Original Article

Morphological and biochemical analysis of 3D scaffold based on biocompatible polymer for tissue engineering

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ABSTRACT

In modern clinical practice, cell therapy is actively used for the treatment of degenerative diseases and malformations, burns, and injuries, as well as during dental and cosmetic operations. Thus, an urgent task is the development of new materials for tissue engineering, which implies, among other things, the study of the interaction of human cells with a three-dimensional synthetic matrix during the formation of tissues. In this scientific work, the regularities of attachment and proliferation of HeLa cells in the polymer of lactic and glycolic acids are evaluated, and some parameters reflecting the state of cells in the polymer and the development of oxidative stress in them are evaluated. It was found that the stereo ultrastructural organization of cells grown in the matrix did not differ from cells grown in vials; the dynamics of cell growth were close to the growth rate under standard conditions in vials; HeLa cells were not exposed to stress in the matrix during the entire cultivation period, which was confirmed by a stable level of intracellular glutathione and the absence of an antioxidant effect.

Keywords: Tissue engineering, Polyester of lactic and glycolic acids, HeLa cells, Serum of cow embryos, Light microscopy

Introduction

One of the main tasks of tissue engineering is the development of new materials for the restoration of tissues lost during transplantation. At the same time, primary importance is given to the study of the interaction of human cells with a three-dimensional synthetic matrix during the formation of tissues. Modern cell therapy involves the use of stem cells, progenitor cells, and differentiated cells. It is used in clinical practice for the treatment of degenerative diseases and malformations, burns, and injuries, as well as dental and cosmetic operations [1]. At the same time, existing treatment protocols require further improvement and development. Modern developments in biomedicine and, in particular, tissue engineering can be used to improve the effectiveness of treatment in restoring lost functionally significant tissues [2].

In recent years, new techniques for obtaining tissue on three-dimensional polymer matrices have appeared, which can be used in transplantation in order to restore lost tissues [3, 4]. Biocompatible matrices for the creation of tissue engineering structures should be characterized by a wide range of parameters. The key parameters are the absence of cytotoxicity, inflammatory and immune response to the material and its decay products, ensuring adhesion, proliferation, and differentiation of cells, as well as mechanical strength in accordance with the purpose [5-7].

To discover new materials in tissue engineering, it is necessary to develop a model that would allow studying the features of the

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interaction of cells with a three-dimensional extracellular matrix during the formation of new tissues [8]. This scientific article describes such a model, as well as several approaches that allow us to study cell proliferation and assess their functional state during cultivation in a three-dimensional matrix. A matrix of polyester lactic and glycolic acids was used as a polymer, which is a biodegradable synthetic material developed for tissue engineering [9]. Currently, matrices made of polyester lactic and glycolic acids are widely used in clinical practice abroad. In the presented work, the regularities of attachment and proliferation of HeLa cells in the polymer of lactic and glycolic acids were evaluated, as well as some parameters reflecting the state of cells in the polymer and the development of oxidative stress in them were evaluated.

Materials and Methods

HeLa cells (human epithelioid carcinoma cells) were grown on DMEM medium with 10% serum of cow embryos in an atmosphere of 5% CO_2 at a temperature of 37℃. Cell viability was at least 98%.

A 3 mm thick sponge made of lactic and glycolic acid polyester was used as a three-dimensional matrix for cell culture. After sterilization and washing, the matrix was filled with a suspension of cells in concentrations from $0.2 \cdot 10^6$ to $2 \cdot 10^6$ cells /ml of medium, and incubated for 12, 48 hours, and 7 days under standard conditions with constant stirring.

The polymer containing the cells was washed with a 10-fold volume of saline solution. Cellular homogenates were prepared by repeating the freezing procedure three times too -115℃ followed by thawing. Then the homogenate with polymer was centrifuged for 5 min at 1500 g, the polymer was removed, resuspended and the homogenate was frozen before measurements. All measurements were carried out on samples prepared from polymer pieces with a volume of about 150 µl.

The amount of protein in cellular homogenates was estimated by the Bradford method [10]. The intracellular GSH content was determined using the ThioGlo1TM maleimide reagent in a 100 mM phosphate buffer (pH 7.4) [11]. Further, the content of glutathione was normalized to protein measured by the Bradford method in the same aliquot of the sample.

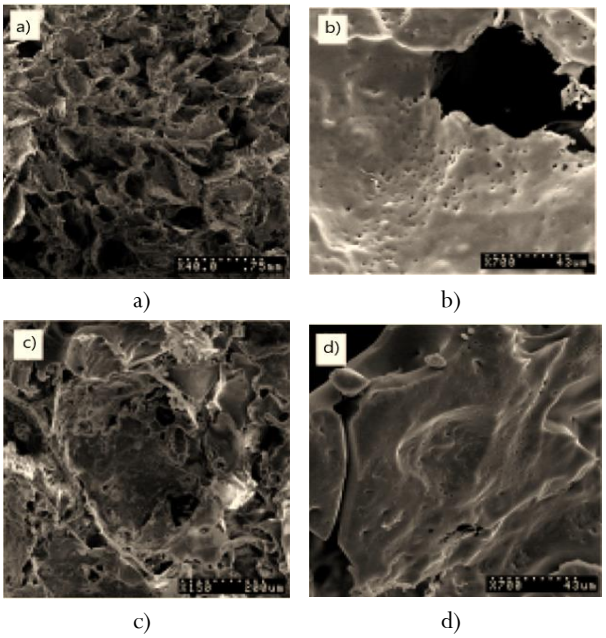
For scanning electron microscopy, the samples were washed with Hanks solution, and fixed with 2.5% glutaraldehyde solution for 24 hours. After dehydration, the samples were dried using hexamethyldisilazane and coated with a layer of gold (10 nm) in the EIKO IB 3 apparatus (Japan) [12]. The analysis of the obtained preparations was carried out in a scanning electron microscope S-570 (Hitachi, Japan) at an accelerating voltage of 15 kV.

For light microscopy, some samples were fixed in 4% paraformaldehyde for 4 hours. The other part of the sample was impregnated with 10% BSA solution and fixed with 2.5% glutaraldehyde solution for 24 hours. All samples were dehydrated and poured into paraffin. Sections with a thickness of 5 µm were stained with Ehrlich hematoxylin and eosin [13]. The analysis of the obtained preparations was carried out using a Motic B3 microscope with the Mekos-C hardware-software complex.

Results and Discussion

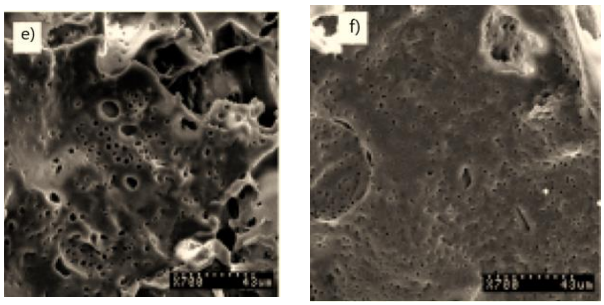
The study of polyester of lactic and glycolic acids and cells in a polymer using a scanning electron microscope is a technically difficult task. The relatively large thickness of the matrix (3 mm), the good solubility of the polymer in solvents, and the lack of sufficient thermal stability (glass transition temperature 37-39℃) do not allow the use of such traditional methods of preparing biological samples as drying by the critical point transition method of liquid CO_2 and freeze-drying [14]. We obtained good results using the so-called "chemical" drying method using hexamethyldisilazane [15]. With this approach, it was possible to ensure the preservation of the polymer microstructure and stereoultrastructural characteristics of cells as was shown by Rzhepakovsky *et al*. [16].

According to the results of the scanning electron microscope, the polyester matrix of lactic and glycolic acids was a porous material formed by hollow cells of oval flattened shape **(Figures 1a and 1b)**. The presence of rounded or oval depressions with a diameter of 1-10 µm and a depth of up to 10 µm was noted on the surface of the cell walls **(Figure 1c)**. The cell sizes ranged from 120 to 900 µm. The thickness of the cell walls varied from 20 to 200 µm, amounting to an average of 60 µm. The volume fraction of polymer in the matrix was about 25%. Investigation of the albumin-pretreated matrix using light microscopy showed empty cells not filled with albumin solution, whose volume was about 20% of the total cell volume. These are completely isolated cells in which cell culture cannot take place. Analysis of the morphology of the matrix incubated without cells in DMEM medium with 10% of cow embryo serum for 12, 48 hours, and 7 days showed no structural changes during the entire observation period **(Figures 1d-1f)**.



a) b)

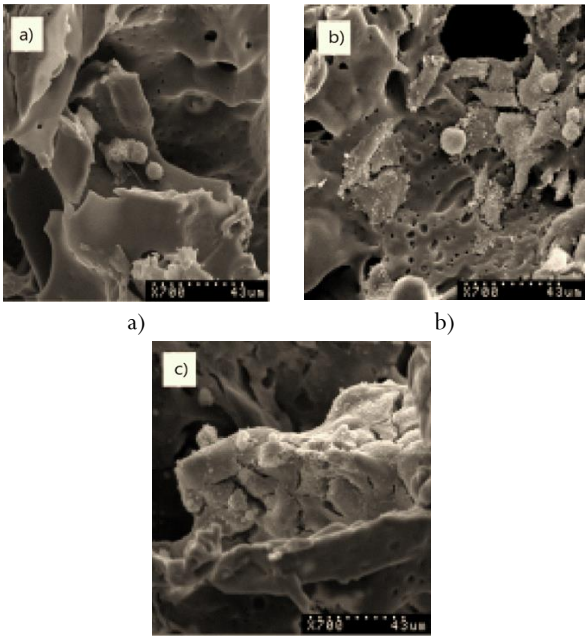
c) d)



e) f)

Figure 1. SEM micrographs of ultrastructural organization of a matrix made of polyester lactic and glycolic acids: a, b, c — matrix before incubation in DMEM medium (magnification ×350, 1300, 6000, respectively); d, e, f — matrix after incubation in DMEM medium for 12 h, 48 h and 7, respectively days (magnification, × 6000 times).

Scanning electron microscopy of matrix samples with cells showed that after 12 hours of cultivation, the presence of single-rounded cells was noted on the surface of matrix cells **(Figure 2a)**. After 48 hours of cultivation of matrix samples with cells, groups of 4 to 8 cells were located on the surface of the matrix cells. Most of the cells had a flattened polygonal shape characteristic of cells in the synthesis stage, in addition, among them there were cells with a rounded shape characteristic of the division stage **(Figure 2b)**. On the 7th day of cultivation, the surface of the walls of individual cells is covered with a large number of flat, polygonal cells tightly arranged to each other **(Figure 2c)**. It is noteworthy that there are significantly more cells on convex cell surfaces than on concave ones. Some cells are completely free of cells, which is consistent with the presence of closed cells. Cells with cells are distributed relatively evenly in the thickness of the matrix.



a) b)

c)

Figure 2. Stereoultrastructure of HeLa cells during cultivation in a matrix of polyester lactic and glycolic acids for 12 hours, 48 hours, and 7 days, respectively (magnification, ×6000)

The light microscopy data confirmed the results of electron microscopy both with respect to cell growth over seven days and with respect to their distribution in the matrix. It should also be noted that at all periods of cultivation, light and scanning electron microscopy did not reveal significant differences in the morphology of cells in the polymer from cells grown in vials.

To estimate the number of cells in the polymer, we homogenized the cells in the polymer and then estimated their number by the total protein content in the resulting homogenate. In this regard, the relationship between the total protein content in the homogenate and the number of cells during cultivation under standard conditions was previously studied. The data obtained in this way allowed us to quantify the dynamics of the division and growth of HeLa cells.

Cultivation of cells for a week showed that they successfully grow in the polymer **(Figure 3)**. Moreover, the growth dynamics in the first 2-3 days were close to the rate of doubling of cells of this line under cultivation in vials (doubling time ~ 20 h). By the seventh day, cell growth slowed down significantly. The average doubling time was about 60 hours. Knowing the doubling time and growth dynamics, we underestimated the conditional number of actively dividing cells ("proliferating cells"). At the same time, the range of cell densities in the polymer, in which good growth was observed, turned out to be quite wide and varied from relatively low cell concentrations (0.2 million/ml polymer) to high concentrations (30 million/ml), close to the density of cells in living tissues.

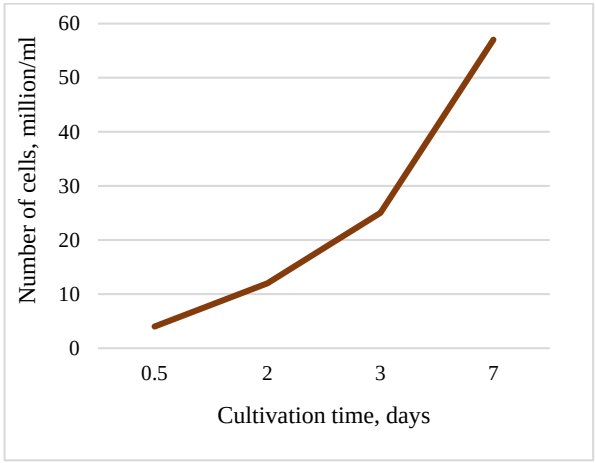


Figure 3. Dynamics of cell growth in the polymer. The polyester matrix of lactic and glycolic acids was filled with a suspension of HeLa cells at a concentration of $2 \cdot 10^6$ cells/ml of medium. Cell culture was carried out in DMEM medium with 10% serum of cow embryos at 37°C in an atmosphere of 5% CO_2 with constant stirring. The number of cells in the polymer was estimated by the total protein

The decrease in the rate of division by the seventh day of cultivation could be due, firstly, to a lack of free area for cell growth in the polymer, and secondly, to the deterioration of cultivation conditions (hypoxia, lack of nutrients, accumulation of metabolic products) caused by a relatively large number of cells. And, finally, thirdly, with the toxic effect of polymer decomposition products. In order to check whether the cells

were under stressful conditions caused by the two-second factors, we studied the "redox"-the state of HeLa cells in the polyester polymer of lactic and glycolic acids

It is well known that the overwhelming number of intracellular processes associated with oxidative stress (including toxic effects, aging, programmed death, and so on) are reflected in a decrease in the level of reduced glutathione (GSH), the main reducing agent in mammalian cells, which maintains intracellular proteins in a reduced state and is used by intracellular enzymes to metabolize derivatives of active forms oxygen [17]. Thus, the GSH level is a parameter sensitive to the occurrence of oxidative stress caused by a wide range of factors.

We assessed the level of intracellular GSH during cell culture in a polymer of lactic and glycolic acids using a highly sensitive fluorescence method. It turned out that the GSH level did not significantly change during the entire cultivation time **(Table 1)**. To make sure that the cells are not exposed to stress, additional experiments were conducted on cell culture in the presence of N-acetylcysteine (NAC). NAC freely penetrates cells, is a precursor of glutathione synthesis, and, in addition, has a pronounced antioxidant effect. However, the intracellular level of GSH and cell growth did not change under the action of NAC **(Table 1)**.

Table 1. Effect of N-acetylcysteine on cell growth and intracellular glutathione content

Cultivation time, days	Number of cells, million/ml		Glutathione content, pmol/μg of protein	
	Control	NAC*	Control	NAC*
0.5	4.41±0.82	3.31±0.38	19.62±4.65	19.2±4.25
2	11.32±0.73	13.78±0.12	19.84±2.48	24.84±3.56
7	55.12±1.81	56.82±3.42	22.87±3.45	23.47±4.65

It should be noted that electron micrographs also did not reveal morphological signs of the appearance of apoptotic cells. Thus, neither signs of oxidative stress nor obvious signs of cell death were found. The slowing down of cell growth by the seventh day is not due to a large number of cells in the polymer and not a nutritional disorder or toxic effect of the polymer but is due to the influence of local factors. The fact is that the area occupied by HeLa cells on the surface of the substrate (approximately 800 μm) is relatively large in relation to the surface area of the matrix cell. Water cells have space for 50 to 1000 cells. HeLa cells are relatively difficult to spread on any surface, which does not allow them to go beyond the cell, and leads to the fact that after filling, cell growth slows down. This interpretation is consistent with the fact that cell growth was weakly correlated with their total number in the polymer. Electronic microphotographs clearly show that polymer cells have a limited size and have a relatively isolated character. It can also be seen that on the seventh day, individual cells in the polymer are either filled with cells or simply empty **(Figure 3)**. Thus, the slowing down of cell growth is determined by the size of the cell and the ability of cells to spread through the polymer [18].

Conclusion

As a result of the conducted scientific research, several approaches to the evaluation of implantable three-dimensional matrices made of polymer materials based on the analysis of growth, morphology, and functional state of cells in vitro have been developed. It was concluded that incubation of the polymer matrix for seven days in a cell culture medium did not cause changes in the polymer structure. The stereoultrastructural organization of cells grown in the matrix did not differ from that of cells grown in vials. In addition, the dynamics of cell growth were close to the growth rate under standard conditions in vials. The range of cell densities in the polymer in which active growth was observed varied from relatively low cell concentrations (0.2 million/ml polymer) to high concentrations (30 million/ml). HeLa cells were not subjected to stress effects in the matrix during the entire time of cultivation, which was confirmed by a stable level of intracellular glutathione, as well as the absence of the effect of an antioxidant, N-acetylcysteine, added to the culture medium.

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Conflict of interest: None

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Ethics statement: The work with cells was carried out considering requirement of ethics commission of Rostov State Medical University.

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