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METHODS

Original Method for Evaluating Endothelial Vasomotor Function of Isolated Vessel in Experiment

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We describe a method for experimental assessment of endothelial dysfunction in an isolated using extracorporeal machine perfusion and vasoactive substrates agents (norepinephrine and acetylcholine). Quantitative assessment of endothelial vasomotor dysfunction was proposed, based on registration of changes in volumetric perfusate flow rate through the isolated vessel. Functional activity of the isolated aorta endothelium and total content of stable NO degradation products in blood plasma were studied in outbred white male rats after subtotal liver resection. Impaired vasodilation ability of the aorta and increased content of NO degradation products in blood plasma observed after surgery indicate the development of vasomotor endothelial dysfunction associated with oxidative stress, cholemia, and endotoxemia.

Key Words: *endothelial dysfunction; extracorporeal perfusion; experiment; modeling*

It is known that endothelial dysfunction is the initial stage and sometimes the key element in pathogenesis of some pathological processes [11]. Considering the integrative role of endothelium in vascular tone regulation and implementation of pro- and anticoagulant, immune, transport, metabolic, and other reactions of the body, the mechanisms of endothelial dysfunction are still the subject of active study. Due to a variety of functions of endothelial cells, endothelial dysfunction in a narrow sense is an imbalance between the production of vasoconstrictors and vasodilators impairing the vasomotor properties of the endothelium, which is referred to as vasomotor endothelial dysfunction (VED) in the literature [1].

Known methods of VED assessment that were developed for solving fundamental problems can be divided into biochemical and functional. Biochemical methods for evaluation of endothelial damage are based on the analysis NO metabolites, prostaglandins and interleukins, vascular endothelial growth factor, and intercellular adhesion molecules. The main limitation of biochemical methods is the need for complex reactions for the analysis of vasoactive metabolites that require expensive reagents and special equipment [7]. In addition, the interpretation of the results is often difficult due to the increased expression of inducible NO synthase in some diseases [10].

A modern functional method for assessing vasomotor endothelial reactions is registration of the responses of an isolated vessel using an isobaric myograph and vasoactive substrates. However, the implementation of this method in experimental practice is limited due to complexity of methodological approaches and high cost associated with the use of a thermostatically controlled myograph, a videomicroscope attachment, and a computer program for processing the results [6].

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Due to limitations of the currently known methods for VED evaluation, lack of clear diagnostic criteria, and the need in further study the mechanisms of endothelial dysfunction, the development of alternative reproducible methods for determining the VED in experiment is a pressing problem.

The aim of the study was to develop original method for evaluating endothelial vasomotor function of an isolated vessel in the experiment.

MATERIALS AND METHODS

The experiments were carried out on outbred white male rats in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The study was approved by the Local Ethics Committee.

The animals were kept under standard vivarium conditions. The rats were anesthetized with diethyl ether, and after left-sided thoracotomy, the thoracic aorta was isolated, and 2-cm aortal segments were prepared. The segments were placed in a Krebs—Henseleit solution cooled to 2-4°C. Next, the isolated vessel was cannulated with a 20G catheter, placed in an extracorporeal perfusion machine (the length was equal to that *in vivo*), and antegrade perfusion was performed with Krebs—Henseleit solution (pH 7.33-7.36) saturated with carbogen (95% O_2+5% CO_2) under constant pressure of 100 mm Hg and at 37°C maintained with a VT-8 ultrathermostat (Termeks-2). The scheme of perfusion is shown in Figure 1.

After 30-min normoxic perfusion (stabilization of the tone of the isolated vessel), norepinephrine 10^{-6} M was added to the perfusate, the volumetric perfusate flow rate through the isolated vessel was recorded for 1 min using a Dyna ultrasonic flow sensor (DUD-400 type). For evaluation of the functional activity of the endothelium, acetylcholine 10^{-5} M was added to the perfusate. The volumetric perfusate flow rate through the isolated vessel was recorded again for 1 min. The severity of VED was evaluated quantitatively by calculating the degree of vasodilation according to our formula:

$$(Q_{ACH}\text{-}Q_{NE}/Q_{NE})\times 100\%,$$

where Q_{ACH} and Q_{NE} are volumetric perfusate flow rates after addition of acetylcholine and norepinephrine, respectively.

The degree of vasodilation ≥1.6 corresponded to normal vasomotor function of the endothelium; vasodilation ≥0.8 to 1.6 indicated moderately expressed VED and from 0 to 0.8 attested to severe VED; in case of negative values, extreme degree of VED and the effect of paradoxical vasospasm in response to acetylcholine were diagnosed.

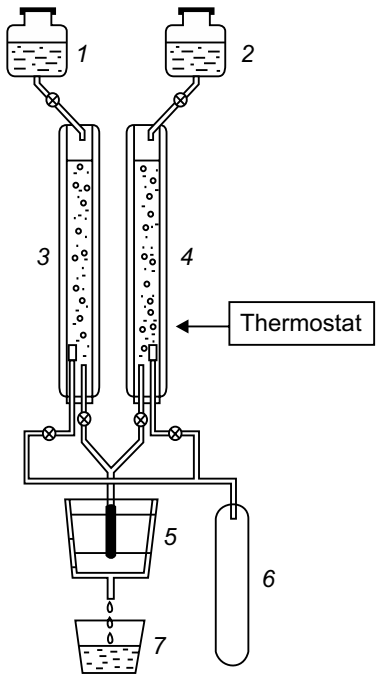

Fig. 1. Scheme of isolated vessel perfusion. *1*, *2*) containers with perfusion solutions; *3*, *4*) thermostatic oxygenators; *5*) thermostatic chamber with an isolated vessel; *6*) a tank with carbogen; *7*) graduated container for measuring volumetric rate of perfusion.

The method developed by us for evaluation of VED in an isolated vessel [9] was validated on the model of subtotal liver resection (SLR) in rats. We have previously demonstrated the formation of circulatory failure in the early postoperative period in this model caused not only by action of extracardiac factors of extensive surgery, but also by developing myocardial damage [5]. The functional state of the endothelium was studied to elucidate the role of vascular tone disorders in the pathogenesis of heart failure after SLR.

The rats (n=70) were anesthetized with diethyl ether (Medkhimprom) and atypical 80% SLR was performed by our original technique [8]. In survivors (n=56), functional activity of the endothelium of the isolated aorta was studied at different terms after SLR: 1, 3, 6, 12 h and 1, 3, 7 days (8 animals per point). Rats of the control group were anesthetized, but no surgery was performed (n=8).

VDE was verified on this model by determining the total content of stable products of NO degradation (NO_2^-/NO_3^-) in the blood plasma at the same time after surgery using the Griss reaction [7], the optical density of the solution was measured on an SF-46 spectrophotometer at λ=540 nm.

Statistical analysis of the results was carried using Statistica 6.0 software (StatSoft, Inc.). The sig-

nificance of differences in independent samples was determined using the Mann—Whitney test. The data are presented as Me (LQ; HQ) quartiles. The differences were significant at $p<0.05$.

RESULTS

In 1 h after SLR, the degree of vasodilation of the isolated aorta decreased by 9% in comparison with the control; in 3 h, the degree of aortic vasodilation further decreased by 38% ($p<0.05$), and a moderate VED was observed: 1.04 (0.95; 1.13) arb. units (Fig. 2). In 6 h after SLR, we observed a progressive decrease in the degree of aortic vasodilation by 87% ($p<0.05$) and severe VED. During the interval from 6 to 24 h after SLR, the decrease in the vasodilation capacity of the aorta reached a certain stable level (plateau). On day 3, a tendency to an increase in the degree of vasodilation was noted, however, this indicator remained by 35% below the control ($p<0.05$), functional activity of the endothelium after SLR did not recovered completely.

The content of NO degradation products in blood plasma of control animals was 15.2 (14.0; 15.8) μmol/liter; in 1 h after SLR, this parameter increased by 37% in comparison with the control (to 20.8 (20.3; 22.4) μmol/liter; $p<0.05$). In 3 and 6 h after surgery, their further increase by 62% (up to 24.7 (22.0; 25.1) μmol/liter) and by 87% (up to 28.5 (26.7; 30.1) μmol/liter) was revealed ($p<0.05$ in comparison with the control). Some stabilization of the level of NO degradation products was observed in 12 and 24 h after CRP: 27.3 (26.2; 28.8) and 26.1 (25.6; 27.4) μmol/liter, respectively ($p<0.05$ in comparison with the control). On day 3 after SLR, the concentration of nitrite/nitrate anions decreased to 19.3 (16.5; 20.0) μmol/liter; by day 7, no differences from the control values were observed. The increase in the concentration of NO degradation products after SLR indicates activation of various NO synthase isoforms and, first of all, the expression of an inducible enzyme isoform by endothelial and other cells [10].

Correlation analysis revealed an inverse correlation between the degree of vasodilation and the total content of nitrite/nitrate anions in the plasma (r=-0.76, $p<0.05$) at all terms after SLR, which can indicate mutual dependence of changes in functional activity of the endothelium and NO production due to progressive damage to endothelial cell membranes [10]. It was previously established that NO excess exhibits prooxidant properties, acts as a powerful proapoptotic factor, and produces a cytotoxic effect on the endothelium [10]. In addition, NO under conditions of overproduction does not exhibit the vasodilator properties [10].

Our findings that an imbalance of the vasomotor reactions develops in the isolated aorta at the early stages after SLR with a predominance of vasoconstriction, despite enhanced production of NO metabolites. In our previous experimental studies, the development of circulatory failure was established and the pathogenetic relationship of functional metabolic myocardial disorders with hypoxia, oxidative stress, coagulopathy, and cholemia was shown at the early stages after SLR [2-5].

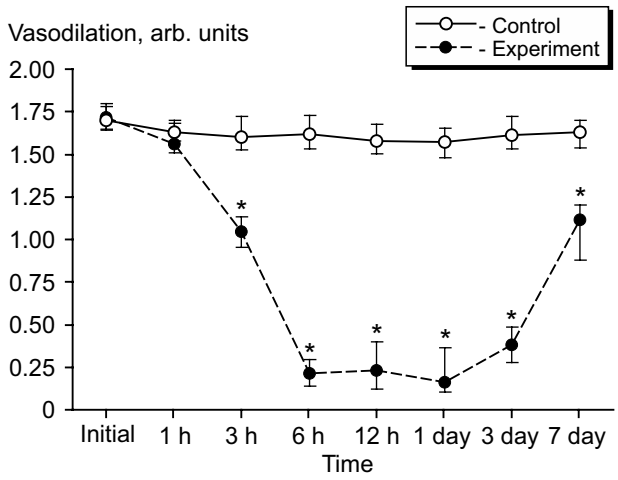

Fig. 2. The degree of vasodilation of the isolated aorta after SLR in rats. *$p<0.05$ in comparison with control.

When comparing published data with our results, we hypothesized that hypoxia that initiates activation of free radical oxidation, toxic effects of bile acids released into systemic circulation from the surgical field, and endotoxemia developing against the background of enhanced catabolism and reduced detoxification function of the remaining part of the liver can be the leading pathogenetic factors causing damage to endotheliocyte membranes and the development of endothelial dysfunction after SLR.

Thus, the developed method for evaluation of endothelial vasomotor function of an isolated vessel in experiment is characterized by relative simplicity and reproducibility. Validation of the method on the rat model of SLR showed that endothelial dysfunction developed at the early terms after SLR manifested by impaired vasodilation of the isolated aorta along with accumulation of NO degradation products in blood plasma of experimental animals. The proposed method can be used in translational studies to identify the cellular and molecular mechanisms of endothelial dysfunction and to develop methods for its pharmacological correction in modeling the pathology in the experiment.

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