EXPERIMENTAL WOUND DRESSINGS OF DEGRADABLE PHA FOR SKIN DEFECT REPAIR

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Abstract

The present study reports construction of wound dressing materials from degradable natural polymers such as hydroxy derivatives of carboxylic acids (PHAs) and 3-hydroxybutyrate/4-hydroxybutyrate [P(3HB/4HB)] as copolymer. The developed polymer films and electrospun membranes were evaluated for its wound healing properties with Grafts – elastic nonwoven membranes carrying fibroblast cells derived from adipose tissue multipotent mesenchymal stem cells. The efficacy of nonwoven membranes of P(3HB/4HB) carrying the culture of allogenic fibroblasts was assessed against model skin defects in Wistar rats. The morphological, histological and molecular studies revealed the presence of fibroblasts on dressing materials which facilitated wound healing, vascularization and regeneration. Further it was also observed that cells secreted extracellular matrix proteins which formed a layer on the surface of membranes and promoted the migration of epidermal cells from the neighboring tissues surrounding the wound. The wounds under the P(3HB/4HB) membrane carrying cells healed 1.4 times faster than the wounds under the cell-free membrane and 3.5 times faster than the wounds healing under the eschar (control).The complete wound healing process was achieved at Day 14. Thus the study highlights the importance of nonwoven membranes developed from degradable P(3HB/4HB) polymers in reducing inflammation, enhancing angiogenic properties of skin and facilitating better wound healing process.

Introduction

The need for novel materials in effective regeneration of injured skin is one of the serious concerns in reconstructive medicine. Management of skin injuries owing to burns, traumas, surgical interventions is carried out with several surgical and therapeutic devices which heal skin defects. These devices are developed using different materials and drugs. The principle and methods of replacing the integrity of skin is measured by various factors like depth and severity of the injury, phase of the wound healing process, wound location, degree of microbial invasion, health conditions of patient and drugs taken by the patient. The basic principle of wound therapy relies on debridement and optimal conditions for wound healing. The ideal wound dressing must fulfill normal functions of healthy skin which includes antimicrobial potential, promote moist wound environment, permit gaseous exchange, provide mechanical protection and be sufficiently elastic to fit the wound shape.

The fundamental studies underpinning cell biology coupled with key clinical landmarks led to invention of novel biomaterials which paved their important roles in tissue-engineering of skin which envisioned better understanding of skin biology [1]. Studies also revealed three important factors in development of tissue-engineered materials including the safety of the

patient, clinical efficacy and convenience of usage. Perusal of scientific studies have reported considerable progress in development of tissue engineered skin for both clinical and experimental medicine [1-3]. The composition tissue engineered skin includes the culture of epidermal cells and biomaterials with cultured cells for replacing damaged epidermis or deeper skin layers. Initially, during development of tissue engineered skin, it is necessary to study the properties of the biomaterial being used and determine its function in defect repair [4]. Earlier suspensions of cell cultures (fibroblasts, keratinocytes) were used to treat skin wounds. However, administration of cell suspensions is not sufficiently effective. Later studies revealed that the application of cells pre-cultured on the scaffold was more effective than using cell suspensions [5]. Thus, in recent decade there has been tremendous interest towards using tissue-engineered constructs: scaffolds carrying cells and drugs and bioactive compounds [2, 6-9]. Tissue-engineered skin has been successfully used to treat various diseases and skin defects such as vitiligo, melanoma, psoriasis, and blistering disorders [1].

Significant progress has been achieved since 25 years at creating tissue-engineered skin, yet there is a need for advancement such as providing a barrier layer of renewable keratinocytes (the cells that form the upper barrier layer of our skin), which is securely attached to the underlying dermis, is well vascularized and provides an elastic structural support for skin [2]. Recent studies demonstrate the role of biomaterials used to create tissue-engineered skin. One of the major challenges is to choose an ideal material for constructing grafts, which can be shaped as 2D and 3D constructs. Wound dressings described in the literature include tissue-engineered systems of keratinocytes or fibroblasts seeded onto scaffolds of various materials such as composites of polylactide/glycolide [10], polyethylene and silicon, collagen and glucosaminoglycan [11, 12].

These models can be used in clinical investigation of various skin diseases [13]. The effective production and cultivation of keratinocytes in laboratory provided a basis for the clinical use [14]. One of the main factors determining successful skin regeneration is the adequate scaffold. Among the most promising cell scaffolds, nonwoven polymer membranesproduced by electrospinning demonstrate better advantages, as they have similar structure to that of collagen fibers of living tissues [2, 3, 14-17]. Electrospinning can be used to produce 3D open porous structures and it is also possible to electrospin natural materials such as collagen and chitosan or synthetic materials such as poly-L-lactide (PLLA), polycaprolactone (PCL) and polyglycolic acid (PGA) [18].

The design and construction of tissue engineering can be achieved with various synthetic and natural polymers whose efficacy is tested for their potential use as carriers of cells for wound healing. Construction of atraumatic wound dressings is among the most promising lines of research. Non-adherent dressings are constructed by using hydrophobic polymer material or by forming a hydrophobic wound contact layer. But some of the major constraints of many wound dressings is that they stick to the wound surface, injuring the regenerating tissues and causing pain. The adhesion of the dressing materials to the wound surface may be caused by various reasons, the most frequent of which is “gluing” of the dressing to the wound surface. The “glue” is the exudate, which dries and forms an eschar. As the hydrophilicity of the polymer material constituting the dressing is increased, it adheres to the wound surface more firmly. During the granulation phase, the adhesion of the wound dressing is associated with the growth of the granulation tissue into the pores of the dressing material. This problem is usually resolved by fabricating wound dressings from hydrophobic polymer materials or by using hydrophobic synthetic polymer to make the wound-facing surface of the dressing. However, such dressings do

not adhere properly to the wound surface and their absorption rate is low, causing exudate accumulation on the wound. Atraumatic absorptive wound dressings can be designed by using resorbable materials. This line of research has not been sufficiently developed yet, as very few biodegradable materials meet all the requirements for wound dressing materials.

Hence the need for biocompatible materials has instigated research on natural degradable polyhydroxyalkanoates (PHAs) as materials for reconstructive surgery, including tissue engineering. There is much scientific evidence already reported that PHAs are biocompatible and functional polymers [19-22]. Scientific reviews highlight that scaffolds of PHAs facilitate proliferation of various cells, including fibroblasts, osteoblasts, chondrocytes, neurons, and stem cells [33-41]. Moreover, some data suggest that fibroblasts and keratinocytes grow better on scaffolds of PHAs than other materials, including polylactides [42]. Thus, there is a promising future for PHAs as materials for constructing tissue-engineered grafts for healing skin wounds. Our previous findings have been reported in production of PHA films, membranes, microparticles, microgranules, tubes, fibers, etc. and have been successfully used to suture musculofascial wounds and perform intestinal anastomosis, as bile duct stents, osteoplastic materials, drug delivery systems, etc. [23-32]. In the present investigation, the main focus is to construct experimental wound dressings from degradable PHAs intended for skin wound repair and evaluate their effectiveness in experiments on laboratory animals.

2. Materials and Methods

2.1. Polymer preparation

Synthesis of polymers was carried out at the Institute of Biophysics SB RAS according to the protocol described in the previous report [43]. In the present study, PHA copolymers comprising monomers of 3-hydroxybutyric and 4-hydroxybutyric acids [P(3HB/4HB)] were used.This is the best PHA for fabricating biomedical products: P(3HB/4HB) has a low degree of crystallinity, the highest elasticity, and the fastest rate of biodegradation *in vivo*. The polymer was synthesized in *Cupriaviduseutrophus*B10646 microbial culture (the strain is registered in the Russian Collection of Industrial Microorganisms) [44]. Cells were batch-cultured in a 14-L New Brunswick Scientific BioFlo 110 fermentor filled with 40 % of its volume with standard mineral salts medium: $Na_2HPO_4 \cdot H_2O$ – 9.1; KH_2PO_4 – 1.5; $MgSO_4 \cdot H_2O$ – 0.2; $Fe_3C_6H_5O_7 \cdot 7H_2O$ – 0.025; NH_4Cl – 0.5 (g/L); the medium was supplemented with glucose in amounts corresponding to cell concentration in the medium. A solution of iron citrate (5 g/L) was used as a source of iron. Hoagland's trace element solution was used with composition 3 ml of standard solution per 1 L of the medium. The standard solution contained H_3BO_3 – 0.288; $CoCl_2 \cdot 6H_2O$ – 0.030; $CuSO_4 \cdot 5H_2O$ – 0.08; $MnCl_2 \cdot 4H_2O$ – 0.008; $ZnSO_4 \cdot 7H_2O$ – 0.176; $NaMoO_4 \cdot 2H_2O$ – 0.050; $NiCl_2$ – 0.008 (g/L). In order to achieve synthesis of P(3HB/4HB) copolymer, the culture medium was supplemented with additional carbon substrate (γ-butyrolactone).

2.2. Analysis of composition and physicochemical properties of P(3HB/4HB)

Polymer was extracted with chloroform and then precipitated with hexane. The composition of PHAs was analyzed with a GC-MS (6890/5975C, Agilent Technologies, U.S.) and NMR spectrometry. ^{1}HNMR spectra of copolymer were recorded at room temperature in$CDCl_3$ on a BRUKER AVANCE III 600 spectrometer operated at 600.13 MHz.

Molecular weight and molecular-weight distribution of PHAs were examined using a gel permeation chromatograph (Agilent Technologies 1260 Infinity, U.S.) with a refractive index detector, using an Agilent PLgel Mixed-C column. Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning calorimeter (Mettler Toledo, Switzerland). Samples were preheated to 60 °C and cooled to 25 °C. The specimens were heated to

temperatures from -20°C to 300 °C, at 5 °C×min^{-1} (measurement precision 1.5 °C). The thermograms were analyzed using the STARe v11.0 software. X-Ray structure analysis and determination of crystallinity of copolymers were performed employing a D8 ADVANCE X-Ray powder diffractometer equipped with a VANTEC fast linear detector (Bruker, AXS, Germany). Calculations were done by using the Eva program of the diffractometer software.

2.3. Preparation of films and nonwoven membranes

Thenonwoven membranes made from ultrafine fibers were prepared by electrospinning from P(3HB/4HB) solutions using a Nanon 01A automatic set-up (MECC Inc., Japan) according to the previously developed technique [45]. A chloroform solution with polymer concentration 6% was poured into a plastic syringe (13 mm inside diameter). The syringe was fixed horizontally in the set-up; the solution feeding rate was 8 ml/h, the applied voltage 25 kV, and the working distance 15 cm. Randomly oriented ultrafine fiberswere collected on a flat steel plate. Films were prepared by casting a chloroform solution (2 % w/v) on degreased glass and subsequent drying at room temperature for 2-3 days in a dust-free box (Labconco, U.S.) [23].

2.4. Physical and chemical characteristics of nonwoven membranes

The thickness of films and electrospun nonwoven membranes was measured with a LEGIONER EDM-25-0.001 electronic digital micrometer (Legioner, China); accuracy of measurement was 1 μm. The microstructures of the surfaces of films and membranes were analyzed using scanning electron microscopy (TM 3000, S 5500, Hitachi, Japan). Prior to microscopy, the samples were sputter coated with platinum (at 10 mA, for 40 s) with an Emitech K575X sputter coater (Quorum Technologies, U.K.).

The surface properties of the polymer films and membranes were examined using a DSA-25E drop shape analyzer (Krüss, Germany) and software DSA-4 for Windows. Each drop of water and diiodomethane were alternately placed on the sample surface with microsyringes, and moments of interaction between each liquid and sample surface were video recorded. The contact angles of these liquids were measured by processing the frame of a stabilized drop in a semiautomatic mode by the “Circle” method, which is embedded in the software package. The results of measurements were used to calculate surface free energy and its dispersive and polar components (mN/m) by the Owens, Wendt, Rabel and Kaelble method [46, 47]. A minimum of six measurements were taken for each surface; means and standard deviations were calculated.

The physical/mechanical properties of films and membranes were investigated using an Instron 5565 electromechanical tensile testing machine (U.K.). Dumbbell-shaped samples 50 mm long, 6.1 mm wide, and 30-40 μm thick were prepared for studying physical/mechanical properties of the films and 3D constructs. The thickness of films was measured prior to testing, using a “LEGIONER EDM-25-0.001” electronic digital micrometer. The samples were maintained under normal conditions for at least two weeks to reach equilibrium crystallization. A minimum of five samples were tested for each type of films and 3D constructs. Measurements were conducted at room temperature; the clamping length of the samples was 30 mm. The speed of the crosshead was 3 mm/min at room temperature. Young’s modulus (E, MPa), tensile strength (σ, MPa) and elongation at break (ε, %) were automatically calculated by the Instron software (Bluehill 2, Elancourt, France). To obtain Young’s modulus, the software calculated the slope of each stress-strain curve in its elastic deformation region. The measurement error did not exceed 10 %.

2.5. An in vitro study

2.5.1. In vitro degradation of films and nonwoven membranes

Biodegradation kinetics of the samples was investigated with *in vitro* model systems. In brief, phosphate buffer solutions with different pH (pH 4.5, 5.9, and 7.0) and human blood stabilized with sodium citrate were used. Polymer films and electrospun nonwoven membranes with 10 mm-diameter disks were prepared and samples with known physical/mechanical properties were weighed and sterilized in a Sterrad NX medical sterilizer (Johnson & Johnson, U.S.). Later, under aseptic conditions, the discs were placed into 20-ml dark-glass vials with the model medium supplemented with gentamycin (100 μg/ml). The samples were maintained at 37 °C for 100 days, with the medium replaced with a fresh one every week. The biodegradation of the films and membranes was evaluated by measuring the mass of the samples. Every 25 days, the samples (three of each type) were removed from the model environments, washed with distilled water, brought to a constant weight, and weighed on an analytical balance of the first grade of accuracy and any changes in mass (X) were determined using the formula:

$$X\,[\%] = \frac{Y_1 - Y_2}{} \times 100,$$

2.5.2. Isolation of MSCs from adipose tissue

MSC s were derived from the adipose tissue of Wistar rats which were euthanized by using an overdose of an anesthetic ether. The adipose tissue was isolated under aseptic conditions from the rats'abdominal and femoral regions; later it was excised into small pieces to prepare a homogeneous mass and rinsed thrice with physiological saline buffered with phosphates containing antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin). The suspension was supplemented with type 1 collagenase solution and incubated for 60 min in a CO_2 incubator at 37 °C, with shaking every 15 min. The adipose tissue was thoroughly mixed to prepare a homogeneous suspension, and the cells were precipitated by centrifugation for 10 min at 1500 *g*. The supernatant was removed and the sediment was resuspended in a complete DMEM medium. The cell suspension was transferred into 90-mm-diameter Petri dishes and placed into CO_2 incubator for 24 h. After 24 h, the medium was replaced by a fresh solution and unattached cells were removed. MSC differentiation into fibroblasts was induced by using the DMEM medium with 10 % fetal bovine serum and a solution of antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin). In DMEM medium, adipose tissue cells showed fibroblast-like morphology. After 3-4 passages, the cells were removed from Petri dishes with a trypsin solution and seeded onto sterile PHA membranes at a concentration of 10^5 cells per scaffold. Gene expression of collagen type I (Col-1) was determined by real time RT-PCR, to confirm the fibroblast phenotype of the cells and their viability. Fibroblast marker gene expression was determined by cell lysis and isolation of mRNA, using an RNA-Extran reagent kit (Sintol, Russia). The encoding DNA was obtained by reverse transcription reaction, using the reverse transcriptase MMLV (Sintol, Russia). RT-PC-reaction was performed using specific primers, with a Real-time CFX96 Touch amplifier (Bio-Rad LABORATORIES Inc., U.S.) according to the manufacturer's guidelines. During the reactionprocess, β-actin was used as a housekeeping gene. The relative level of expression of the marker genes was determined by the relative threshold cycle method ($2^{-\Delta\Delta Ct}$).

2.5.3. Seeding MSCs onto scaffolds

Polymer films and electrospun nonwoven membranes with 10 mm-diameter disks were placed into 24-well culture plates (TPP Techno Plastic Products AG, Switzerland) and sterilized in a Sterrad NX medical sterilizer (Johnson & Johnson, U.S.).Scaffolds were seeded with cells at a concentration of 10^5 for 7 days. Cells were cultured on the DMEM medium with 10 % fetal bovine serum and a solution of antibiotics (Sigma-Aldrich reagents) in the 5 % CO_2 atmosphere at 37 °C, with the medium replaced by a fresh one every three days.

Cell viability was evaluated using MTT assay. A 5 % MTT solution (50 μl) and a complete nutrient medium (950 μl) were added to each well of the culture plate. After 3.5 h cultivation, the medium and the MTT solution were replaced by DMSO to dissolve MTT-formazan crystals. After 30 min, the supernatant was transferred to the 96-well plate, and optical density of the samples was measured at a wavelength of 540 nm, using a Bio-Rad 680 microplate reader (Bio-Rad LABORATORIES Inc, U.S.). The number of viable cells was determined from the calibrated graph.

Cell cytoplasm and nuclear DNA molecules were stained with phalloidin conjugated with fluorescein (FITC) and DAPI respectively (Sigma–Aldrich, U.S.), to observe cell morphology and to count the cells attached to scaffolds.The scaffolds with cells were fixed in a 3.7 % formaldehyde solution for 60 min and then rinsed in phosphate buffered saline to remove the fixative. Later the scaffolds with attached cells were incubated in a Triton X-100 solution (0.1 %) (GERBU Biotechnik GmbH, Germany) for 5 min followed by a 1 % bovine serum albumin solution (Ampresco, U.S.) for 30 min at room temperature. Cell cytoplasm was stained with a FITC solution (a 1/100 dilution) for 60 min at room temperature; nuclei were contrasted with DAPI (a 300 nM solution, for 5 min). The cells were examined using a Leica fluorescence microscope (Leica Microsystems).

2.6. An in vivo study

The efficacy of nonwoven polymer membranes prepared by electro-spinning from P(3HB/4HB) copolymers was studied on model skin defects induced in Wistar rats. Experiments were conducted in accordance with the Animal Welfare Act and the Guidelines for Ethical Treatment of Animals.

2.6.1. Surgical protocol

The rats were given an in halation anesthetic and a 2×2-cm section in the inters capular region of each rat was shaved and then excised (both epidermis and dermis, to its full thickness) under aseptic conditions and average wound area of 4.0-4.5 cm^2 was selected. The experiment was conducted with three sets of animals: two treatment groups, one with the grafts of nonwoven membranes and second with membranes carrying epidermal cells derived from adipose tissue. The third group served as control whose wound was covered with sterile gauze. The dressings were fixed with 4 sutures and covered with sterile gauze. The state of the animals (motion activity, feeding behavior) was assessed daily throughout the experiment. The wounds were photographed and the area of the wound and the rate of healing were determined in order to monitor wound healing.

2.6.2. Histologic examination

For histologic examination, skin samples were excised after the animals were euthanized, at Days 3, 7, and 14 of the experiment. The specimens were fixed in 10 % neutral formalin and conventional histological techniques were used. During investigation, evaluation of the inflammatory reaction and acanthosis (an indicator of increased proliferation of basal and spinous cells), counting of horn cysts (a marker of the lagging cell differentiation in the epidermis under the conditions of accelerated regeneration), quantification of sebaceous glands

and hair follicles (a source of undifferentiated epithelial cells that can be involved in regeneration of the epidermis in the case of vast shallow wounds) were carried out.

2.6.3.Analysis of expression of regenerative factors using real-time PCR (RT-PCR)

To get an insight into the mechanism of the wound healing process, gene expression studies were carried out to detect the factors that characterized the degree of inflammatory process, vascularization, and formation of the new connective tissue at the defect site. Real-time RT-PCR was performed in the region of quantification of gene expression for factors of rat angiogenesis (VEGF), inflammation (TNF-a), collagen type I (Col-1) (an indicator of connective tissue formation), keratin 10 (K10), and keratin 14 (K14) (indicators of the formation of the spinous and granulosa layers of the epidermis during regeneration) to determine the quantification level of transcription of genes responsible for inflammation, vascularization, and connective tissue formation. Total RNA was isolated from the granulation tissue of the treatment and control groups by extraction with a guanidinium thiocyanate-phenol-chloroform mixture from the RNA-Extran reagent kit. Later, from RNA template by using reverse transcriptase, cDNA was synthesized with several types of oligonucleotide primers: with a mixture of random primers – hexa primers – and oligo-dT primer, following the procedure recommended by the manufacturer (Sintol, Russia). Negative controls of reverse transcription reaction were prepared for all samples to confirm the absence of DNA contamination in the initial RNA. Real-time PCR-amplification for quantification of cDNA fragments of rat VEGF, rat TNF-a, rat Col-1, rat K10, and rat K14 were performed by using a CFX-96 thermocycler, according to the manufacturer's protocol, using the color channel of HEX.

2.7. Statistics

Statistical analysis of the results was performed by conventional methods using the standard software package of Microsoft Excel. Arithmetic means and standard deviations were found. The statistical significance of results was determined using Student's test (significance level: $P \leq 0.05$). Statistical analysis of surface properties of the samples was performed by using embedded methods of the DSA-4 software.

3. Results

3.1. Characterization of cell scaffolds based on P(3HB/4HB)

The characterization of cell scaffolds results showed the presence of 3HB and 4HB monomer units, 90/10 mol. %, which was confirmed with ^{1}H NMR spectrum of P(3HB/4HB) (Fig. 1). P(3HB/4HB) displayed the following: degree of crystallinity (C_x) 50 %, average molecular weight (M_w) 473 kDa, polydispersity (Đ) 3.26, melting point (T_{melt}) 154 °C and thermal decomposition temperature $T_{(degr)}$ 286 °C.

Similarly, morphological characteristics were studied with SEM analysis which revealed the micrograms with different surface morphology of wound dressings prepared by different techniques. The solution cast P(3HB/4HB) films with a 9:1 ratio had round pores up to 2 μm in diameter (Fig. 2). The nonwoven membranes composed of randomly oriented ultrafine fibers showed distinct fibers with an average diameter of 1.7 μm and the distance between them was found to be 1.3 – 1.67 μm. The comparative study of the surface properties and physical/mechanical properties of the wound dressings are given in Table 1. It was also found that the films and nonwoven membranes had similar water contact angles at around 75.7-76.9 ° with different diiodomethane contact angles at 55.1 and 43.5 °. Interestingly, when their surface properties were compared it was observed that they differed in their physical/mechanical characteristics. The elongation measurement at broken end indicated the elasticity of products

with order of magnitude higher in membranes than in films, displaying higher parameters of mechanical strength including Young's modulus and tensile strength. The water vapor permeability of films and membranes P(3HB/4HB) was determined, and this parameter was higher by a factor of more than 20 in the electrospun membranes than in the solution cast films.

3.2. An in vitro study

An in vitro analysis was carried out by measuring the mass of the films and membranes at pH 5.9. (Figure 3), which showed no change in their mass nor tensile strength throughout the observation period of 100 days. Similar results were also obtained at pH values (4.5 and 7.0). The obtained result indicated that polymer samples prepared from P(3HB/4HB) were not degraded in the phosphate buffer solutions at different pH used in the experiment. The hydrophobic films and membranes maintained in the phosphate buffer solution for 24 h did not show any swelling which was confirmed by measuring their mass. In order to confirm further, a chromatographic analysis of the medium was carried, which showed the absence of 3HB and 4HB monomers. Thus, the stability of the product was proved and there was no sign of hydrolysis. However, samples incubated in the biological medium (stabilized blood) were degraded by enzymes (Fig. 3). The mass reduction was first detected after 25 days of incubation and during 100 days of incubation, the mass reduction of the nonwoven membranes reached up to 34 %, while the mass of the films was decreased by 25 %. The physical/mechanical parameters of the films and membranes measured after incubation in the phosphate buffer solution for 100 days is described in Table 1. The mechanical strength of the films and membranes incubated in blood showed a gradual decrease from Day 50, as the samples were degraded. After 100 days of incubation, all parameters decreased by 26-32 % of their initial values (Table 1).

The prepared films and membranes were seeded with cells isolated from the rat adipose tissue. Adipose tissue cells growing on the polystyrene of the culture plates were used as the control. The process of growth was measured with MTT assay (Fig. 4 a) which showed that at Day 7 after seeding, the number of viable cells on the nonwoven membranes was 2.4×10^5 cells/cm^2, which was 25 and 30 % higher than the number of cells on polymer films and control (polystyrene), respectively. In the DMEM medium, adipose tissue cells acquired the fibroblast phenotype and synthesized Col-1, which was confirmed by expression studies using real-time RT-PCR in the region of the quantitative evaluation of gene expression to the factors of Col-1 (Fig. 4 b). The DNA copy number was also higher on the nonwoven membranes. The results of quantification of Col-1 by real-time PCR were confirmed by immunocytochemical staining as shown in figure 5 indicating the immunocytochemical staining with Col-1 antibodies at Day 14 of adipose tissue cell culture. Interestingly, cells growing on films and nonwoven PHA membranes showed higher rate of collagen synthesis than in the cells growing in the DMEM medium on polystyrene (control).

Thus, as the cells showed better proliferative and synthetic activities on nonwoven membranes than on the smooth films, the nonwoven membranes were chosen for constructing hybrid tissue-engineered grafts carrying fibroblast cells. The electrospun membranes (scaffolds) used for constructing grafts were composed of randomly oriented ultrafine fibers and during the electrospinning the fibers were collected on a flat plate.

3.3. An in vivo study

Throughout the postoperative observation period, all animals were healthy and active with normal eating behavior and moved on their own. After the treatment group animals awakened from anesthesia, they did not show any signs of pain. Complete blood counts showed

the following: until Day 4, the leukograms of all animals had demonstrated slight lymphopenia, an increase in band neutrophils to 4% and there was an increase in segmented neutrophils up to 1.2%. The day after the defect was created, counts of erythrocytes and hemoglobin decreased and the animals developed leukocytosis. The counts of erythrocytes and hemoglobin had returned to their initial levels by Day 3 in the treatment groups and by Day 7 in the control. Thus, insignificant changes in some of the parameters of peripheral blood (elevated ESR and moderate leukocytosis) in the first week post surgery were part of the natural response of the body to surgical intervention.

Measurements of the wound area suggested that in the treatment groups, the defects repaired faster (Fig. 6). Photographs of the wounds taken during the experiment are shown in Figure 7. Histology of the skin samples excised at the wound regions showed usual tissue response to surgical intervention, characteristic of the healing process including phases of post-traumatic inflammation, connective tissue formation, cicatrix formation and restructuring along with wound epithelialization. Inflammation was observed in the wound region in all animals but the hyperemia of the dermis vessels and cell infiltration of the dermis were pronounced to different extents depending on the group. In the control group, large amounts of seropurulent exudate containing leukocytes were localized under the surface necrotic masses. In the group with nonwoven P(3HB/4HB) membranes seeded with epidermal cells, hyperemia of the vessels was less pronounced and there was no purulent exudate. That was a usual post-traumatic tissue response with moderate aseptic inflammation, which subsided rather quickly and was followed by tissue formation processes.

Measurements of the wound surface area showed significant changes in the group with membranes seeded with epidermal cells. During the initial days, the wound decrease rate was the highest: 0.4-0.65 cm^2/d; at Day 3 the wound area was 3.6 cm^2 (81 % of the initial wound area). Later at Day 5, the wound area constituted 62.7 % of its initial area, similarly at Day 7 the wound area was found to be 57 % and at Day 14 it was 9 %. The group consisting of PHA membranes showed lower healing rate of 0.3-0.4 cm^2/d. At Day 3, the wound areadecreased by 9 % (91 % of the initial area) and at Day 7, the wound area constituted 78 % of the initial area and at Day 14 it was found to be 9.1 %. This result was comparable with the results obtained from the group with membranes carrying cells. In the control group, during the first 24 h post-surgery, the healing rate was 0.16-0.23 cm^2/d.

In the group with nonwoven membranes without cells, the average healing rate was lower: 0.45 cm^2/d. At Day 5, the area of the wound decreased to 62.7 % in comparison with the initial wound area and at Day 7 it decreased to 57 % and at Day 14 it was found to be 9.1 %. In the control group, the wound healing rate was lower in comparison with the treatment groups which showed 0.16-0.23 cm^2/d and the area of the wound decreased by 4 % by Day 5. At Day 7, a decrease in the wound area reached 23 % and at Day 14 it was found to decrease by 30 % of the initial wound area.

In order to elucidate the mechanism of the wound healing process, a study was carried out to determine the factors that characterized the degree of inflammatory process, vascularization, and formation of the new connective tissue at the defect site. Real-time RT-PCR was performed in the region of quantification of gene expression to the factors of rat angiogenesis (VEGF), inflammation (TNF-a), collagen type I (Col-1) (an indicator of connective tissue formation), keratin 10 (K10) and keratin 14 (K14) (indicators of the formation of the spinous and granulosa layers of the epidermis during regeneration). Total RNA content was isolated from the granulation tissue of the treatment and control groups. The extraction of RNA was carried out

using guanidinium thiocyanate-phenol-chloroform mixture from the RNA-Extran reagent kit. Later, from the RNA template, using reverse transcriptase, cDNA was synthesized using several types of oligonucleotide primers which included a mixture of random primers –hexa primers – and oligo-dT primer according to the protocol described by the manufacturer (Sintol, Russia). Negative controls of reverse transcription reaction were prepared for all samples to confirm the absence of DNA contamination in the initial RNA. Real-time PCR-amplification for quantification of cDNA fragments of rat VEGF, rat TNF-a, rat Col-1, rat K10 and rat K14 were performed by using a CFX-96 thermocycler, according to the manufacturer's protocol using the color channel of HEX.

During the first 24 h, all animals developed edemas, which were more pronounced in the control group. The histology of the new tissue collected from the defect site at Day 3 showed that in the control group, there was formation of connective tissue with very few collagen fibers and blood vessels. Vascularization was more pronounced under the experimental wound dressings, which was confirmed by the PCR of VEGF gene expression as an indicator of blood vessel formation (Fig. 8 a).

The wounds covered with the polymer membrane dressings of P(3HB/4HB) displayed the density of newly formed collagen fibers which was higher than the control. Further, at Day 7 the total cell counts in the dermis under the wound surface were slightly lower in the treated groups which were studied under the microscope. It was also observed that there was a clear shift towards fibroblast cells, suggesting faster reduction in the inflammatory process, which had developed at the defect site. The faster inflammation reduction in the treatment groups was confirmed in the PCR-based tests of TNF-α gene expression (inflammation factor) (Fig. 8 b). The results of planimetric measurements (Fig. 6) and analysis of histological sections (Fig. 9 a), which suggested faster regeneration of the defects in the treatment groups, were confirmed by counting hair follicles, sebaceous glands, and horn cysts, as objective indicators of wound healing (Table 2).

Regeneration started earlier in the treatment groups, in which hair follicles and sebaceous glands were detected at Day 7, whereas only horn cysts, a marker of the lagging cell differentiation in the epidermis was detected in the control group.

The results of PCR-based assays of gene expression of collagen type I (Col-1) (an indicator of connective tissue formation), keratin 10 (K10) and keratin 14 (K14) (indicators of the formation of the spinous and granulosa layers of the epidermis during regeneration) showed that new tissues developed were considerably better and wound healing rate was faster in the treated groups under experimental polymer membranes. The numbers of Col-1, K10, and K14 cDNA copies were higher than those in the control by factors of 4.0-5.0, 3.5-4.0 and 4.0–4.5, respectively (Fig. 9 b).

Counts of hair follicles, sebaceous glands, and horn cysts as objective indicators of wound healing process are listed in Table 2. Sebaceous glands and hair follicles are sources of undifferentiated epithelial cells (structurally the same as basal cells), which take part in skin regeneration.

4. Discussion

Reconstructive medicine needs novel materials for effective regeneration of skin injured due to burns, traumas, and surgical interventions. Skin replacements in the form of films have been increasingly used recently. These are fabricated from various natural and synthetic materials: collagen, polyvinylchloride, polyethylene, polypropylene, polyethylene terephthalate,

poly-epsilon-caprolactone, etc. Skin grafts made of resorbable polymers such as polyvinyl alcohol (PVA), poly(lactide-co-glycolide) (PLGA) blended with chitosan and collagen, polycaprolactone (PCL), gelatin, and glucosamine glycans were tested as skin wound dressings in experiments on laboratory animals [1-3, 48-51]. Many of the films are sufficiently strong and supple; they are convenient to use. However, these wound dressings do not have sufficient vapor permeability. Hydrophilic polyurethane films hold much promise; the best known representatives of these films are Op-Site and Tegaderm. These wound dressings are convenient to use, elastic, and transparent; they adhere well to the wound surface. Fixomull Stretch is a highly adhesive, water and vapor permeable wound dressing consisting of a white nonwoven polyester substrate coated with a skin-friendly polyacrylate adhesive. At the same time, vapor permeability of the majority of such dressings is too low. Omiderm, which is made of elastic hydrophilic polyurethane, has much better vapor permeability and, which is very important, can be used in combination with antibacterial drugs. A special group of wound dressings used in management of burn injuries comprises non-adhesive dressings. Non-adhesive wound dressings Adaptic (Johnson & Johnson) and Damo (Damor America Inc.) are impregnated with non-smearing ointments, emulsions or creams. Skintact (Robinson) is a wound dressing that shows low adherence and high absorptive ability.

Wound dressings are used in the treatment of various wounds, but traditional bandages are growing out of use. Scientific achievements enabled creation of modern wound dressings, which have different properties depending on the phase of wound healing in which they are used. Resorbable wound dressings meet all biomedical requirements and can be useful in both early and late phases of wound healing. Prolonged-action formulations are usually based on high-molecular-weight polymer materials which are degraded in the body without releasing toxic substances. Designing resorbable polymer wound dressings with different biodegradation rates is one of the important objectives of biomedical research. There is great activity in searching for new functional materials and scaffolds.

The present study is an investigation of experimental wound dressings prepared from the P(3HB/4HB) copolymer — the most promising representative of PHAs for biomedical applications — which has a low degree of crystallinity, the highest elasticity, and the fastest rate of biodegradation *in vivo* [32, 45]. It is not by chance that the first medical products for which Tepha (U.S.) obtained permission from FDA for clinical use were fabricated from PHAs composed of 4-hydroxybutyrate monomers.

The first stage of the present study deals with degradability of casting films and nonwoven membranes composed of randomly oriented ultrafine fibers of P(3HB/4HB) *in vitro*, in the phosphate buffer solution and in stabilized blood. In contrast to polylactide/glycolide nonwoven membranes, which were degraded in Ringer's solution at different rates, depending on the component ratio [52]. In the present study, P(3HB/4HB) films and membranes were only degraded in the biological medium (blood), with just inconsiderable strength losses (between 26 and 32 % of the initial values), but were stable in the phosphate buffer solution. These results were consistent with the data obtained in our previous studies reporting monofilament fibers of P(3HB) and P(3HB/3HV) [53].

Therefore, in the present study, both smooth films and nonwoven membranes of P(3HB/4HB) were used as scaffolds for culturing adipose tissue MSCs. The characterization studies using electron microscopy and staining of cells with fluorescent dyes and MTT assay confirmed the elasticity of nonwoven membranes containing ultrafine fibers which were produced by electrospinning and also had more functional cell scaffolds. The study also

confirmed more effective MSC differentiation into fibroblasts which was supported with gene expression studies of collagen type I (Col-1) using real time RT-PCR techniques. The cells cultivated on P(3HB/4HB) membranes synthesized 20 % more extracellular matrix proteins, such as collagen type 1, than cells on films. These results are consistent with the data reported in previous studies, which showed that nonwoven electrospun membranes had interconnected porous structure and a high surface area to volume ratio and acted as better scaffolds for fibroblast, keratinocyte and epithelial cell cultures than films [17, 54-56]. According to the study conducted by Blackwood et al. [52], the nonwoven membranes of polylactide/glicolide effectively facilitated the growth of human keratinocytes, fibroblasts and endothelial cells in mono- and bi-culture enabling collagen production on scaffolds of PLGA with the component ratios of 75:25 and 85:15. Similarly, Sharma et al. [14] reported successful construction of grafts for skin regeneration consisting of two-layer nonwoven membranes with a donor's skin flap as interlayer. These results were in accordance with the present investigation of the *in vitro* study using P(3HB/4HB) films and nonwoven membranes in the MSC culture. In the present study, the use of nonwoven electrospun membranes to construct biotechnological wound dressings (grafts) was evaluated on experimental laboratory animals.

The scientific studies reveal less reports available on PHA polymers as material for constructing wound dressings. In recent years, much more attention has been focused on grafts of polylactides and polylactide/glycolide copolymers [1-3, 14]. Studies also highlight that membranes of 3-hydroxybutyrate-co-3-hydroxyhexanoate [P(3HB/3HHx)] were investigated as scaffolds for tarsal repair in eyelid reconstruction in rats; the results were compared with implantation of commercial dermal matrices (ADM) purchased from ReDerm (Jieya Company, China), and the authors reported that the P(3HB/3HHx) implants repaired the defect, causing a less pronounced tissue response than the commercial matrices. They concluded that the P(3HB/3HHx) membrane was proven to be a suitable candidate for tarsal repair [57]. In a study conducted by Murueva et al. [58] it was reported that P(3HB) microparticles loaded with nonsteroidal anti-inflammatory drugs (diclofenac and dexamethasone) were used in experiments on laboratory mice with experimental skin wounds caused by chemical burns. Treatment with anti-inflammatory drugs encapsulated in polymeric microparticles made of degradable polymer accelerated wound healing compared with routine therapy (estimated by the area of burn wound, wound healing activity, the number of acanthotic cells, and the number of hair follicles and sebaceous glands). The results showed that drug-loaded degradable polymeric microparticles could be promising candidates for treatment of skin defects.

In the present study, the efficacy of using nonwoven membranes of P(3HB/4HB) was studied on model skin defects in Wistar rats. The results displayed insignificant changes in the parameters of peripheral blood in the initial days. The post-surgery effects were part of the natural response of the body to surgical intervention. Histologic examination and measurements of the wound surface area showed that both cell-free and cell-seeded membranes facilitated wound healing. Histology of the skin samples excised at the wound regions showed usual tissue response to surgical intervention characteristic of the healing process. The nonspecific inflammation observed in all groups of animals was a typical posttraumatic tissue response and part of the natural healing process. In the group with nonwoven P(3HB/4HB) membranes seeded with epidermal cells, hyperemia of the vessels was less pronounced and there was no purulent exudate. The wounds under the P(3HB/4HB) membrane carrying cells healed 1.4 times faster than the wounds under the cell-free membrane and 3.5 times faster than the wounds healing under the eschar (control). In the "membrane+cells" group, complete healing was achieved at

Day 14. A similar study was carried out with films of 3-hydroxybutyrate-co-3-hydroxyvalerate [P(3HB/3HV)] at the V.I. Shumakov Federal Research Center for Transplantology and Artificial Organs of Rosmedtekhnologii. The films were found to be suitable scaffolds for culturing MSCs and epidermal cells for various applications, including skin wound repair [59]. Similarly, polymer films of P(3HB/3HV) blended with polyethylene glycol (ElastoPOB) carrying bone marrow mesenchymal stem cells were studied. Experiments on laboratory animals with model defects of the skeletal muscle and burn wounds showed that the polymer material effectively immobilized the cells and delivered them to the defect site for therapy [60]. Overall, the study reported that nonwoven polymer membranes P(3HB/4HB) and membranes carrying epidermal cells (grafts) on laboratory animals showed that they conformed to the wound surface in all areas, protected the wound, and facilitated reparative processes, enabling the wounds to heal faster than the wounds covered with the gauze dressing. The polymer membranes prepared and tested in this study can be used to reduce inflammation, enhance angiogenic properties, and facilitate skin regeneration.

During histologic examination of tissue sections, we counted hair follicles, sebaceous glands, and horn cysts at the defect site as objective indicators of the healing process. The number of hair follicles and sebaceous glands, which are sources of undifferentiated epithelial cells taking part in skin regeneration, was greater in the group with nonwoven polymer membranes than in the control group even at Day 7 post surgery, and at Day 14 it was 3-5 times as large. The number of horn cysts, which indicate lagging differentiation of dermal cells, reached 7 in the control group at the end of the experiment, while in the groups with experimental membranes, no horn cysts were observed not only at Day 14 but also at Day 7.

By using molecular methods, we managed to characterize the wound healing process and mechanism. The faster inflammation reduction in the treatment groups, with experimental wound dressings, was confirmed in the PCR-based tests of TNF-α gene expression (inflammation factor). The results of PCR-based test of gene expression of collagen type I (Col-1) (an indicator of connective tissue formation), keratin 10 (K10) and keratin 14 (K14) (indicators of the formation of the spinous and granulosa layers of the epidermis during regeneration) also showed that new tissues developed considerably better and wound healing rate was faster under experimental polymer membranes. The numbers of Col-1, K10, and K14 cDNA copies were higher than those in the control by factors of 4.0-5.0, 3.5-4.0, and 4.0–4.5, respectively.

Thus, using morphological, histological, and molecular methods, we showed that nonwoven electrospun membranes of P(3HB/4HB), especially when combined with fibroblasts, facilitated wound healing.

Conclusion

Solution-cast films and nonwoven electrospun membranes composed of ultrafine fibers were prepared from P(3HB/4HB) solutions and tested as experimental wound dressings. The films and membranes did not swell in the phosphate buffer solution and were not hydrolyzed in it, but they were slowly biodegraded, without dramatically losing their mechanical strength, in stabilized blood. Nonwoven membranes were more effective than smooth films as scaffolds for MSC culture and MSC differentiation into fibroblasts. The nonwoven polymer membranes and grafts based on them were investigated in experiments on laboratory animals with model skin defects. The experimental membranes fitted the wound shape, protected the wound from external influences, and facilitated wound healing, promoting fast wound repair. Employing morphological, histological and molecular methods, we showed that nonwoven membranes

combined with cells synthesizing growth factors facilitated wound healing, neovascularization and regeneration and enabled complete wound healing by Day 14 post surgery. The use of nonwoven membranes of degradable P(3HB/4HB) as atraumatic wound dressings can reduce the inflammation, enhance the angiogenic properties of the skin, and facilitate its healing.

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