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METHODS

Effect of Circulation Parameters on Functional Status of HepaRG Spheroids Cultured in Microbioreactor

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We studied the relationship between microcirculation parameters and functional status of HepaRG cells in spheroids and chose an optimal regimen within the physiologically permissible limits of mechanical impact for the cells that maintains the expression of functional genes of the liver.

Key Words: *microcirculation; liver-on-a-chip; HepaRG spheroids*

Creation of adequate models of human organs and tissues is essential for the development and effective preclinical evaluation of drug effects (toxicity, search for molecular targets, biotransformation routes, *etc.*). The latest progress in cell biology, microtechnologies, and microfluidics resulted in creation of engineering models of human organ functional units, so-called organs-on-a-chip. This technology can in future provide the base for preclinical trials with high predictive efficiency [1,2,8].

The liver is one of the key organs for analysis of drug effects and toxicological studies. In order to achieve a correct response to a xenobiotic, it is essential to create the microenvironment simulating the basic biomechanical parameters, characteristic of liver tissue in human organism.

The liver is an organ with a dense vascular network. About 25-30% of total blood volume (approximately 100 ml blood) passes through 100 g of the liver tissue (or ~1.5 liters through 1.5 kg adult human liver tissue) every minute. From the hemodynamic viewpoint, the sinusoid is the basic unit of active transport and metabolism. The sinusoid diameter usually varies from 7 μ (in the lobular periportal region) to 15 μ (in the lobular pericentral region).

The shear stress in the liver is evaluated at ~0.1-0.5 dyne/cm^2 (0.01-0.05 Pa). These values correspond to the lowest shear stress in human capillary system (typical values for a capillary are 15 dyne/cm^2, or 1.5 Pa). The shear stress in the sinusoids increases significantly during reperfusion or partial hepatectomy, and can play a role in triggering liver regeneration processes. The physiological shear stress is essential for the maintenance of functional phenotypes of tissues with dense vascular network in health and disease. However, the shear stress applied to hepatocytes is difficult to measure, as sinusoidal epithelial cells directly contacting with blood and the Disse space are smoothened by circulating blood [6].

The sinusoidal epithelium has pores of ~100 nm in diameter occupying 2-3% of sinusoidal area and supporting plasma flow from the sinusoid to the Disse space filled with interstitial (intercellular) fluid. The flow from the sinusoids to the intercellular space is realized at the expense of difference in the mechanical and oncotic pressure between the two spaces. Oncotic

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pressure is determined by the presence of proteins in the plasma, but normally, it is low in comparison with the mechanical pressure [13]. Despite the fact that the intercellular fluid velocity is low (0.1-2 μ/sec) in comparison with the capillary blood flow (10-20 cm/sec), it can be the cause of mechanical stress, and changes in this parameter can lead to liver fibrosis. Disturbances in intercellular fluid flow can affect secretion of growth factors, *e.g.* TGF-β and modulate structure and organization of the extracellular matrix (collagen) [14]. Hydrostatic pressure created by intercellular fluid in the liver is approximately 2.86±1.04 mm Hg (381.3±138.6 Pa) [9]. In order to simulate these effects *in vitro*, we used a microfluidic system (MFS) previously described in detail [8,11] that allows reproducing various microcirculation conditions by varying external pressure and pumping frequency.

The aim of our study is to determine the microcirculation mode not surpassing the physiologically permissible levels of mechanical impact on HepaRG cells in spheroids and maintaining the expression of functional genes of the liver.

MATERIALS AND METHODS

HepaRG cells were cultured in complete nutrient medium (CNM): William's E with L-glutamine (Gibco), 10% fetal calf serum (HyClone), 5 μg/ml human recombinant insulin (Gibco), 5×10^{-5} M hydrocortisone hemisuccinate (Sigma), and 1% penicillin/streptomycin (Gibco) in a CO_2 incubator (37°C, 95% air, 5% CO_2, humidity 98%).

After formation of a confluent monolayer, 2% DMSO was added in CNM in order to induce differentiation. In 14 days after the beginning of differentiation, spheroids (aggregates consisting of 5000 HepaRG cells) were formed in CNM without DMSO over 5 days in low adhesion round-bottom 96-well plates.

After formation, the spheroids were transferred into wells of a 96-well plate or in MFS (30 spheroids in 200 μl CNM/well) and cultured for 1 day more. Three pumping regimens were used during spheroid culturing in MFS: at ±30 kPa pressure on the pump membranes and 2 Hz frequency of valve triggering, at ±30 kPa and 5 Hz, and at ±10 kPa and 5 Hz.

The geometry of MFS contour and the forces acting on the spheroid in MFS are are shown in Figure 1. The microcontour channel is formed by a layer of polydimethylsiloxane (PDMS) and the slide. The section of the channel is rectangular, 100 μ high. The width of the channel in rectangular compartments is 500 μ. The spheroids are located in cell compartments. Circulation of the nutrient medium through the compartments is realized by means of volume pump consisting of a working chamber and two valves. The valves and working box are thin-walled elastic membranes set to motion with an external pressure and vacuum source. A cycle of the pump work includes a succession of five steps of the working box membranes and valve positions maintaining nutrient fluid delivery to cells. The control block allows regulation of air pressure on the pump membranes and modulation of the frequency of alternation of the pumping cycle steps. The characteristics of MFS and the flow created by the pump have been described previously [11].

After culturing in a plate or in MFS the spheroids were lyzed in Qiazol (Qiagen), the RNA was isolated, and the gene expression was analyzed by the real time PCR as described previously [7]. Primers used for evaluation of gene expression were presented in Table 1.

The data were statistically processed by nonparametric Mann–Whitney *U* test. The differences were considered significant at $p<0.05$.

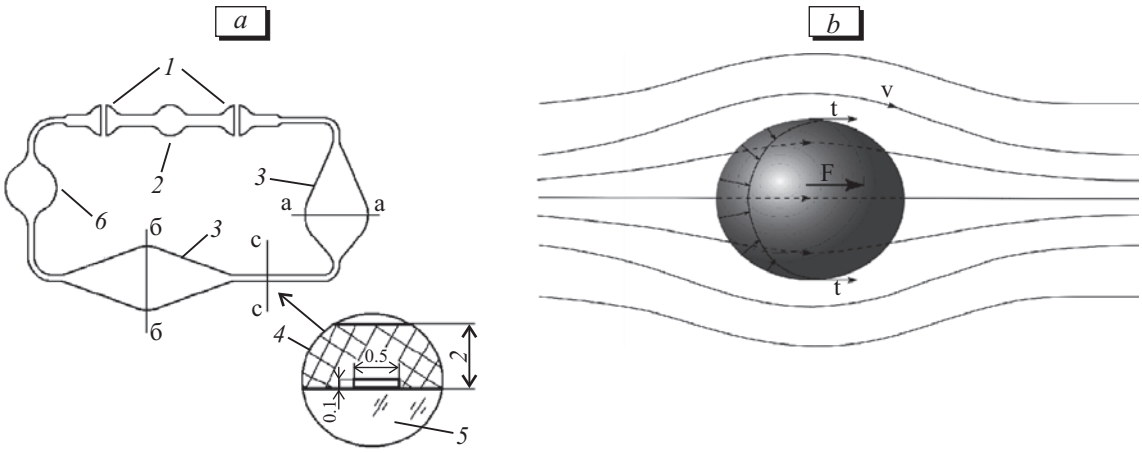

Fig. 1. Geometry of the microchannel (*a*) and forces acting on the spheroid in MFS (*b*). *a*: *1*) valve; *2*) working box; *3*) cell compartment; *4*) PDMS layer; *5*) slide; *6*) extension box. Sections a-a and b-b: sites of pressure pickup location; c-c: transverse section of MFS channel. *b*: F – force acting on the spheroid from the fluid flow; τ – shear stress; ν – velocity of fluid flow.

TABLE 1. Primers Used in the Study

Gene	Forward primer	Reverse primer
Cyp3A4	3'-GATTCCAAGCTATGCTCTTCAC-5'	3'-TTCTTGCTGAATCTTTCAGGG-5'
ALB	3'-GAGACCAGAGGTTGATGTGATG-5'	3'-AGTTCCGGGGCATAAAAGTAAG-5'
MDR1	3'-ACCGTTGTTTCTTTGACTCAG-5'	3'-CAAAGAGTTTCTGTATGGTACCTG-5'
ROCK1	3'-AACATGCTGCTGGATAAATCTGG-5'	3'-TGTATCACATCGTACCATGCCT-5'
RPL23A	3'-CTGGAAGAGGCTGTGTATGAA-5'	3'-TAGTAGATGGGTGTGTGAGGAC-5'

RESULTS

The function of the spheroid in MFS compartment depends on various factors associated with pulsatile motion of the medium. An obvious factor is more intensive metabolism due to constant inflow of fresh culture medium and removal of vital activity products. However, the reaction to mechanical factors of various kinds and nature, reproducing the *in vivo* exposure, is of particular interest.

The flow of nutrient medium around the spheroid improves nutrient delivery and removal of metabolites. The pressure differences in MFS compartments and the mean volume expenditure and hence, the time over which the medium completes a cycle, were different at different pumping regimnes (Table 2).

It is shown [4,6] that the liver perfusion velocity is 1.5 liter/min/1.5 kg tissue or 1 ml/min/g tissue (1 μg/min/μg). If HepaRG cell in the spheroid is a cube with a side of 10-15 μ and 1 g/ml density, the weight of a cell is 1-3.5×10^{-9} g and the weight of a spheroid of 5000 cells is 5-17.5 μg and the physiological volume consumption corresponding to this spheroid weight varies from 5 to 17.5 μl/min. The volume consumption for ±10 kPa, 5 Hz is within this range (Table 2).

Generally, the forces acting on the spheroid in the flow of viscous fluid are described by the stress tensor:

$$s_{ij}=-p\delta_{ij}+\mu(\partial_i v_j+\partial_j v_i),$$

where μ is the fluid viscosity, p – hydrostatic pressure, $\partial_i v_j$ – derivative of component j of fluid velocity by coordinate i. Diagonal components of this tensor (δ_{xx}, δ_{yy}, δ_{zz}) are forces directed perpendicularly to the spheroid surface, nondiagonal ones are forces applied along the spheroid surface (shear stress).

Changes in the diagonal components along the spheroid surface in fact mean a pressure differential. If we consider the spheroid as a porous body, this differential, depending on its magnitude, leads to (1) movement of the spheroid in general, (2) to its volume deformations, and (3) to fluid filtration through the spheroid.

For tentative quantitative evaluation of these phenomena on the basis of digital simulation of a stationary flow at the maximum velocity in a cycle we evaluated the maximum pressure differentials and the shear stresses for the spheroids in an MFS compartment. The velocity of fluid filtration through the spheroid changed with changes in the pressure in the compartment. It differed from the zero during the pressure increase and drop. The maximum values for various modes over the period were evaluated on the base of Darcy's law:

$$v_\Phi=\frac{k\Delta p}{\mu d},$$

where k was permeability coefficient for porous medium, μ is fluid viscosity, Δp is pressure differential on spheroid, and d is spheroid diameter. For given the hepatocyte size and number of cells in a spheroid, permeability coefficient was k=0.7×10^{-12} m^2. The respective values were presented in Table 3.

TABLE 2. Immediate Volume Consumption, Time of Nutrient Medium Circulation, and Maximum Velocity in the Compartment at Different Pump Regimens

Parameter	Mode		
	±30 kPa, 2 Hz	±30 kPa, 5 Hz	±10 kPa, 5 Hz
Consumption, μl/min	25.68	60.7	11
Circulation, min	15	6	36
Maximum velocity in compartment, mm/sec	0.2	0.2	0.08

TABLE 3. Main Mechanical Factors Acting on the Spheroid at Different Pumping Regimens

Pumping pressure, kPa	Pressure differential on spheroid, Pa	Velocity of filtration through spheroid, μ/sec	Shear stress, Pa	Minimum pressure in compartment for the cycle, Pa	Maximum pressure in compartment for the cycle, Pa
±10	0.38	1.33	0.09	-880	608
±30	0.9	3.1	0.2	-1400	1608

According to a previous study [3], shear stress in human liver does not exceed 0.05 Pa. The regimen ±10 kPa is closer to the physiological shear stress values, while at ±30 kPa, it 4-fold surpasses the normal.

The compression is evaluated by Hooke's law:

$$\Delta p = E\varepsilon,$$

where Young's modulus is taken for 600 Pa [14]. The maximum deformation ε values are 0.06% for ±10 kPa and 0.15% for ±30 kPa. For soft tissues (brain and liver), the threshold linear viscoelastic deformation values are very low ~0.1-0.2% [5]. At pumping regimen ±30 kPa, the deformation values approximate the threshold values of linear viscoelastic deformation.

In addition to shear stresses, the cell is exposed to changing hydrostatic pressure; the amplitudes are presented in Table 3. In rat liver, *in vivo* the pressure values are 2.86±1.04 mm Hg (381.3±138.6 Pa) [9], which means that ±10 kPa regimen generates pressure values most close to physiological.

The characteristic velocities of interstitial (intercellular) fluid flow in the liver are within 0.1-2 μ/sec

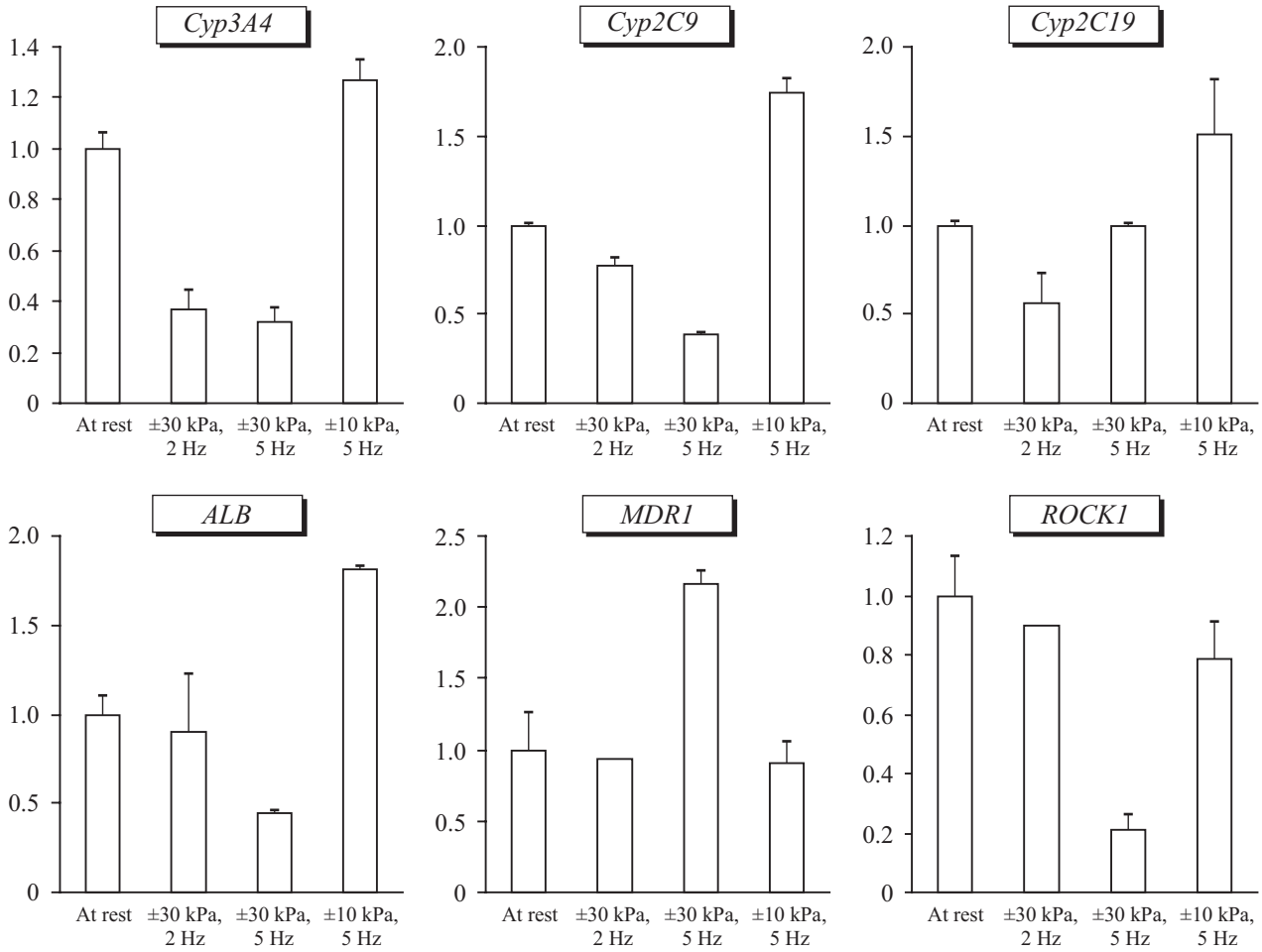

Fig. 2. Expression of genes of cytochromes 3A4 (*Cyp3A4*), 2C9 (*Cyp2C9*), 2C19 (*Cyp2C19*), albumin (*ALB*), bile duct transporter (*MDR1*), and protein involved in the maintenance of cell-cell contacts (*ROCK1*) in HepaRG cells in spheroids cultured in MFS at various pumping regimens.

[10,14], this indicating that ±10 kPa mode is associated with more physiological velocities of fluid filtration through the spheroid.

In order to evaluate the inflluence of microcirculation regimen on the function of HepaRG cells in spheroids, we analyzed the expression of the main hepatic functional genes: cytochromes 3A4 (*Cyp3A4*), 2C9 (*Cyp2C9*), 2C19 (*Cyp2C19*), albumin (*ALB*), bile duct transporter (*MDR1*), and protein involved in cell-cell contact maintenance (*ROCK1*) (Fig. 2). The ±10 kPa pumping regimen (best approximating MFS culturing to physiological conditions by all parameters) maintains high expression of main hepatic cytochrome 3A4, cytochromes 2C9 and 2C19, and albumin (functional marker of the liver) (Fig. 2).

The cell–cell contact is essential for hepatocytes. In case of failure of this contact as a result of trauma or disease, the hepatocyte looses its differentiated status and high expression of cytochromes P450 and acquires capacity to proliferation [6]. The contacts between HepaRG cells in the spheroids seem to be disturbed by pumping at ±30 kPa and 2 Hz and ±30 kPa and 5 Hz, as the expression of cytochrome 3A4 decreased significantly and expression of albumin and ROCK1 reduced (Fig. 2). *ROCK1* gene encodes protein involved in the maintenance of cell-cell contacts and cell contact with the matrix [12]. In addition, expression of MDR1 transporter increases at these regimens, high level of this expression being characteristic of the tumor and undifferentiated cells (Fig. 2).

Hence, MFS pumping regimen at ±10 kPa is preferable for culturing of HepaRG spheroids, as it does not surpass the physiologically permissible levels of mechanical exposure of liver cells in humans and maintains high expression of hepatic functional genes.

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