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Normalization of Blood Biochemistry Parameters Using Experimental Hybrid Plasma Filter for Bioartificial Liver

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HIGHLIGHTS

- A prototype of a hybrid plasma filter for an extracorporeal bioartificial liver has been developed.
- Laboratory tests of the prototype were carried out.
- Achieved normalization of the rejected biochemical parameters.

Abstract: Auxiliary bioartificial liver (BAL) is a promising direction for supporting liver functions in acute liver failure. However, clinical trials of BAL have not shown convincing results so far. We present a prototype of a hybrid biocomposite plasma filter containing living immortalized hepatocytes. This filter is designed to integrate into the biological circuit of the extracorporeal blood purification system with the aim of mimicking some functions of the liver. Due to the presence of hepatocytes in the filter, adequate metabolism of plasma substrates is ensured when blood contacts with special perforated membranes. We used HepG2 cell line as working cells. For evaluating the performance of the filter, we applied plasma from patients with liver failure who had abnormalities in biochemical indicators of blood. We analyzed concentrations of albumin, total and direct bilirubin and urea as criteria for effectiveness. Our results indicate normalization of these parameters after passing plasma through 7 filters and improvement after passing through 1 filter. This confirms the effectiveness of our prototype and creates a basis for developing a new extracorporeal BAL.

Keywords: artificial liver; hepatocytes; plasma filtration; liver; cell cultivation.

INTRODUCTION

The liver is one of the most complex and multifunctional organs in the human body. It performs a variety of functions that are essential for life but difficult to categorize by importance or specificity. Liver diseases are among the most prevalent and serious health problems worldwide. According to WHO and GBD (Global Burden of Disease) researchers, four main factors account for most cases of liver damage and death: viral hepatitis C, viral hepatitis B, alcoholic liver disease, and non-alcoholic fatty liver disease [1]. In 2019, there were 1.69 billion cases of liver disease globally [1]. By 2030, it is projected that chronic viral hepatitis C infections will increase annually and result in 14-17% more deaths, cirrhosis and HCC (hepatocellular carcinoma), while liver disease mortality will double. In Russia, liver cirrhosis prevalence and mortality rates are among the worst in the world [2]. Currently, there are no effective treatments for liver diseases except for donor organ transplantation when irreversible liver failure occurs.

However, one-third of patients die while waiting for a donor organ [3], partly due to organ shortage. The treatment during this period aims to eliminate contraindications for surgery and immunosuppressant use, as well as provide etiotropic antiviral therapy and hepatoprotective therapy. There are also methods of plasma diafiltration that remove toxins by separating and adsorbing fractionated plasma or using hemodialysis. However, these methods do not synthesize proteins, blood coagulation factors, lipids, enzymes, hormones or bile, or perform numerous metabolic processes and biochemical transformations, because they lack a biological component - hepatocytes.

A promising technology for plasma diafiltration with a biological component is the Bioartificial Liver (BIP) systems that use cultured hepatocytes to provide liver functions outside the body. The main resource of this system is liver cell cultures that have enough proliferation and survival potential for use in special conditions. The essence of this system is to demonstrate specific functions of liver cells and correct abnormal biochemical parameters of the patient's blood, then return it to the body after filtering it through special systems that enable safe and effective interaction between cells and plasma. To test the systems at the R&D stages, special cell lines are used that have maximal biological properties and a large or unlimited growth potential [4]. This requires the use of animal or human blood plasma as a study object and measuring the changes in plasma component concentrations.

The selection of cell lines for the cell system is an ongoing research topic. Primary cultures of human hepatocytes are frequently used for pharmacological and toxicological studies in vitro, but they are not suitable for long-term experiments because of their limited functional qualities. They have a short life span and division capacity (up to 7 days) and tend to lose their specific functions over time [5,6]. A more effective alternative is hepatocytes derived from reprogramming (hiPSC-Heps) [7,8], which are currently applied in the first extracorporeal liver function support system that has entered the first phase of clinical trials [9]. For R&D purposes, cell lines with moderate liver-specific properties are used, such as HepG2 cells [10-16], their subclone HepG2/C3A [17], NKNT-3 [18], tumor and xenogeneic cells.

Several extracorporeal liver support systems (ELSS) have been developed that use hepatocytes as a biological component: ELAD, HepatAssist, AMC-BAL, SRBAL, hiHep-BAL and BBALS [8,19-26]. These systems aim to simulate the hepatic lobule structure and the directional flow of substances for their proper metabolism by hepatocytes, as well as their elimination and return to plasma. However, none of these systems can provide continuous bile and pigment secretion.

We present a system model for extracorporeal contact of separated human plasma with hepatocytes, which enables the control of fluid flow direction on both sides of hepatocytes: sinusoidal (venous) and biliary (biliary). The system model is based on a patented plasma filter that can form a scalable biological circuit in extracorporeal plasma filtration systems. The plasma filter has a unique feature of allowing regulated bile and pigment secretion into the biliary duct. However, this study focuses on testing sinusoidal flow.

MATERIALS AND METHODS

Study Design

We use plasma with abnormal biochemical parameters from patients with liver failure as the input for the system we developed. We evaluate the effectiveness of the system by measuring the normalization of these parameters after perfusion through the system. We also verify the metabolic interactions between plasma and hepatocytes during perfusion by monitoring the biochemical parameters before and after the procedure (Figure 1). We collect the blood plasma of patients in a reservoir and do not require veno-venous bypass. We perform a control test in an empty plasma filter without hepatocytes.

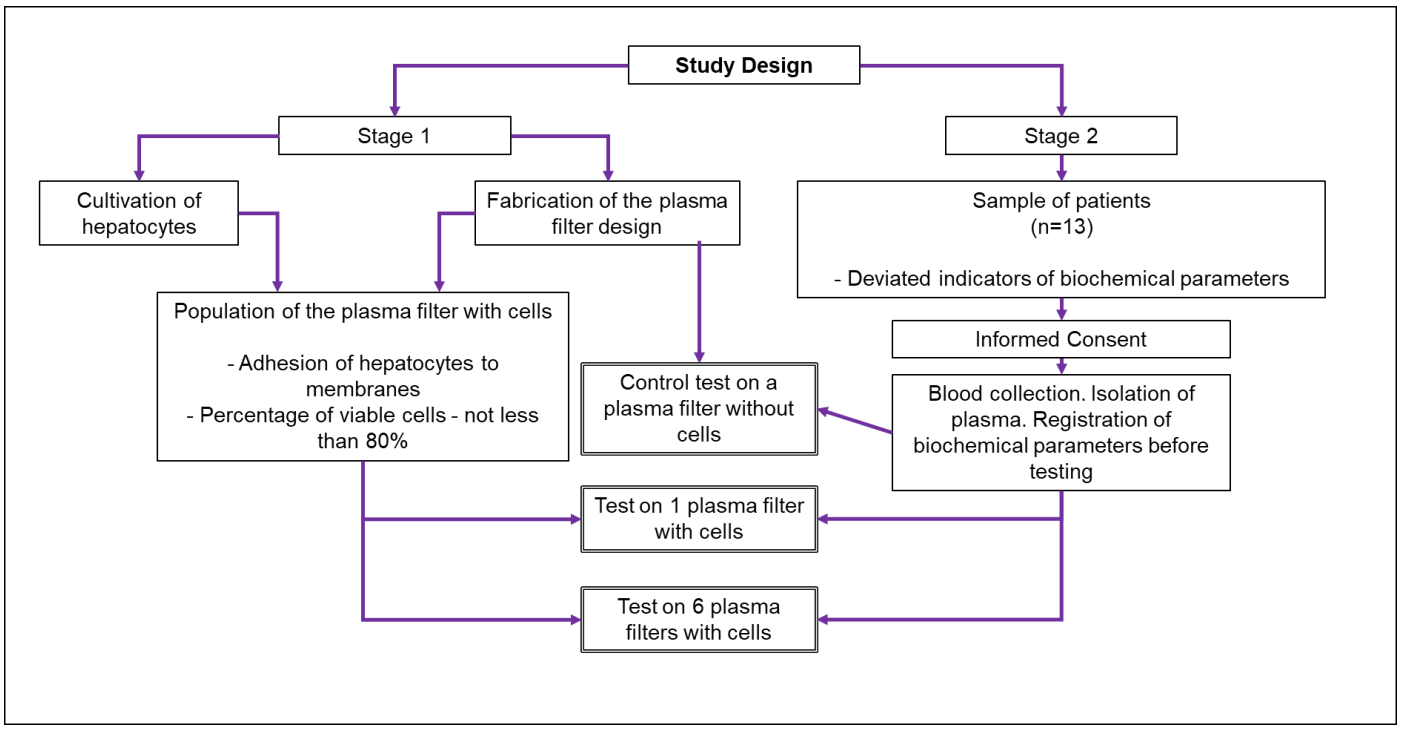

Figure 1. Study Design

Construction manufacturing

The system model consists of a plasma filter - a plastic biochip - that can be used as a component of the biological circuit for extracorporeal liver function support (EVF). The biochip has three cavities: one for perfusing dialysate or nutrient solution, one for culturing hepatocytes on a semi-permeable membrane, and one for circulating plasma [27]. The biochip is made of a biocompatible polymer and assembled by connecting layers of parts. Three of these parts form the cavities, while the lid and the bottom have holes for liquid inlet and outlet, as shown in Figure 2. The diameter of each biochip is 84 mm, the channel width is 3 mm, and the usable area is 2 x 10^3 mm^2.

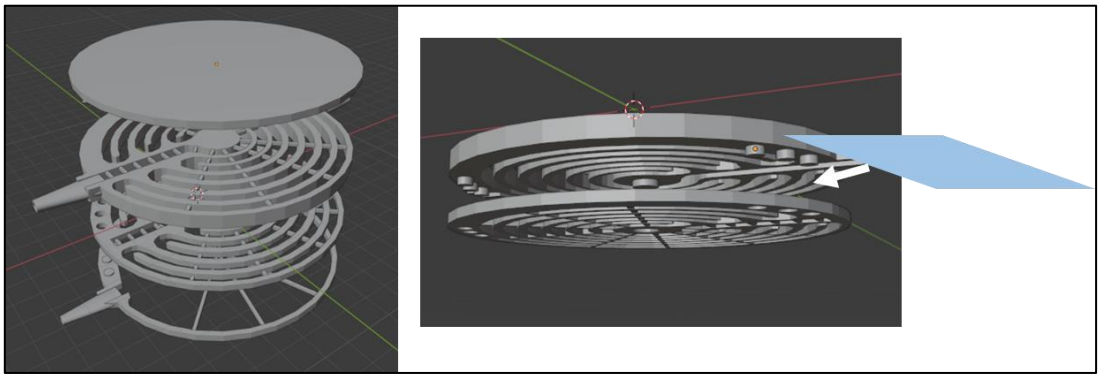

Figure 2. A 3D model of the biochip assembly. The spiral shape enables one-way flow of liquids through the whole available area of the biochip.

The biochips are designed to be connected in series by tubes to external sources and drains of liquids without air gaps. The fluids are blood plasma (Figure 3 A1) and culture media (Figure 3 C1). They flow into opposite directions in parallel cavities-channels as shown in Figure 3. Between these channels, there is another cavity with identical shape but without outlets for culturing hepatocytes (Figure 3 B1) on a semi-permeable membrane (Figure 3 B2). The height of this cavity allows efficient diffusion of plasma into it, washing hepatocytes for full metabolism and returning to the plasma channel (Figure 3 A2) for further exit from the biochip to systemic circulation. For this experiment, we used polylactide as material for this cavity with a height of 1 mm.

The semi-permeable membrane separating this cavity from plasma channel has pores that are large enough to let plasma components pass through but small enough to block hepatocytes from entering plasma

circulation (Figure 3 A4). Similarly, this cavity communicates with dialysate medium (Figure 3 C2) through another semi-permeable membrane at its bottom side, where cells attach and grow. The layers are aligned so that their edges match along their contour and form a single arch. The membranes between layers make up lower and upper surfaces of channels, creating a unified set of cavities on each layer. We printed this design on a 3D printer with precise dimensions, proportions and shapes. We secured membranes between layers using grooves and holes systems (Figure 3 A3).

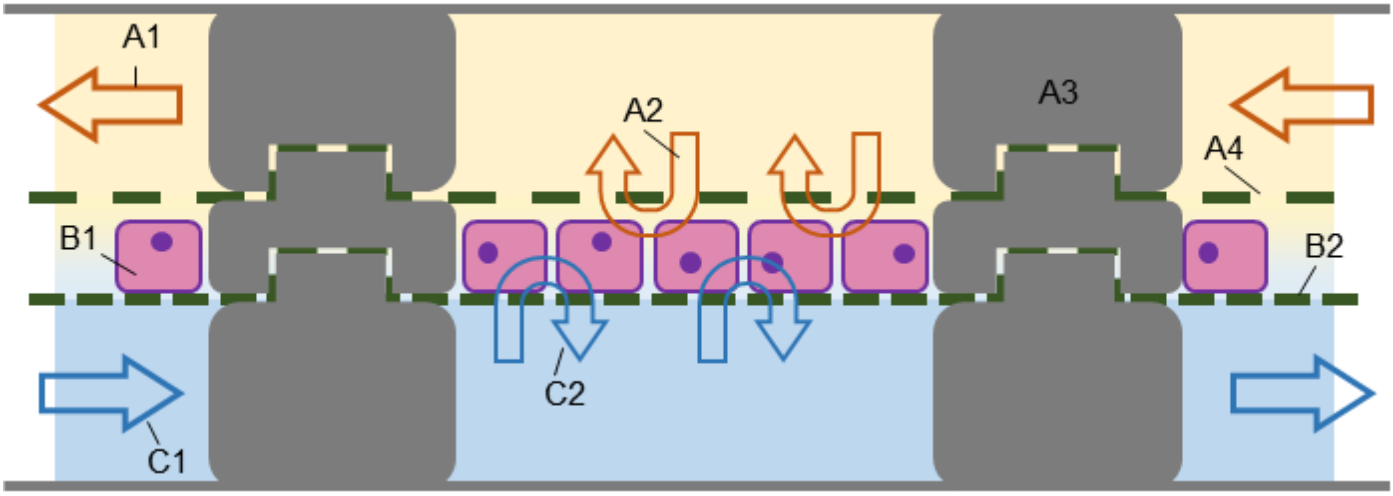

Figure 3. Scheme of the vector flow of liquids. Description in the text.

Track membranes (Reatrek, Russia) were used as substrates and fenestrated partitions between layers. The lower membrane had pores of 5 μm for metabolic exchange, while the upper membrane had pores of 15 μm for mechanical barrier, since hepatocytes have sizes ranging from 20 to 30 microns.

The biochip model (plasma filter) was modified for this experiment by making the lower layer similar to the upper layer for better sealing of the membrane by the tube walls.

Biochip settlement hepatocytes

Biochip settlement hepatocytes Immortalized hepatocyte lines HepG2 were used for this experiment. They were obtained from the Institute of General Genetics named after N.I. Vavilov of RAS (Moscow). They were cultured in a humid atmosphere with 5% carbon dioxide at 37°C in DMEM/F12 medium with L-glutamine (Sigma, Japan), sodium pyruvate (PanEco, Russia) at 1%, fetal calf serum Research Grade (Dia-m, Russia) at 10%, and antibiotic-antimycotic (Merck, Germany) at 1%. The medium was changed every 3 days and the cells were subcultured into flasks by enzymatic dissociation when they reached confluence. Flasks with ventilated caps (Corning, USA) were used.

The membranes were coated with an adhesion factor (Gibco, USA) for 30 minutes before the experiment following the manufacturer's instructions.

The cells were detached with a 0.05% trypsin solution (PanEco, Russia) when they formed a monolayer and monitored visually. Trypsin activity was stopped with the medium itself because it contained serum. The cells were seeded on the biochip by adding the cell suspension in medium to the biochip structure without its top layer for better visual control. The cells settled on membranes with pores of 5 μm.

Cell viability was evaluated by checking their attachment to the membrane surface after two days of seeding. Cell density was measured using Figure 4. Dead cells were removed from culture on day 5 with versene solution and resuspended manually. A 0.4% trypan blue solution was added at 0.1 ml per 1 ml of suspension and incubated at 37°C for 10 minutes before counting them on a Goryaev chamber. The viability rate was 87%.

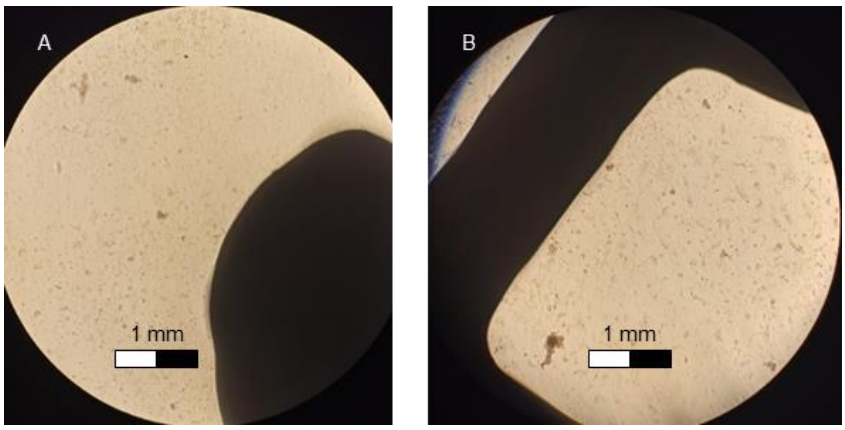

Figure 4. Culture of hepatocytes on a polylactide scaffold and in the field of view under a microscope at 4x magnification: A- on day 2; B - on day 5.

Seven biochips were prepared for each patient. Each biochip had functional channels with an area of 2 x 10^3 mm^2 and contained about 1.2 million hepatocytes. Nutrient medium was delivered to the lower layer below hepatocytes through artificial fittings. Medium filled up and reached cells was visually checked.

Nutrient medium was replaced with serum-free one (PanEco, Russia) one day before experiment. Medium in dialysate channel was also replaced with saline solution ten minutes before experiment. Saline solution from upper plasma channel was removed before experiment.

Sample of patients

Plasma samples from thirteen patients diagnosed with liver failure at hepatitis department of Yakutsk City Clinical Hospital (Yakutsk, Republic of Sakha (Yakutia)) were tested. Nine patients had hepatitis C virus infection, two had fatty liver disease, one had acute toxic hepatitis, and one had acute liver failure of unknown cause. Four patients were women and nine were men. Patients were selected based on four abnormal biochemical markers indicating liver dysfunction: albumin below 35 g/l, total bilirubin above 20.4 µmol/l, direct bilirubin above 5.1 µmol/l, urea below 3 .2 mmol/l in men and below 2.6 mmol/l in women. Patients were between 18 and 50 years old and had no acute immune system problems (allergies, autoimmune diseases, respiratory infections, etc.). Plasma samples were obtained after patients gave informed consent. Fifty milliliters of venous blood were drawn in tubes with EDTA, centrifuged at 1300 g for 12 minutes and separated plasma from blood cells. Plasma samples were loaded into polymer containers for blood preparation and single-use components without additives. Containers were connected to biochips through donor tubes with fittings.

Experimental test

A layer for plasma delivery was prepared in biochip design. A new layer with a perforated membrane and tubes fixed to artificial fittings on both sides of previous layers was added. Cavities were filled with saline solution to check their seal.

High-precision peristaltic pumps were used in the experiment. The flow rate of liquids was 1 ml/min. Liquids were supplied to the biochip from a container through container tubes installed in the peristaltic pump head according to the manufacturer's instructions, and tight connections of the tubes with a simulated fitting, the dimensions of which made it possible to ensure hermetic fastening. The flow of liquids had an opposite direction to each other. The liquids were set in motion at a rate of 100 ml/h.

To establish the scale, a portion of plasma was divided into two parts of 10 ml in two stages of the experiment: at the first stage, the plasma was passed through one biochip with hepatocytes, a separate plasma volume through one biochip without hepatocytes for control comparison, at the second stage, through 7 biochips. The duration of plasma flow in one biochip was 43 minutes, the duration of the second stage was from 4 hours 43 minutes to 5 hours 11 minutes.

The concentration of substances was determined by taking 1 ml of plasma from each sample before the procedure and the entire volume that passed through the biochips. Samples were provided to the biochemical laboratory, where the study was carried out using an automatic biochemical analyzer Horiba ABX Pentra 400 (Horiba ABIx SAS, France) using colorimetric, photometric technologies to determine the concentration of individual chemicals and metabolic products.

RESULTS AND DISCUSSION

An important point reflecting the functional efficiency of the biochip is the metabolism of substances freely circulating in the plasma and the synthesis of proteins. These include, in particular:

- bilirubins, which are converted into bilirubin esters with glucuronic acid by the enzyme uridine diphosphate glucuronyltransferase, interacting with glucuronic acid, and are excreted in the bile;
- ammonia, which is neutralized by binding in the ornithine cycle in mitochondria hepatocytes, ending with the synthesis of urea;
- albumin, which provides 75-80% of plasma oncotic pressure, is synthesized in hepatocytes, and is the main indicator of the synthetic ability of hepatocytes.

All obtained quantitative data are presented in absolute values and means with a standard deviation. Statistical analyzes were performed manually.

Albumen

Plasma biochemical parameters were improved towards normal levels by dynamic plasma perfusion into the hybrid biochip. The parameters were significantly normalized by plasma perfusion through 7 biochips (Figure 5). Albumin concentration increased and was normalized in 11 out of 13 samples. Detailed data for each sample are presented in Table 1.1 of Annex 1. The hypothesis was confirmed by the nonparametric Wilcoxon test, which compared the indicators and showed that the empirical value of T was in the significance zone: T_{emp} = 8, T_{cr} =12 ($p \leq 0.01$), T_{cr} =21 ($p \leq 0.05$), Temp < T_{cr} (0.01). Data for calculations and results are presented in Table 2.1 and 2.2 of Annex 2. Plasma perfusion through the plasma filter without cells did not affect albumin concentrations.

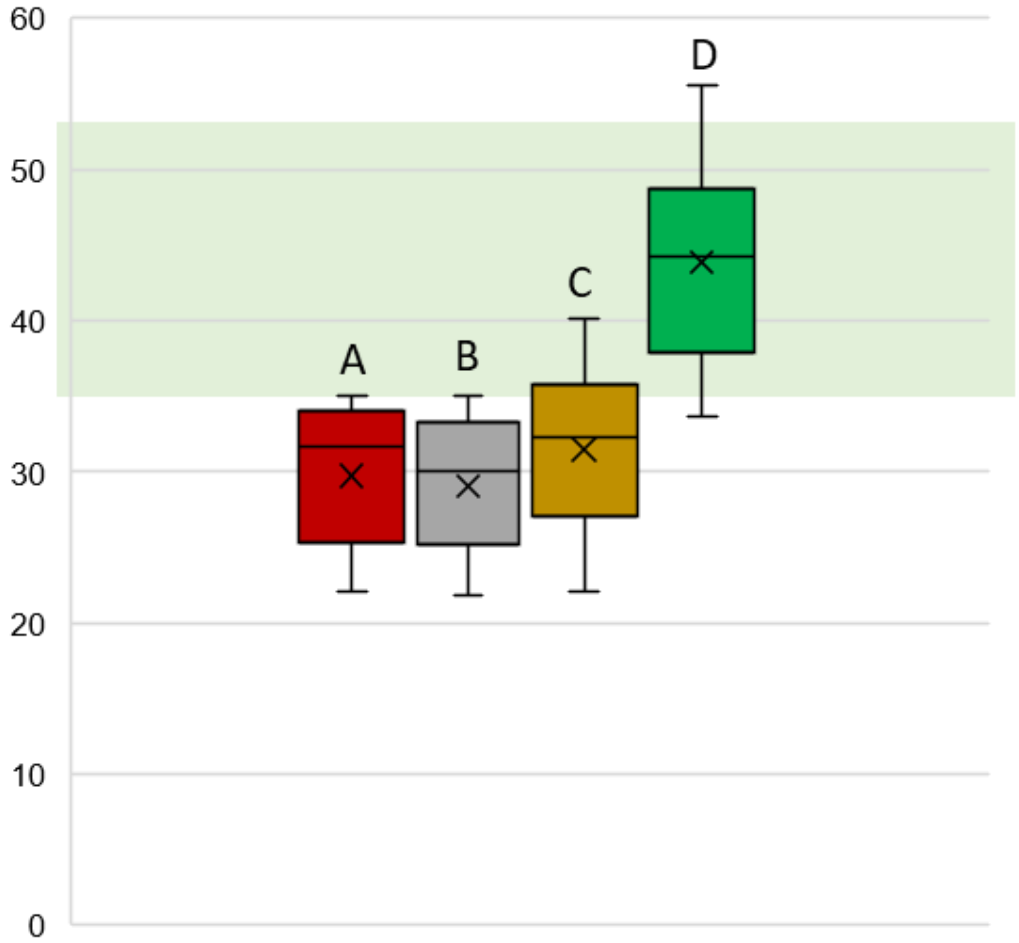

Figure 5. Dynamics of albumin concentration in the studied plasma. Against the background, the area is shaded, indicating the limits of the norm - 35-53 g / l. A) The concentration of albumin before the procedure. The mean value is 29.7 g/l ± 4.62 (standard deviation). B) Albumin concentration after perfusion through 1 biochip without hepatocytes. C) Plasma albumin concentration after perfusion through 1 biochip. The average is 31.5 g/L ± 5.29. D) Plasma albumin concentration after perfusion through 7 biochips. The average is 43.8 g/L ± 6.68.

Bilirubins

Total bilirubin was normalized in 7 patients after perfusion through 7 biochips. Unbound (direct) bilirubin also decreased to normal levels in 7 patients. Total and direct bilirubin levels decreased after perfusion through 7 biochips (Figure 6). Control perfusion did not change plasma parameters. Detailed data for each sample are presented in Table 1.1 of Annex 1. The Wilcoxon T-test showed that the empirical value of T for total bilirubin indicators was 0 and for direct bilirubin indicators was 1 after perfusion through one biochip and 0 after perfusion through seven biochips. T_{cr} =12 (p≤0.01), T_{cr} =21 (p≤0.05). The empirical value of T was in the significance zone: $T_{emp} < T_{cr}$ (0.01). Data for calculations and results are presented in Table 2.3, 2.4, 2.5 and 2.6 of Annex 2. The hypothesis was accepted - the indicators were significantly reduced after perfusion through biochips.

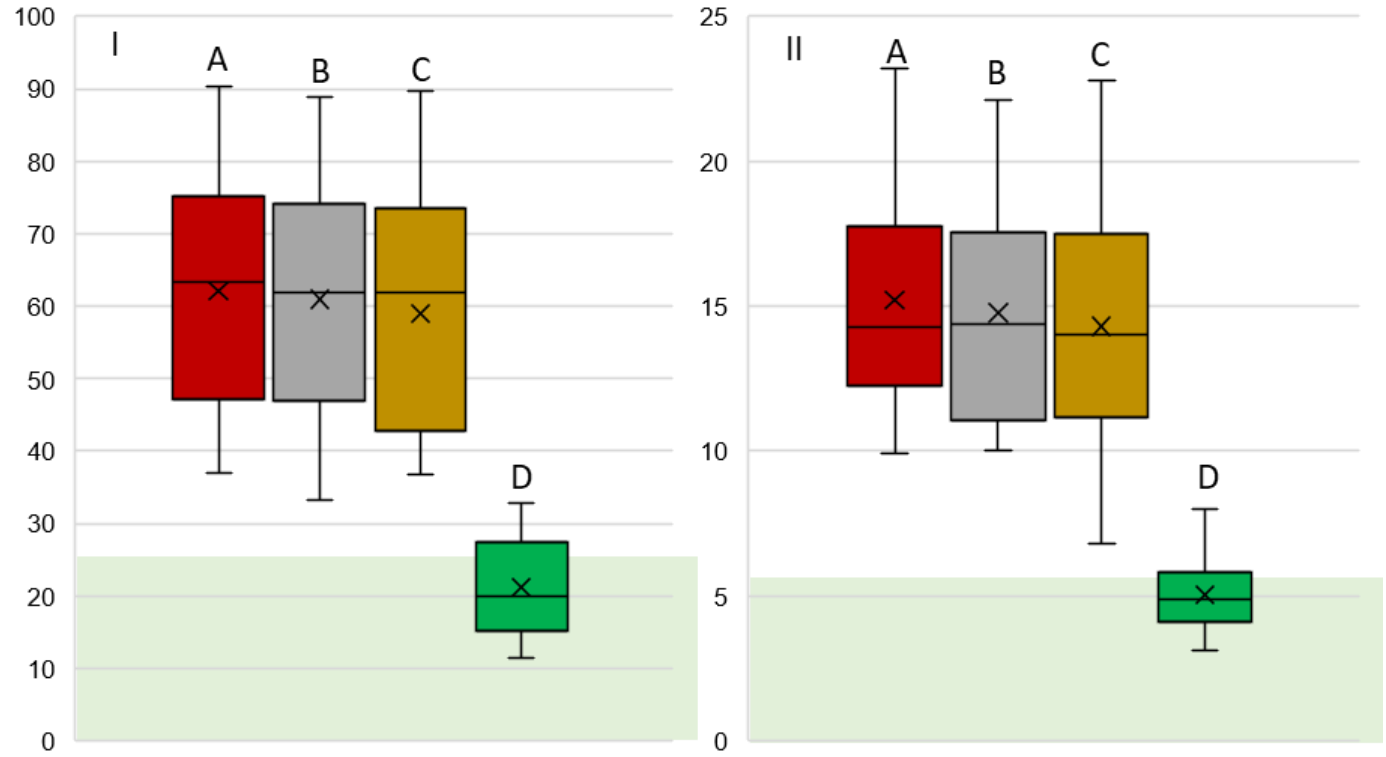

Figure 6. Dynamics of the concentration of total bilirubin (I) and direct bilirubin (II) in the studied plasma. Against the background, an area is shaded indicating the limits of the norm: up to 20.4 µmol/l (I), up to 5.1 µmol /l (II). I /A) Concentration of total bilirubin in plasma before the procedure. The average value is 62.1 µmol/l ± 15.87. I/B) Plasma total bilirubin concentration after perfusion through 1 biochip without hepatocytes. I/C) Concentration of total plasma bilirubin after perfusion through 1 biochip with hepatocytes. The average value is 59.0 µmol / l ± 17.03. I/D) Plasma total bilirubin concentration after perfusion through 7 biochips with hepatocytes. The average is 21.2 µmol/l ± 6.76. II/A) Concentration of direct bilirubin in plasma before the procedure. The average is 15.2 µmol/l ± 3.79. II/B) Plasma direct bilirubin concentration after perfusion through 1 biochip without hepatocytes. II/C) Concentration of direct bilirubin in plasma after perfusion through 1 biochip with hepatocytes. The average value is 14.3 µmol/l ± 4.33. II/D) Plasma direct bilirubin concentration after perfusion through 7 biochips. The average value is 5.0 µmol/l ± 1.37.

Urea

Urea level was normalized in 12 patients after perfusion through 7 biochips, indicating that hepatocytes metabolized toxic ammonia. Urea concentration increased, indicating that hepatocytes could transform ammonia into urea (Figure 7). Detailed data for each sample are presented in Table 1.1 of Annex 1. The Wilcoxon T-test showed that the empirical value of T was 0. Critical values for the Wilcoxon T-test for n=13: T_{cr} =12 (p≤0.01), T_{cr} =21 (p≤0.05). The empirical value of T was in the significance zone: $T_{emp} < T_{cr}$ (0.01). Data for calculations and results are presented in Table 2.7 and 2.8 of Annex 2. The hypothesis was accepted - the indicators were significantly increased after perfusion through biochips.

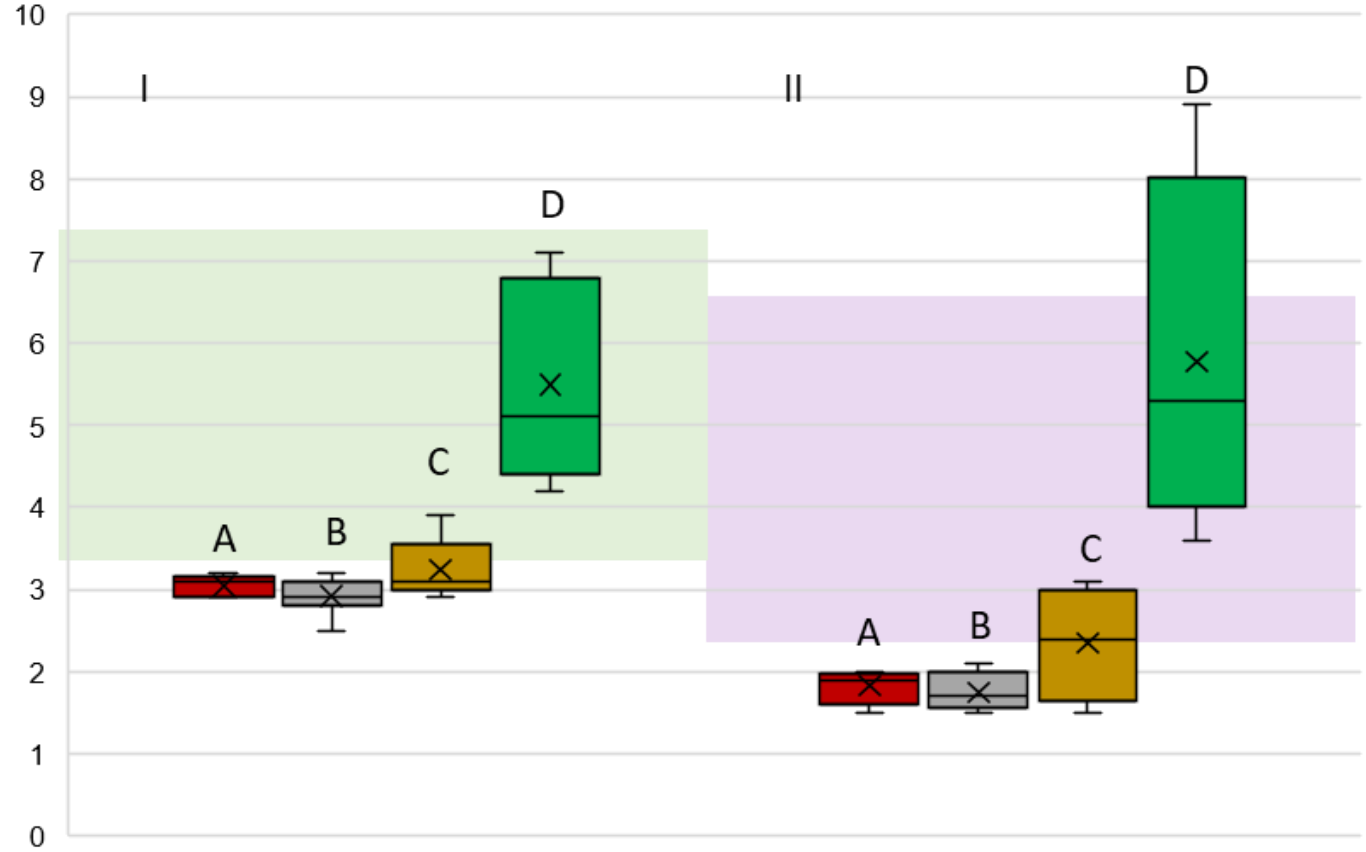

Figure 7. Dynamics of urea concentration in the studied plasma. Against the background, areas are shaded indicating the limits of the norm: for men (I) - 3.2-7.3 mmol/l, for women (II) - 2.6-6.7 mmol/l. A) Plasma urea concentration before the procedure. The average indicator: for men (I) - 3.0 mmol/l ± 0.12 (standard deviation), for women (II) - 1.8 mmol/l ± 0.22. B) Plasma urea concentration after perfusion through 1 biochip without hepatocytes. C) Plasma urea concentration after perfusion through 1 biochip with hepatocytes. Average: in men (I) 3.2 mmol/l ± 0.35, in women (II) - 2.4 mmol/l ± 0.7. D) Plasma urea concentration after perfusion through 7 biochips with hepatocytes. The average value: in men (I) - 5.5 mmol/l ± 1.15, in women (II) - 5.8 mmol/l ± 2.23.

CONCLUSIONS

Liver failure is a life-threatening condition with a mortality rate of 60 - 80% according to various sources. EVP systems are an alternative method to maintain liver function in patients waiting for an organ donor and may improve survival and well-being. Similar devices have been successfully tested and shown to provide not only detoxification, but also regeneration of the residual liver after resection of a part [8].

This study evaluated a patented alternative model of the hybrid plasma filter as a functional unit of the extracorporeal auxiliary liver system. HepG2 immortalized cells were used as a biological component.

The blood plasma of patients with liver failure was perfused through a single biochip with sufficient biomass of HepG2 hepatocyte cultures and some changes in blood biochemical parameters towards normalization were observed. However, this scale was insufficient to normalize all deviated parameters.

The perfusion of plasma through 7 biochips with cells connected in series reliably normalized some blood biochemical parameters deviated in liver failure: albumin, total and direct bilirubin, urea. The data indicated that biochips, with adequate scaling and increased functional biomass, which directly contacted the plasma of patients, could metabolize biochemical components of blood plasma and normalize them, deviated in liver failure.

The plasma filtration procedure was performed by connecting several biocomposite plasma filters in series into a single biological circuit. The number of plasma filters was determined individually depending on the biomass of the functional hepatocyte cultures obtained.

This study showed that EVP systems with layered plasma filters improved the biochemical composition of the blood and represented a promising model for developing new therapeutic approaches for liver failure treatment.

PATENTS

The paper mentions a patent for a utility model of a biocomposite filter for the biological circuit of an extracorporeal hemoperfusion system obtained by our team in 2021: No. RU 204 435 U1, priority date: 12/11/2020.

The Russian Patent Office (Rospatent) decided to issue a patent for an invention of a method for normalizing the biochemical parameters of the blood of patients with irreversible liver failure. Application No. 2020140915/14(076161). Application date: 12/11/2020.

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Conflict of interest. The authors have declared that there are no conflicts of interest.
Supplementary Material: Supplementary material, Table 1.1 to 2.8, is available in: <https://www.documentador.pr.gov.br/documentador/pub.do?action=d&uuid=@gtf-escriba-tecpar@82867fc9-5160-40e4-80db-e12a398c5c63>

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