μm and around catgut – 272.14 ± 4.11 μm.

Further monitoring of the state of tissues in animals with implanted polymeric sutures did not reveal any significant changes in the state of the implants. Thirty-six weeks after the surgery, no unfavorable events were observed at the site of implantation. In some animals the TC around PHA threads was 20–40 μm. The implants were surrounded by healthy tissues of the newly formed

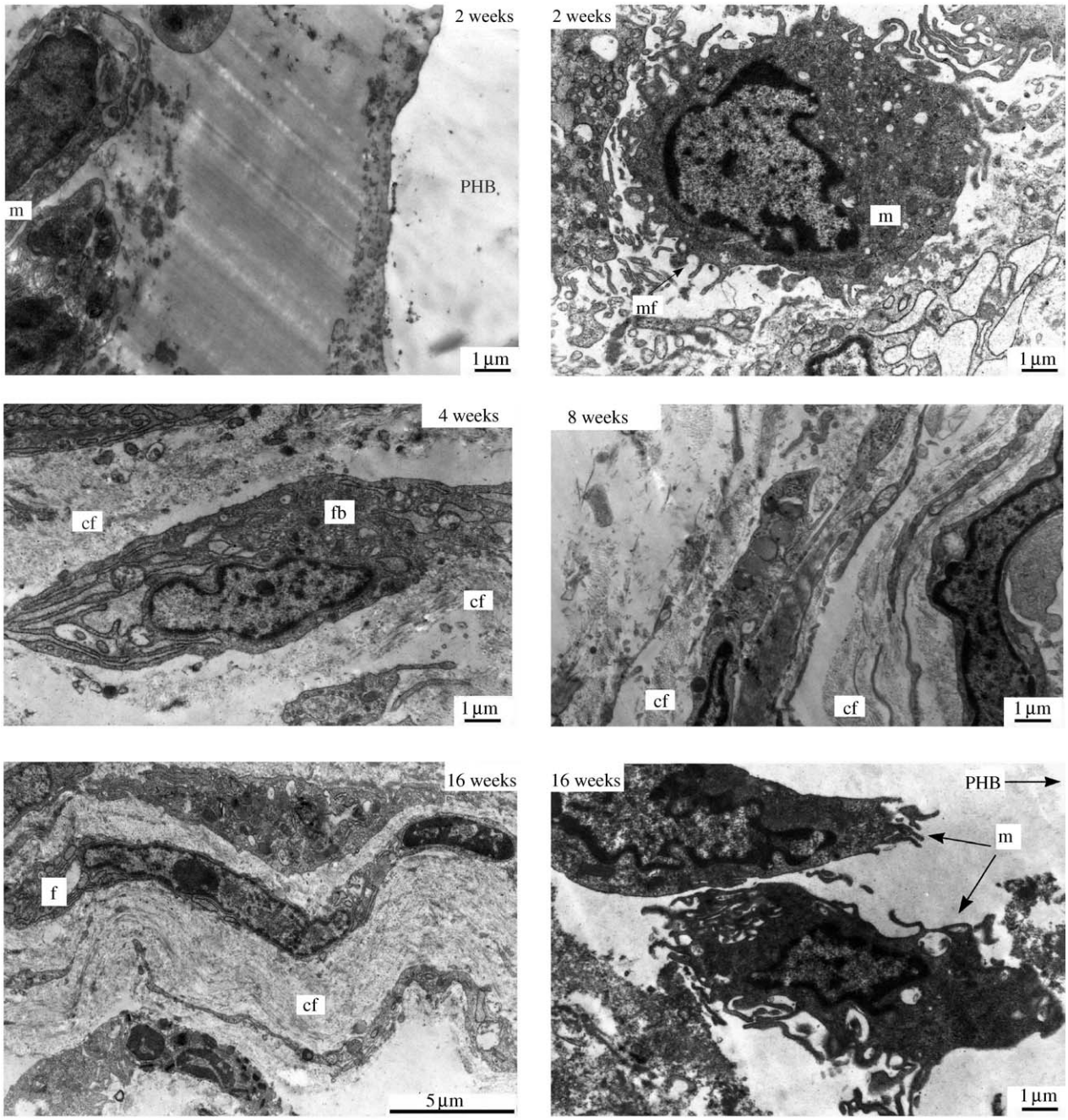

Figure 3 Ultra-thin sections of the FC forming around the implanted PHA sutures at different times after the implantation. Labeling: m, macrophages; fb, fibroblasts; f, fibrocytes; cf, collagen fibers.

fibers arranged in parallel with the polymeric implants (Fig. 9(a)). Forty-eight weeks after the surgery, the FC around the implants were minimal (Fig. 9(b)). In the immediate proximity to the polymeric thread, around it, and in the adjacent tissues, mono- and poly-nuclear M were still abundant. At that time, tissues did not exhibit any adverse reaction to the presence of a foreign-body – the polymeric thread.

Thus, we found out that the tissue response in the animals with implanted PHA sutures followed the same pattern throughout the experiment, irrespective of the polymer chemical composition. Differences between the thicknesses of the FC around the PHB and PHB/PHV sutures and between the numbers of their FR were not statistically significant. Four and eight weeks after the surgery, the TC around silk was not significantly different from the TC around PHB and PHB/PHV sutures. PHB and PHB/PHV sutures implanted intramuscularly for an extended period (up to one year) did not cause any acute vascular reaction at the site of implantation or any adverse events, such as suppurative inflammation, necrosis, calcification of FC, or malignant tumor formation. It is noteworthy that the presence of hydroxyvalerate in the polymer did not influence the duration and intensity of the inflammation phase and the character of development of the FC around sutures. After four to eight weeks the number of M in tissues adjacent to copolymer implants was significantly ($p < 0.05$) larger than their number around PHB. The TC around silk implants was similar to that of the capsules around the tested PHA sutures, but it changed very little until after 24 weeks; the number of M did not increase. Later (16 and 24 weeks after the surgery), the capsules around silk implants were significantly thicker than those around the tested PHA sutures. Tissue response to the implantation of catgut was most pronounced: the coarse FC around the

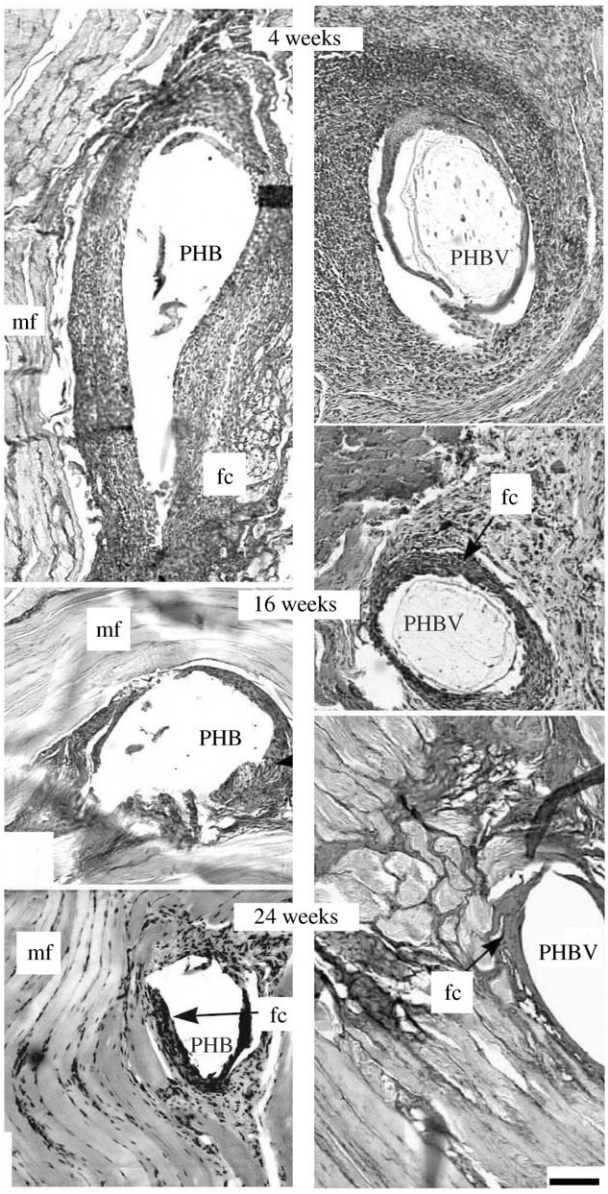

Figure 4 Morphology of tissues surrounding PHB and PHB/PHV (PHBV) sutures after the surgery. Hematoxylin–eosine. Labeling: fc, fibrous capsule; mf, muscle fibers. Marker is 0.01 mm.

implant was more than twice thicker than the capsules around PHA and silk sutures. Since week eight after the surgery, the number of M around the catgut had become significantly smaller than their number around the PHAs. The formation of blood microvessels in tissues around the implants was similar in all variants.

4. Discussion

The diversity of PHAs, the wide range of their physicochemical and physical–mechanical properties, and the feasibility of producing composites with various materials make them materials of the 21st century. PHAs are promising candidates for biomedical applications. The elastic copolymers PHO/PHHx have great potentials for tissue-engineering applications and for the reconstruction of elements of the cardio-vascular system [5, 7]. Long-duration experiments (lasting up to 40 weeks) on animals showed that various types of specially processed constructions produced from purified PHO/PHHx possess all the properties necessary for biomaterials to be applied in tissue-engineering. A new PHA polymer – a copolymer of hydroxybutyrate and hydroxyhexanoate (PHB/PHHx) – which has only recently been described, seems to be a promising material. The first evaluation of biocompatibility of this polymer shows that fibroblasts adhere to and grow on PHB/PHHx films an order of magnitude better than on PLA and PHB [26].

The two PHA polymers that have been most thoroughly investigated so far and that are mostly dealt with now are polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-valerate (PHB/PHV). The biocompatibility of these polymers was first shown in the early 1970s. The polymer films implanted into rabbits subcutaneously and intramuscularly for a period up to eight weeks caused a typical reaction of the surrounding tissues to a foreign-body – formation of a FC; no negative changes were observed in the surrounding tissues. That work resulted in obtaining the first patent on biomedical applications of PHAs [27].

The data obtained in the investigation of PHB/PHV biocompatibility are contradictory. For instance, polyhydroxybutyrate copolymers containing various amounts of hydroxyvalerate were tested as nerve conduits implanted in rats during a four-month experiment, and no adverse effect of hydroxyvalerate was revealed in the process of axon regeneration [9]. Insignificant inflammatory reactions recorded in the period immediately after implantation did not affect tissue regeneration. Professor Gogolewski *et al.* [10] investigated *in vivo* tissue reaction to PHB and PHB/PHV compared with polylactides (PLA), when implanted intraperitoneally to mice. No complications, such as abscesses or necroses of tissues, were observed in the experiments. The tissues surrounding polymer implants gave a typical response involving mononuclear M phagocytizing PHAs and the formation of a FC. However, with PLA implants the signs of inflammatory reactions vanished by the end of the first month after the surgery, while in the animals with PHA implants the signs of inflammation persisted during three months, and the presence of hydroxyvalerate made the reaction stronger. Nevertheless, later (three to six months after the surgery) unwanted events in response to the presence of hydroxyvalerate in the implants ceased to occur, and the state of tissues was the same in all groups of animals. Similar results were obtained when PHB/PHV films containing various amounts of hydroxyvalerate (7–22 mol %) were implanted intramuscularly to sheep for a period up to 90 weeks [11]. Although, generally speaking, the tissue response to the implantation of any PHA was comparable (postoperative inflammation involving neutrophils, lymphocytes, fibroblasts, and M and the formation of a well-vascularized FC around the foreign-body), the number of inflammation cells was larger and the strength of the response was more pronounced in the cases of higher hydroxyvalerate content.

It is important to note that inflammatory, pyrogenic, and other unwanted reactions to the *in vivo* implantation of new biomaterials and constructions can be determined by the chemical composition, implantation form and site, and, to a large extent, the degree of chemical purity of the material.

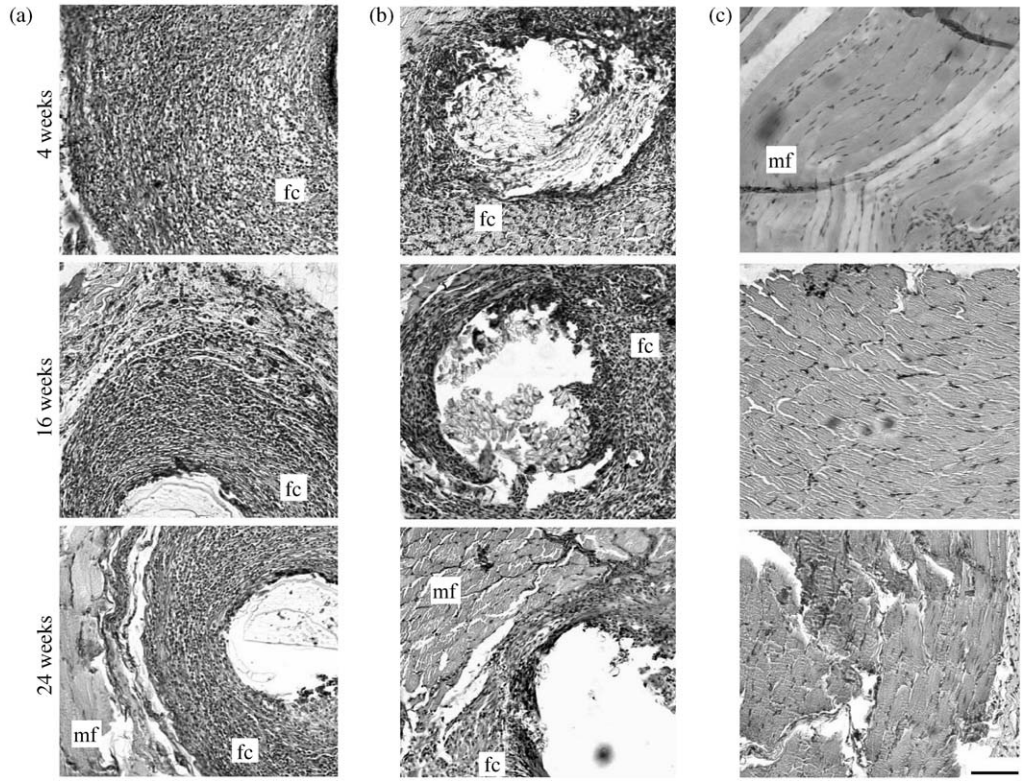

Figure 5 Morphology of tissues surrounding catgut (a) and silk (b) sutures after the surgery. Structure of the muscle tissue of an intact animal's right femur (c). Hematoxylin–eosine. Labeling: fc, fibrous capsule; mf, muscle fibers. Marker is 0.01 mm.

The analysis of the literature shows that toxicological investigations are often conducted on commercially available PHAs rather than on PHAs of medical purity [7]. It has been ascertained that commercial PHB, PHB/PHV, and other PHAs can contain fragments of microbial cells with lipopolysaccharide and other complexes, which may induce adverse reactions of cells in the *in vitro* systems and pyrogenic reactions *in vivo* [28–31]. The endotoxin content in commercial PHAs (PHB and PHB/PHV) can amount to 120 U/g [5, 30]. In one of our recent investigations we have found that insufficiently purified PHA samples contain long-chain hydroxy acids, including β-hydroxytetradecanoic acid (C_{14}), which are components of bacterial cell walls. As a result, these PHAs activate blood enzyme systems, accelerating the recalcification and the reaction of complement [32].

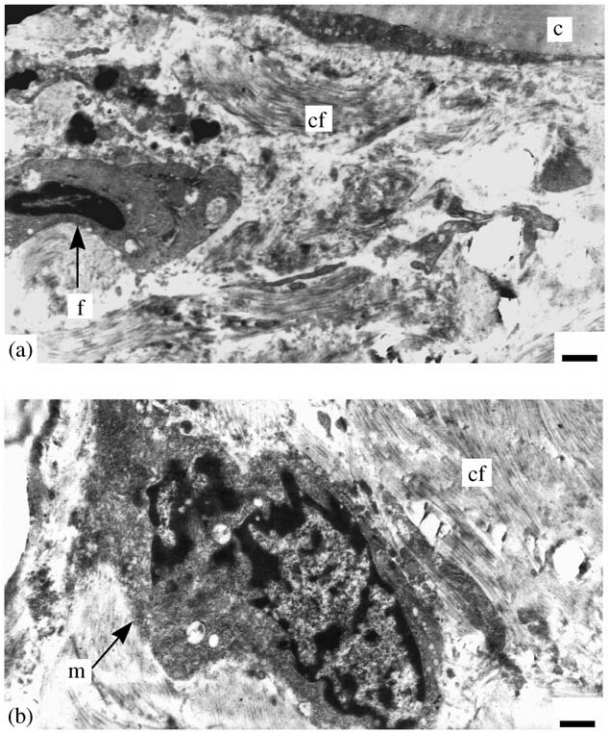

Figure 6 Ultra-thin sections of the FC forming around the implanted catgut sutures at different times after the implantation: (a) four weeks, (b) 16 weeks. Labeling: m, macrophages; c, catgut; m, macrophage; f, fibrocyte; cf, collagen fibers. Marker: (a) 1 μm, (b) 5 μm.

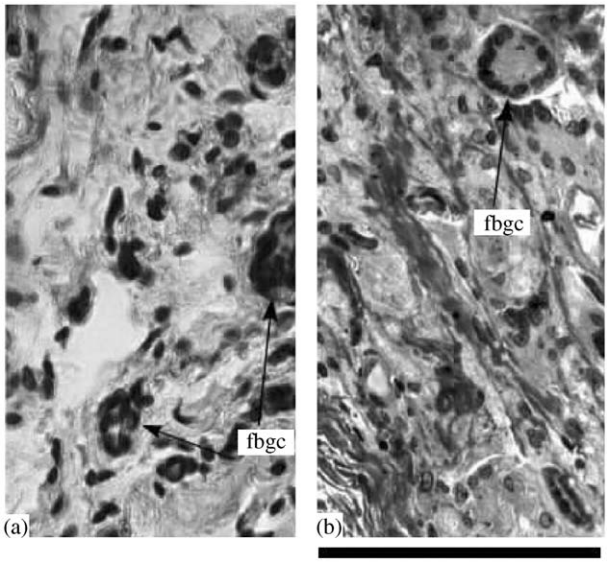

Figure 7 Foreign-body giant cells with an ordered ring-shaped position of the nuclei (FBGC) in tissues surrounding implants: (a) two weeks (b) 16 weeks after surgery. Semi-thin section. Toluidine blue. Marker is 0.01 mm.

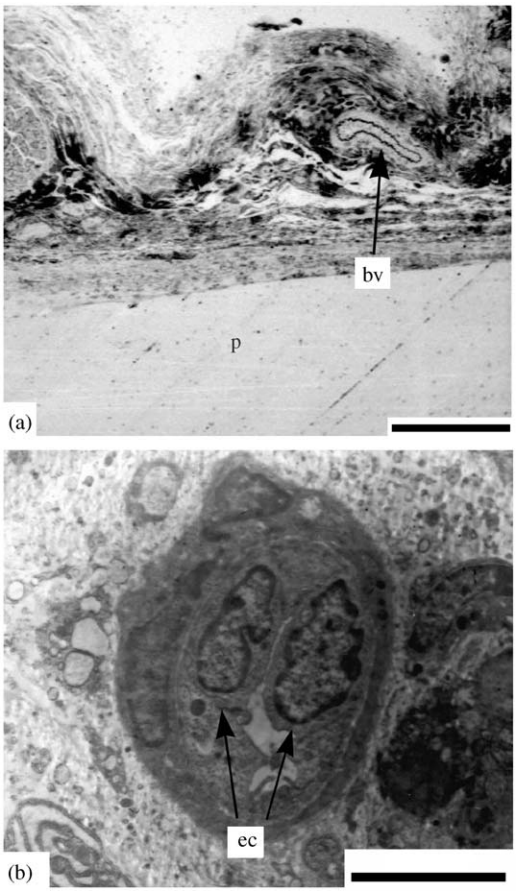

Figure 8 Vascularization of capsules: (a) semi-thin section (four weeks after implantation of PHB); p, polymer; ec, endothelium cells. Marker is 0.01 mm; (b) ultra-thin sections of the FC forming around the implanted PHB sutures; bv, a forming blood vessel (eight weeks after the surgery). Marker is 5 μm.

Thus, in our work we investigated PHB and PHB/PHV of high purity (99.9%), which were free of organic impurities of protein, carbohydrate or lipid nature and long-chain hydroxy acid microimpurities. It was proved earlier that such PHB and PHB/PHV (containing up to 30 mol % hydoxyvalerate) do not produce any irritative, immunotoxic, and sensitizing effects and can be used in contact with blood [32, 33]. The physiological–biochemical investigations of some integrated parameters, conducted in the six-month experiment, and estimates of the local tissue reaction, presented in this paper, have not detected any deviation in the test animals relative to control groups. PHB and PHB/PHV implants did not produce any adverse effect on physiological, biochemical, and functional characteristics of the animals, irrespective of the chemical composition of the material and the duration of its contact with the internal environment. The presence of 15 mol % hydroxyvalerate did not affect the postoperative condition of the animals and the investigated parameters [33]. In the course of our investigations, we analyzed the growth and development of the animals; estimated the condition of the peripheral blood and its biochemical parameters as well as parameters of protein metabolism and functions of the kidneys and the liver; and examined the reaction of lymphoid tissue and postmortem state of internal organs. The absence of significant differences among animals in 26 physiological–biochemical parameters (masses of animals and their internal organs, blood morphology and biochemistry, reaction of lymphoid tissue, and local tissue reaction at the site of implantation) throughout the experiment was confirmed by the cluster analysis by the one-link method. The same results were obtained in the initial postsurgery period (weeks one, two, and four), when significant differences were recorded between groups of animals in the largest number of parameters [34, 35]. The estimates of the general condition of the animals in combination with the analysis of the local tissue reaction to the implantation of PHB and PHB/PHV (15 mol % hydroxyvalerate) allow a conclusion that high-purity PHA samples produce no adverse effect *in vivo* on the organism and tissues.

5. Summary

It has been proved that monofilament sutures made of high-purity PHB and PHB/PHV (15 mol % hydroxyvalerate) provide necessary strength of suture throughout the period of healing of a muscular-fascial wound. The reaction of tissues to PHA implants is manifested as a transient post-traumatic inflammation (up to four weeks) and the formation of a FC less than 200 μm thick, which becomes as thin as 40–60 μm after 16 weeks. No differences have been revealed in the tissue response to PHB and PHB/PHV sutures. M and giant cells of foreign bodies with a high activity of AcP are actively involved

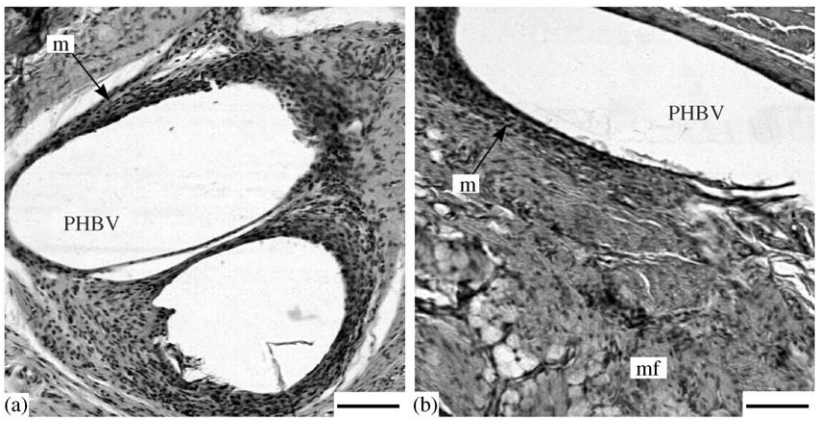

Figure 9 Morphology of tissues surrounding PHB/PHV fibers in 36 (a) and 48 (b) weeks after the implantation. Hematoxylin–eosine. Labeling: m, macrophages; mf, muscle fibers. Marker is 0.01 mm.

in tissue response to PHA implantation. PHB and PHB/PHV sutures implanted intramuscularly for an extended period (up to 1 year) do not cause any acute vascular reaction at the site of implantation or any adverse events, such as suppurative inflammation, necrosis, calcification of FC or malignant tumor formation.

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