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СРАВНЕНИЕ ЦИТОТОКСИЧНОСТИ СИНТЕТИЧЕСКИХ СОСУДИСТЫХ ПРОТЕЗОВ *IN VITRO*

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Цель. Изучить и сравнить цитотоксичность ключевых видов синтетических протезов, используемых в артериальной реконструктивной хирургии, включая политетрафторэтилен (ПТФЭ) и полиэтилентерефталат (дакрон).

Материалы и методы. На культуре первичных эндотелиальных клеток пупочной вены человека (англ. – *human umbilical vein endothelial cells*, HUVEC) 3 пассажа проведен MTS-тест, используемый в лабораторных исследованиях с привлечением клеточных технологий для изучения цитотоксичности лекарственных веществ и медицинских изделий. Тест подразумевает использование реагента MTS, представляющего собой 3-(4,5-диметилтиазол2-ил)-5-(3-карбоксиметоксифенил)-2-(4-сульфофенил)-2H-тетразолиум; дополнительно используется феназина метосульфат (PMS), играющий роль электрон-связывающего реагента. В ходе эксперимента клетки инкубировались с ПТФЭ и дакроном в течение 24 часов при 37°C с содержанием 5% CO_2. Культивированные в стандартной ростовой среде HUVEC выступили в роли контроля. MTS в присутствии PMS восстанавливался митохондриальными дегидрогеназами эндотелиальных клеток в формазан, имеющий синее окрашивание. Супернатант культур клеток фотоколориметрически при помощи анализатора Stat Fax 3200 (microplate reader) Awareness technology Inc. Palm City Fl. (США).

Результаты. Наименьшие средние значения отмечались в группе дакрона – 0,21 (0,20-0,22) единиц оптической плотности, наибольшие отмечены в группе контроля – 0,36 (0,35-0,38); показатели в группе ПТФЭ составили 0,35 (0,33-0,36). При сравнении исследуемых групп статистически значимые различия были обнаружены между группой контроля и дакрона ($p<0,001$), контроля и ПТФЭ ($p=0,037$), дакрона и ПТФЭ ($p<0,001$). Инкубация с дакроном привела к угнетению метаболической активности клеток на 41,7% по сравнению с группой контроля ($p<0,001$). Метаболическая активность клеток, подверженных воздействию ПТФЭ, была близкой к группе контроля, т.е. соответствовала оптимальным условиям культивирования эндотелиальных клеток *in vitro*.

Вывод. В сравнении с полиэтилентерефталатом (дакроном) политетрафторэтилен (ПТФЭ) наименее выраженно угнетал метаболическую активность эндотелиоцитов *in vitro*.

Ключевые слова: *цитотоксичность; ПТФЭ; дакрон; HUVEC; in vitro.*

COMPARISON OF CYTOTOXICITY OF VASCULAR PROSTHESES *IN VITRO*

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Aim. To study and compare cytotoxicity of the main types of synthetic prostheses used in arterial reconstructive surgery, including polytetrafluoroethylene (PTFE) and polyethylene-

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terephthalate (Dacron).

Materials and Methods. On the culture of human umbilical vein endothelial cells (HUVEC) of the 3rd passage, MTS test was conducted that is used in laboratory examinations with attraction of cellular technologies to study cytotoxicity of medical drugs and medical products. The test implies use of MTS reagent that is 3-(4,5-dimethylthiazol-2-il)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; additionally, phenazine methosulfate (PMS) was used that plays the role of electron-binding reagent. In the experiment, cells were incubated with PTFE and Dacron within 24 hours at 37°C with 5% CO_2. For control, HUVEC cultured in the standard growth medium, were used. In the presence of PMS, MTS was reduced by mitochondrial dehydrogenases of endothelial cells to formazan staining blue. Supernatant of cell cultures was evaluated by photocolorimetric method on Stat Fax 3200 analyzer (microplate reader) of Awareness technology Inc. Palm City Fl. (USA).

***Results*.** The lowest mean values were noted in Dacron group – 0.21 (0.20-0.22) optical density units, the highest values were noted in the control group – 0.36 (0.35-0.38); parameters in PTFE group were 0.35 (0.33-0.36). In comparison of the groups statistically significant differences were found between the control group and Dacron group ($p<0.001$), control and PTFE group ($p=0.037$), Dacron and PTFE ($p<0.001$). Incubation with Dacron led to suppression of metabolic activity of cells by 41.7% as compared to the control group ($p<0.001$). Metabolic activity of cells exposed to PTFE, approached that of the control group, that is, it corresponded to the optimal conditions of culturing of endothelial cells *in vitro*.

***Conclusion*.** In comparison with polyethylene-terephthalate (Dacron), polytetrafluoroethylene (PTFE) showed the least suppression of metabolic activity of endothelial cells *in vitro*.

Keywords: *cytotoxicity; PTFE; Dacron; HUVEC, in vitro.*

The optimal method of revascularization in the open reconstructive surgery of peripheral arteries is use of autologous materials, in particular, of large saphenous vein. Rarely, in complicated surgical situations, freshly made or cryopreserved allogenous venous or arterial prostheses are used that may lead to immunosensitization reactions and uncontrolled degradation processes. In cases the autologous material is unavailable, vascular prostheses mostly of polytetrafluoroethylene (PTFE) and polyethylene-terephthalate (Dacron) are used.

Vascular conduits made of PTFE were first introduced into clinical practice in 1976 [1]. Prostheses made of Dacron have been used in cardiovascular surgery for more than 70 years. Nowadays, the most Dacron prostheses are coated with collagen or gelatin, or impregnated with silver; besides, to reduce thrombogenicity, they are coated with heparin [2].

In earlier studies, Dacron grafts showed satisfactory patency within 16 months with use of short sections 3.5 mm x 4 cm for coronary artery bypass [3]. Here, PTFE vascular prostheses used for coronary artery bypass showed only 14% patency within 45 months [4]. Nevertheless, small diameter synthetic grafts practically are not used at present because of high risks for development of complications.

With this, artificial prostheses find a wide use in reconstructive surgery of the aorta and major vessels. Some authors report safety and reliability profile of Dacron prostheses to be not lower than those of PTFE: according to the data of recent studies, structural defects occur not more than in 0.2% of cases in the long-term period after reconstruction [5]. However, in comparison with Dacron, PTFE is a less porous material due to which it is less permeable to blood; despite chemical inertness of the material, proteins and blood cells may deposit on PTFE as

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well [6]. According to some clinical studies, patency of PTFE and Dacron prostheses is comparable [7].

Early complications of reconstructive interventions may often be attributed to unsatisfactory biological compatibility of a prosthesis and a native vessel. Artificial grafts have a tendency to absence of endothelization in the areas outside the zones of anastomoses; finally, on parts of prosthesis deprived of endothelium, blood plasma protein, mainly fibrinogen and platelets, deposit forming the so called «pseudoneointima» reaching 1 mm thickness, which eventually predisposes the prosthesis to thrombosis and to increased risks of infection in bacteremia [8]. More severe late complications include hyperplasia of intima especially in the zone of distal anastomoses which develops by different mechanisms, cell interactions and physical factors associated with implantation of an artificial prosthesis [9]. Many experimental and clinical *in vivo* and *in vitro* studies were devoted to investigation of molecular-genetic aspects of pathogenesis and possible ways of prevention of development of peripheral atherosclerosis as such and of complications of reconstructive surgery including hyperplasia of intima, thrombosis, ischemia-reperfusion [10-13].

In physiological conditions, endothelium has athrombogenic surface on which chondroitin and heparin sulfates are expressed; anticoagulation properties of intima are also ensured by production of prostaglandin I2, nitric oxide (II) and ADP-ase [14]. Impossibility to fully reproduce athrombogenic properties of a native vessel in an artificial vascular prosthesis, on the whole predetermines the destiny of artificial materials in the arteries in surgery of peripheral arteries. An important role in development of complications is played by porosity of the material of vascular prostheses, compliance between an implanted graft and a native vessel, peculiarities of the blood flow in the zone of anastomosis.

Improvement of endothelization and hemocompatibility of vascular prostheses, as well as modification of their surface in general, serve to prevent deposition of different blood plasma proteins and to improve the long-term patency of artificial grafts. The inner lumen of grafts may be changed by application of different compounds, for example, hydrophilic polyethylene glycol, zwitterion polymers, heparin and others. However, excessive hydrophily prevents adhesion of endothelial cells and formation of the optimal inner lining of prostheses. Therefore, many research works are directed to improvement of the functional endothelization of prostheses by molecular-genetic methods [15,16].

In vitro study of the influence of artificial materials used in the vascular surgery, on cells of the vessel wall may provide additional insight into the mechanisms of interaction of cell elements of a native vessel, and also of blood and vascular prosthesis. Under study are peculiarities of adhesion of HUVEC to PTFE material, for example, after modification of the latter with low-temperature plasma; peculiarities of biocompatibility of different materials, for example, of silk fibroin and polyurethane membranes in culturing with HUVEC; cytotoxicity of artificial materials is evaluated *in vitro* for different kinds of exposure [17-19].

Evaluation of cytotoxicity *in vitro* in general is widely used in preclinical trials in studying the influence of different medical drugs and medical products on cell culture. Among the most requested in the routine laboratory practice are MTT and MTS tests.

MTT test is based on the ability of mitochondrial dehydrogenases of living and metabolically active cells to convert water-soluble 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan possessing a different extent of staining. In addition of dimethyl sulfoxide (DMSO) to formazan, the latter dissolves which permits to measure the optic density of the obtained solution, and, in this way, to evaluate metabolic activity of the studied cells and, accordingly, cytotoxicity of the studied substance or medical product.

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A similar method of cytotoxicity uses MTS reagent 3-(4,5-dimethylthiazol-2-il)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium in the presence of phenazine methosulfate (PMS) that plays the role of electron-binding reagent. MTS, like MTT, is reduced by cells to formazan; the extent of staining of the cellular supernatant may be measured by a photocolorimetric method (Figure 1) [20].

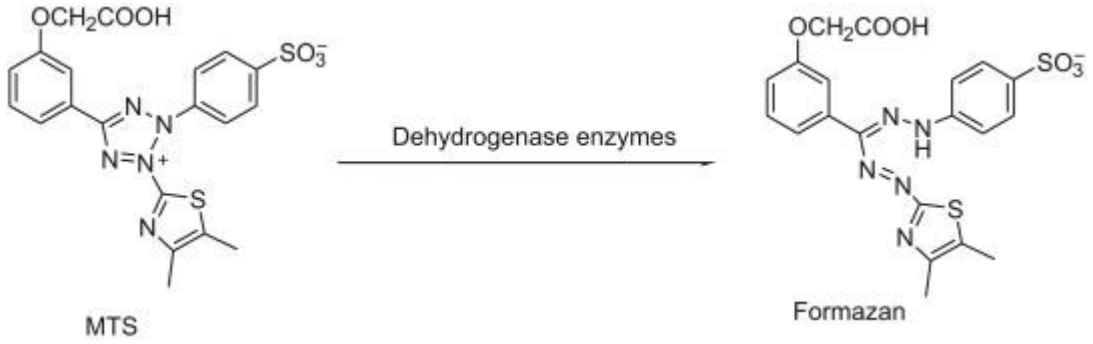

Fig. 1. The scheme of reduction of MTS to formazan under action of dehydrogenases

The most popular object for *in vitro* study of different vascular diseases and pathological conditions is human umbilical vein endothelial cells (HUVEC). HUVEC possess a number of advantages: convenient isolation, relatively low cost, easy culturing in laboratory conditions. HUVEC were first isolated and cultured *in vitro* by E. Jaffe, et al. in 1973 [21]. The HUVEC line is most commonly used in *in vitro* studies in the field of vascular biology. HUVEC are used both to study physiological processes occurring in the endothelium, and to model different pathological processes, in pharmacological research, and in studying effects of medical products.

In fundamental research human umbilical vein endothelial cells are often used as a model of choice for biomedical industry and preclinical experiments.

Thus, the *aim* of the given work was to study and compare cytotoxicity of the key types of synthetic prostheses used in the arterial reconstructive surgery including polytetrafluoroethylene (PTFE) and polyethylene-terephthalate (Dacron).

Materials and Methods

In the experiment, cultures of the primary endothelial cells of the human umbilical vein of 3rd passage were used. Isolation and culturing of cells were carried out in Laboratory of cell technologies of Central Scientific Research Laboratory of Ryazan State Medical University according to the standard accepted protocols. The tested objects were PTFE and polyethylene-terephthalate (Dacron) 4x4 mm in size of the same mass (25 mg). The design size and mass of the materials were selected taking into account the optimal coverage of the surface area of membrane inserts and the results of the preliminary experimental studies. As a control, growth endothelium medium ECGM (Cell Applications Sigma/Aldrich, catalogue number 211-500) was used that was added into a well on the plate in the quantity similar to that in other wells. The experiment was performed three times with different primary HUVEC lines to exclude measurement errors.

In the course of each experiment primary HUVEC were inoculated into the rows of wells of 12-well plate (Corning, catalogue number 3512) (3rd passage). The time of growth of cells in 12-cell plate before addition of the tested objects was 48 h at 37°C in a thermostat with 5% CO_2 (CO_2 incubator WS-180CS, World Science, Korea). On achievement of 80% confluence, the tested objects of 25 mg mass were introduced into the membrane

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inserts of 12-well plate (Corning, 6.5 mm, growth area 0.33 cm², pores 0.4 μm, catalogue number 3413) and were incubated for 24 hours at 37°C with 5% of CO_2 (Table 1).

Table 1

Experimental Design

Time	Control	Dacron	PTFE
0 h	HUVEC 0.1 x 10^6	HUVEC 0.1 x 10^6	HUVEC 0.1 x 10^6
48 h	ECGM	25 mg of Dacron	25 mg of PTFE
72 h	MTS/PMS	MTS/PMS	MTS/PMS
73.5 h	Transfer of contents of wells to 96-well plate for measurement of optical density		

After 24 hours, membrane inserts were taken out of the plates and were exposed to MTS/PMS reagents (Abcam, catalogue number ab223881) for 1.5 hour at 37°C with 5% CO_2. After this period the obtained solutions (cell supernatant) with different extent of staining were transferred to 96-well plates (Corning, catalogue number 3599) for evaluation of the optical density on analyzer ((Stat Fax 3200 (microplate reader), Awareness technology Inc. Palm City Fl., USA)) at 490 nm (reference value 640 nm). From each well not less than 5 samples were taken to exclude measurement errors (Figure 2).

Fig. 2. 96-well plate with cell supernatant after incubation with MTS/PMS before measurement of optical density

Statistical processing of the data obtained was carried out using software package Statistica 10.0 (Stat Soft Inc., USA).

Results and Discussion

The lowest average values of optical density in the supernatant were noted in Dacron group – 0.21 (0.2-0.22) optical density units (ODU), the highest values were noted in the control group 0.36 (0.35-0.38) ODU; parameters in PTFE group were 0.35 (0.33-0.36) ODU. Comparison of the studied groups showed statistically significant differences between the control and Dacron groups ($p<0.001$), control and PTFE ($p=0.037$), Dacron and PTFE ($p<0.001$, Figure 3).

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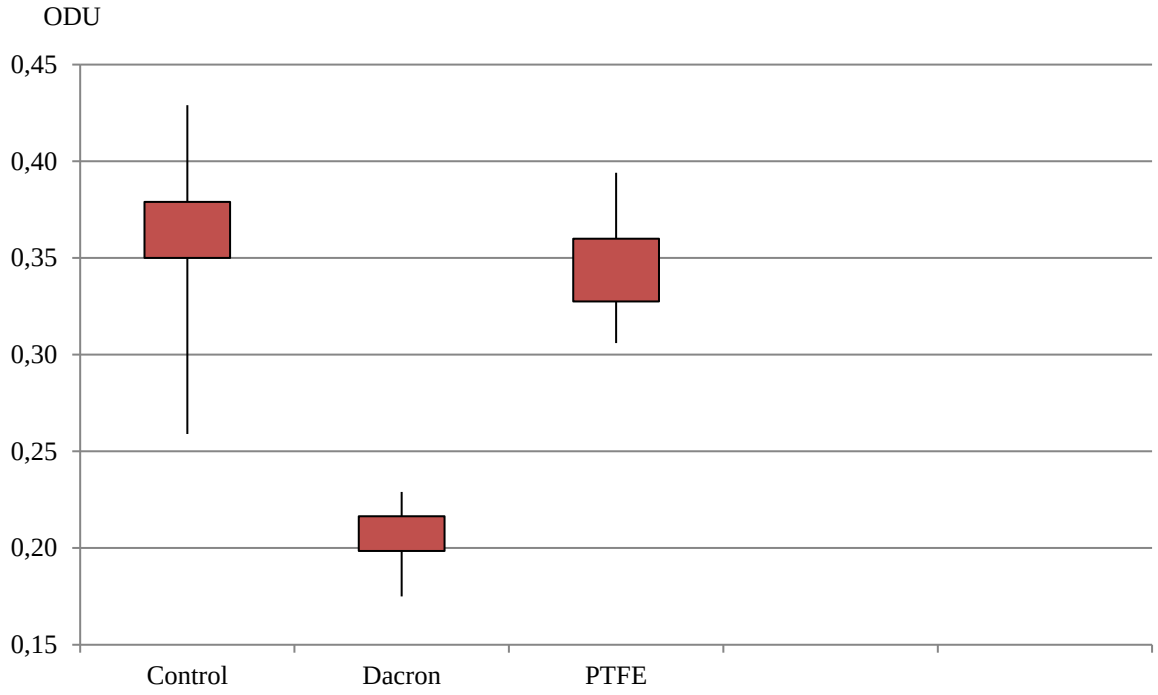

Fig. 3. Comparison of the optical density values in the studied groups in the experiment on cytotoxicity

Note: all comparisons between groups are statistically significant ($p<0.05$)

Analysis of cytotoxicity implies investigation of the probability for the influence of some material on viability of cells, for example, on metabolic activity, integrity of cell membranes, cell growth. Study of cytotoxicity and/or viability of cells i*n vitro* has a number of advantages, such as rapid implementation of examinations, relatively low cost and also a possibility of using human cells with no risk for patient's health; besides, *in vitro* use of human cell lines may give more precise results than some *in vivo* experiments on animals.

There exists a large amount of methods for evaluation of cytotoxicity: 1) method excluding use of any dyes/kinds of staining; 2) colorimetric methods; 3) fluorometric methods; 4) luminometric methods. MTS-test used in the present work, along with MTT, XTT, WST-1, WST-8, LDH, SRB and NRU methods, is referred to colorimetric methods [22].

MTS-test is a rapid, sensitive and specific method to study cytotoxicity *in vitro*. A limitation of the method may be influence of time of incubation and type of cells on the results.

However, the results of studies show that the choice of the optimal time of exposure to MTS gives reliable experimental results [22].

MTS-test may be used to study cytotoxicity of materials applied in different fields of medicine both for evaluation of the direct contact with cells and also of indirect contact, for example, with use of membrane systems [23]. A study of cytotoxicity plays an important role in the cardiovascular surgery as well. Thus, works are under way to study prosthetic valves on the basis of LLDPE, PTFE, Dacron, bovine and porcine pericardium coated with hyaluronic acid, where methods of evaluation of cytotoxicity (LDN-methods) were actively used [24].

The experiment conducted by the authors showed that the most cytotoxic substance in relation to HUVEC was Dacron:

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examination of the optical density of supernatant obtained from cells incubated with this material demonstrated minimal parameters indicating 41.7% suppression of the metabolic activity of cells in comparison with the control group. Metabolic activity of cells exposed to PTFE was close to that of the control group (reduction of the optical density not more than by 2.8%), that is, it corresponded to the optimal conditions of functioning of endothelial cells *in vitro*.

Evaluation of cytotoxicity permits to study the reaction of cells to exposure to different materials. The work for studying cytotoxicity of the key materials used in vascular surgery, with application of MTS-test on the primary human umbilical vein endothelial cells being a convenient and available material for *in vitro* studies, showed a possible relatively simple and available laboratory method of evaluation of the influence of artificial prostheses on the main elements of the vascular wall. The method is reproducible, which was evidenced by the results of repeated experiments.

The approach used in the given work, provides a routine method to study the influence of different conditions of the extracellular environment, prosthetic coatings, chemical agents on metabolic activity of cells that may expand knowledge of the processes of hemocompatibility, endothelization of vascular prostheses and hyperplasia of intima in *in vitro* conditions.

Conclusions

1. Polyethylene-terephthalate (Dacron) possesses a cytotoxic effect for the primary human umbilical vein endothelial cells *in vitro*, significantly suppressing metabolic activity of cells.

2. In comparison with polyethylene terephthalate (Dacron), polytetrafluoroethylene produces a minimal damaging effect on endotheliocytes *in vitro*.

3. MTS-test may be used for routine laboratory study of the influence of materials applied in reconstructive arterial interventions, on the cells of vascular system *in vitro*.

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