Functional Activity of Hepatocytes in Liver Fragments *In Vitro* as a Function of Fragment Size and Duration of Culturing

V. V. Solov'ev, N. A. Onishchenko, V. S. Akatov, and E. I. Lezhnev

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The possibility of using liver fragments in extracorporeal blood detoxication systems is discussed. The detoxication function of liver fragments *in vitro* is studied by assessing metabolism of p-nitroanisole and ethanol and synthesis of urea. Optimal size of liver fragments is 0.4-0.5 mm and the period of active hepatocyte function is about 10 h.

Key Words: *liver fragments; hepatocyte; detoxication; ethanol; urea; p-nitroanisole*

High mortality in hepatic insufficiency of different genesis is largely determined by disturbances of detoxication function of the liver [4]. Usual physicochemical methods of detoxication (hemosorption, hemofiltration, plasmapheresis) are often insufficient [3,4]. There is a possibility of extracorporeal detoxication by passing the blood through an apparatus containing xenogenic hepatocytes. It has been shown that this allows purification of the blood and activation of regenerative processes in the liver [3,12,13]. It should be noted that attachment to a biosubstrate [11], the presence of other liver-derived cells [8], and spatial tissue organization [7] are essential conditions for *in vitro* maintenance of functional activity of hepatocytes. This suggests the possibility of using liver fragments in extracorporeal perfusion systems. However, a comprehensive study of functional activity of hepatocytes in tissue fragments and how long it can be maintained, as well as its dependence on the size of fragments, methods of preparation, and other factors is necessary.

We assessed detoxicating activity of hepatocytes in liver fragments as a function of their size and time of culturing.

Institute of Transplantology and Artificial Organs, Russian Academy of Medical Sciences, Moscow; Institute of Theoretical and Experimental Biophysics, Russian Academy of Science, Pushchino, Moscow Region

MATERIALS AND METHODS

Liver was aseptically obtained from Wistar rats weighing 100-200 g under ether narcosis and transferred to Hanks' solution containing 5 mM glucose (4°C). The tissue was cut either with scissors (0.5 mm and more) and divided into fractions by sedimentation or desintegrated using a soft tissue grinder (designed at the Institute of Theoretical and Experimental Biophysics, Russian Academy of Science) producing fragments of the same size (from 0.1 to 1 mm). The mean size of tissue fragments in the fraction was determined under a microscope using an ocularmicrometer. The fragments were cultured at 37°C in flasks with Williams' medium E (Sigma) containing 1 μM dexamethasone, 0.1 μM insulin, and 100 μmol/ml kanamycin with constant shaking, the pH was maintained within 7.2-7.4 with a mixture of air and 5% CO_2. For evaluation of the detoxication function of hepatocytes 2 mM nitroanisole (NA), 10 mM ammonium chloride, and 10 mM ethanol were added to the incubation medium. The activity of lactate dehydrogenase (LDH) in the medium was measured 15 min after cutting.

Cell viability was assessed by vital staining with luminescent nuclear stains bisBenzimide Hoechst 33342 (staining of live cells, green luminescence) and ethidium bromide (staining of dead cells, red-to-

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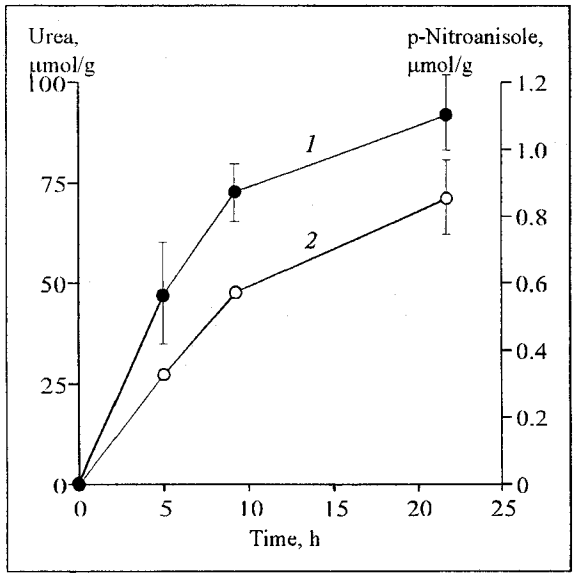

Fig. 1. Kinetics of urea synthesis (2) and p-nitroanisole demethylation (1) by 0.4-mm liver fragments during incubation.

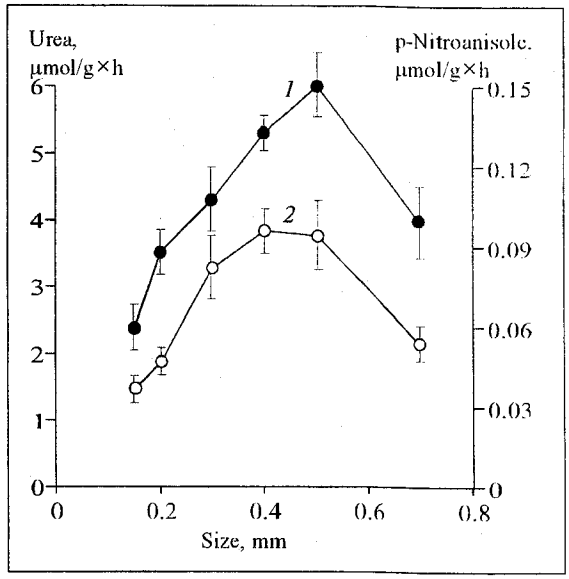

Fig. 2. Specific rates of urea synthesis (1) and p-nitroanisole demethylation (2) by liver fragments of different size.

orange luminescence). Sections of liver fragments were examined under a luminescence microscope, and spatial distribution of dead and live cells was analyzed. Histological analysis of liver fragments fixed with glutaraldehyde and stained with OsO_4 was also performed.

Specific rate of respiration of liver fragment suspended in Hanks' solution was measured by means of a Clarke polarographic electrode in a thermocontrolled cell (37°C). Succinate was added in a final concentration of 10 mM.

The concentrations of urea and ammonium were measured using Lachema and KONE kits, respectively. The concentrations of p-nitrophenol (PN, [14]), ethanol [1], and LDH [5] in the medium were determined as described previously.

The data are presented as $X_{mean} \pm m$, where m is standard deviation.

RESULTS

For evaluating the viability and energy metabolism of hepatocytes we studied respiratory activity of liver fragments as a function of their size and method of preparation. Oxygen consumption by 0.4-1.0-mm fragments constituted 0.5-0.6 μmol O_2/g×min and did not depend on the method of preparation. Succinate stimulation coefficient (ratio of stimulated to basal respiration rate), which correlates with the percentage of damaged cells [2], increased from 1.4 to 2 times as the size of fragments decreased from 1 to 0.4 mm. Under these conditions the release of LDH into the incubation medium rose from 20 to 45% of its total tissue content. In smaller fragments specific rate of oxygen uptake decreased, while the succinate stimulation coefficient rose. For instance, for 0.1-mm fragments the rate of succinate-stimulated respiration was 1-2 μmol O_2/g×min, and stimulation coefficient rose to 7-10; practically all tissue LDH was detected in the medium. These findings indicate minor hepatocyte losses in 0.4-0.5-mm and larger fragments and a reduced number of live hepatocytes in smaller liver fragments.

Functional, in particular detoxicating, activity of hepatocytes in liver fragments was assessed by their ability to synthesize urea and metabolize NA and ethanol.

Since preparation of small fragments is associated with considerable cell damage and, on the other hand, mass transfer in large fragments is restricted due to diffusion limits, we assumed that there should be an optimal size of fragments corresponding to the maximum functional activity of hepatocytes. In view of this we studied detoxication activity of hepatocytes as a function of the size of liver fragments and the time of *in vitro* incubation. Figure 1 demonstrates kinetic curves of urea and PN accumulation in response to addition of 10 mM ammonium chloride and 2 mM NA. For any fragment size, the rate of biotransformation of NA into PN was relatively high during the first 8-10 h and then decreased despite the presence of substrates. Synthesis of urea from exogenous ammonium was confirmed by its decrease in the incubation medium proportional to urea accumulation. Figure 2 shows the rate of urea synthesis and NA demethylation (accumulation of PN in the medium) during a 10-h incubation as a function of fragment size. Similar data were ob-

tained for ethanol oxidation. The maximum rates of urea synthesis (up to 30 μmol/g×h), NA metabolism (0.1-0.2 μmol/g×h) and ethanol oxidation (30-50 μmol/g×h) were observed in 0.4-0.5-mm fragments. This rate of urea synthesis is comparable to those obtained in isolated hepatocytes immobilized in gel within semipermeable fibers (8.5 μmol/g×h [10]), and encapsulated hepatocytes (10.4 μmol/g×h [6]), which are also considered as potential biosorbents for extracorporeal perfusion systems. Fragments above 0.5 and below 0.4 mm exhibited lower functional activity.

Hepatocyte viability was assessed by simultaneous staining with Hoechst 33342 and ethidium bromide immediately after preparation of fragments and during their incubation. Immediately after preparation we observed green luminescence (live cells) in the core of particles and orange luminescence (dead cells) on its surface. This indicates that only surface hepatocytes are damaged during the cutting procedure. A zone of dead cell inside the 0.5-mm fragments appeared during incubation and gradually rose with time. Cell death confirmed by histological study is presumably induced by limited oxygen diffusion. Therefore, impairment of functional activity during long-term incubation (more than 10 h) can be attributed to hepatocyte death.

Thus, hepatocytes in tissue fragments retain a relatively high functional activity *in vitro* for about 10 h, optimal size of fragments being 0.4-0.5 mm. These findings suggest that liver fragments have an advantage over isolated hepatocytes as the components of extracorporeal perfusion systems, since the use of tissue fragments allows to circumvent technological difficulties in isolating hepatocytes and preserving their functional activity.

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