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Hepatocytes isolation from adult rats for liver recellularization

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Abstract

Background: Currently hepatocytes obtaining is prerequisite to create the necessary conditions for medical research, because it is an important tool in developing of new strategies in tissue engineering domain, which represents obtaining functional organs in laboratory conditions.
Material and methods: The study was made on adult Wistar rats liver with body weight 274.66± 2.52 g (n=3) which were used for hepatocytes extraction by perfusion through the upper cave vein with combination of type II collagenase and type I dispase and Hank's 0.9 mM MgCl2, 0.5 mM EDTA and 25 mM HEPES (HiMedia, India).
Results: The cells were counted with trypan blue 0.25% in hemocytometer and cultured in William's E medium (HiMedia, India) with 2 mM L-glutamine, 5% fetal bovine serum (Lonza, Belgium), antibiotic antimycotic solution (HiMedia, India), 100 nM dexamethasone and 100 nM insulin, with 2.5 x 10^5 cells per well in 12-well plates. After isolation were obtained 324, 48 ± 1, 25 x 10^6 hepatocytes, with a viability of 94.7 ± 0.9 % which indicates a high yield of cells viability.
Conclusions: The hepatocyte isolation method by liver perfusion with the combination of collagenase-dispase is feasible for obtaining a large amount of functional hepatocytes intended for the recellularization *in vitro* of decellularized liver scaffolds. The yield and viability of hepatic cells could be increased by enzymatic digestion of liver tissue using combination of collagenase/dispase solution due to the less cytotoxic effect.

Key words: hepatocytes, cell separation, cell survival, collagenases, dispase, in vitro techniques.

Introduction

Liver is the organ with a large functional metabolic profile, being a subject of an impressive number of biochemical investigations. Its main function in the body is the transformation of nutrients into physical and chemical forms that are used in the body, and on the other hand they are excreted. What is important is that the liver functions in the liver are only performed by parenchymal cells (hepatocytes). They constitute 90 – 95%, the majority, from the total weight of the other types of cells and 2/3 of the total cell population. This relative morphological uniformity of the liver as well as the linkage between hepatocytes with its organo-specific function make it possible to use parenchymal cells as a model for investigating various biochemical, biophysical, pharmacological and physiological processes that happen in the liver [1, 2, 3, 4]. The sources of hepatocytes may be from the liver that has been rejected after transplantation, portions of liver resected from patients with cirrhosis or other hepatic diseases and portions of liver resected from healthy patients [5, 6, 7, 8, 9, 10]. The hepatocytes isolated from rats, mice or human liver are a useful technique for studying liver function *in vitro* as well as for the recellularization of decellularized hepatic scaffolds. Also, the hepatocytes obtained after isolation have a major utility to be transplanted, as cell therapy of hepatic diseases for correction of metabolic disorders of the liver in the absence of donors liver for transplantation [11, 12, 13, 14, 15, 16, 17, 18].

Due to multitude possibilities for using isolated hepatocytes, there are various hepatocyte isolation protocols. What is important, is that they should be allowed for obtaining a higher number of hepatocytes with good viability and purity, preservating *in vitro* all functions specific for them *in vivo* [19].

The aim of our study is the isolation and cultivation of viable hepatocytes in large amounts from the liver of an adult rat for the *in vitro* liver recellularization.

Material and methods

The study was done on the liver from Wistar adult rats with body mass 274.66±2.52 g from which the hepatocytes were extracted. Before the isolation process, the substrate for hepatocytes adhesion was prepared. With 3-4 hours prior to initiating the experiment the cell culture surfaces of the culture plates or culture flasks were coated in a thin layer with Type I collagen solution at a concentration of 40 µg / ml. They were subsequently washed with HBSS (Hanks' balanced salt solution) without calcium and Mg (HiMedia, India) and allowed to dry in a Nuve 090 laminar flow hood. The hepatocytes were isolated by superior cava vein perfusion with 0.05% type I collagenase (HiMedia, India), 0.1% type I dispase (HiMedia, India) and HBSS without calcium and Mg (HiMedia, India) with 0.9 mM $MgCl_2$, 0.5 mM EDTA (Ethylenediaminetetraacetic acid) and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HiMedia, India) after washing the intrahepatic blood vessels with Buffer I solution. Prior to initiating the hepatocyte isolation process Buffer I and II solutions were heated

in the water bath at 42° C. The work surface was sterilized with 70% alcohol after which it was left under UV rays for at least 15 minutes. During the perfusion process a 75 watt incandescent light lamp was set at 25-30 cm above the perfused liver area to maintain the temperature at around 37° C, in order to avoid hepatocytes death caused by temperature lowering during liver perfusion with solutions which become colder. Also, to maintain temperature of perfused liver, to prevent drying of its surface and to remove unwanted particles that precipitate from the air, with a syringe the liver surface was wetted continuously with warm 0.9% NaCl solution.

The isolated hepatocytes were counted with triptan blue in the hemocytometer and the biochemical parameters such as ALT (Alanine aminotransferase), glucose, total protein and glucose-6-phosphatase were determined with the Elitech kit.

Results

Isolation of hepatocytes from the liver of adult rat is required to study the recellularization liver process *in vitro*. Prior to sacrificing the animals 5000 IU of heparin was injected intraperitoneally. The general anesthesia was performed with 60 mg/kg ketamine and 5 mg/kg xylazine, then was removed the fur with a trimmer and it was processed with 70% alcohol. With scissors, the toracoabdominal wall was removed and a suprahepatic portion of the inferior cave vein was channeled with 18 G plastic catheter during persisting cardiac contractions, for keeping hepatocytes alive. We used a two step perfusion by collagenase/dispasse solution for hepatocytes isolation from adult Wistar rat liver through the superior vena cava with Buffer I solution (Hank's 0.9 mM $MgCl_2$, 0.5 mM EDTA and 25 mM HEPES) through a 0.22 mm filter for 4-5 minutes at a speed of 15 -20 ml/min. At the same time the inferior vena cava was ligated and the portal vein was sectionated and every 2 minutes it was clipped and released for decompression. This procedure is repeated 6-8 times (fig. 1). The second step was performed by Buffer II solution (collagenase/dispase) liver perfusion at a rate of 25-30 ml/min until it became flaccid, it took about 10-12 minutes, with clamping and realising of the portal vein every 2-3 minutes. Later the liver was extracted from the abdominal cavity and underwent mechanical disintegration for hepatocyte release (fig. 2). It was placed under a laminar flow hood in a Petri box with William E and 5% FBS nutrient solution placed on the ice for 5 minutes and was removed the liver capsule and the hepatocytes were released by shaking. With a pipette the released cells were collected and filtered through a 100 µm strainer placed on a 50 ml tube. After isolation, was appreciated the cells viability, that was 92%. Some of them were used for biochemical parameters tests, and some were preserved to be used in recellularization of decellularized liver matrices.

The cells were counted with 0.25% blue trypan solution in haemocytometer and apreciated the cellular viability. Then the cells were cultured in William E (HiMedia, India) medium with 2 mM of L-glutamine, 5% fetal bovine serum (Lonza, Belgium), antifungal antibiotic solution (HiMedia, India), 100 nM deaxmethasone and 100 nM insulin. The cells were seeded in 12-well plates by 2.5 x 10^5 cells per well and incubated at 37°C with 5% CO_2. Following the hepatocytes isolation were obtained a total of 324, 48±1, 25 x 10^6 cells with a viability of 94.7± 0.9%, indicating a high yield of viable cells (fig. 3).

The hepatocyte isolation is a very delicate process. During the hepatocyte isolation, it is necessary to obtain a maximum number of cells and to preserve their morphology and functionality. The biochemical parameters of the hepa-

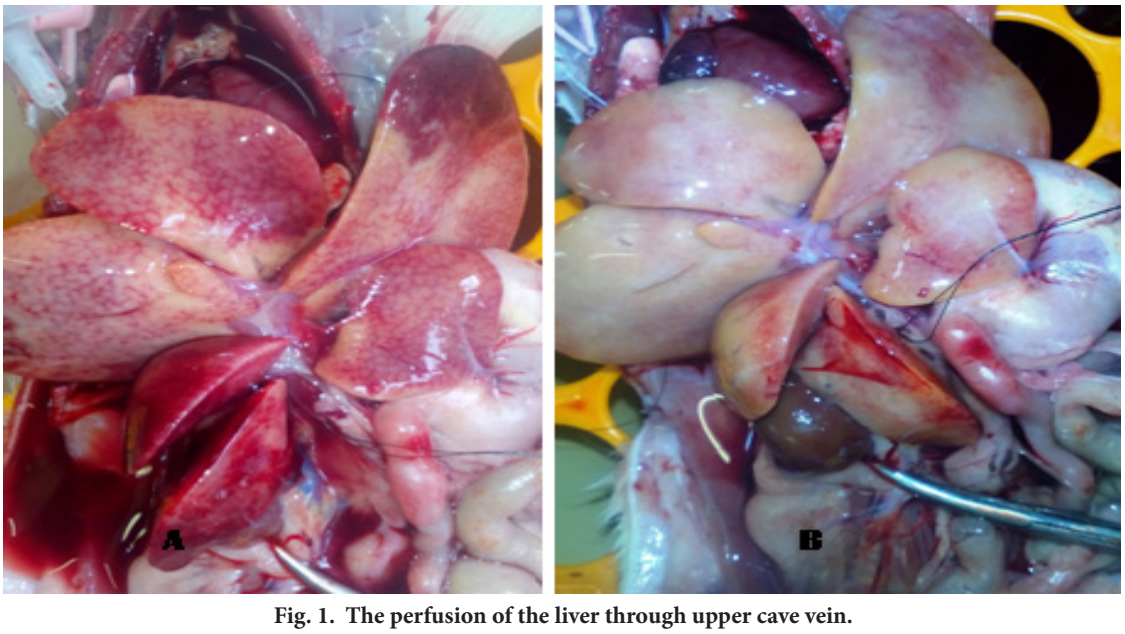

Fig. 1. The perfusion of the liver through upper cave vein.
A – with Hank's solution with 0.9 mM $MgCl_2$, 0.5 mM EDTA and 25 mM HEPES at a rate of 15-20 ml/min,
B – with Hank's solution and type II collagenase / type I dispase displacement with speed of 25 ml/min.

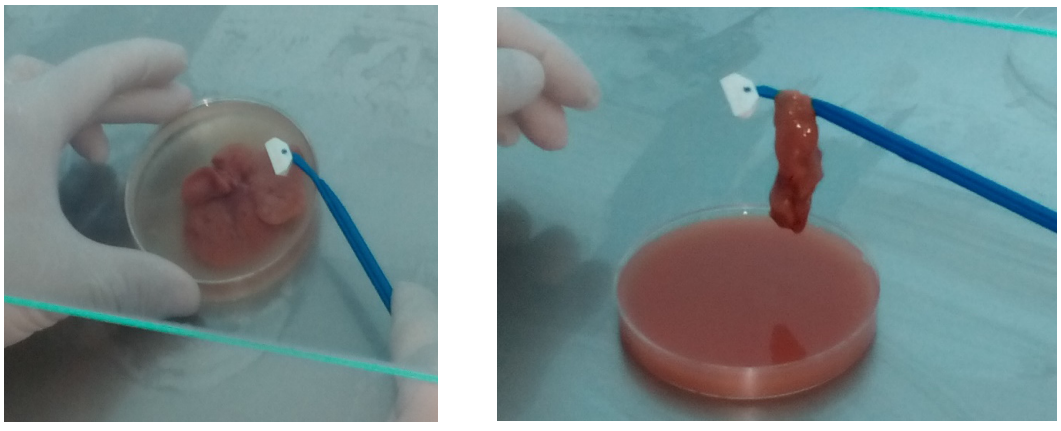

Fig. 2. The mechanical disintegration of the liver (A), liver carcase after isolation of the hepatocytes (B).

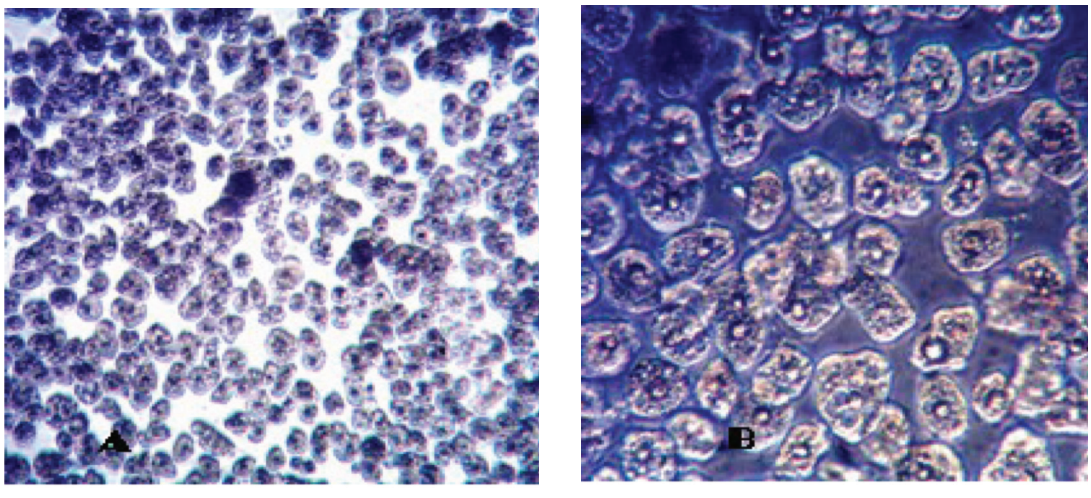

Fig. 3. Liver cells visualized with phase-contrast inverted microscope KZD: A – x10, B – x40.

tocytes that were analyzed show gluconeogenesis process, glucose-6-phosphatase, which is found only in hepatocytes and alanine aminotransferase, and as well as the content of glucose and total protein (tab. 1).

Table 1

Functional – metabolic properties of isolated hepatocytes in culture

Biochemical parameters	Catalytic activity
ALT, u / L	17.83 ±1.06
Glucose-6-phosphatase, u / L	3.41±0.46
Biochemical parameters	**Concentration**
Glucose, mM / L	4.16±0.10
Total protein, g / L	61.86±2.09

Discussion

Since hepatic tissue for liver isolation is limited, improvements in hepatocyte production and cryopreservation protocols are necessary to maintain cell viability during cultivation over a longer period of time and to prevent the hepatocyte number reduction after thawing [20, 21, 22].

According to the literature, there are many protocols for the isolation of hepatocytes with high viability and purity, but they depend on the type of collagenase used which determines significant differences in hepatocyte viability after digestion [23].

In the majority of the methods, collagenase is used to isolate hepatocytes from liver tissue. But Ricky H. Bhogal et al. used a combination of collagenase / protease / hyaluronidase and deoxyribonuclease [24].

Another study by MN. Berry shows that after buffered perfusion containing 0.05% collagenase and 0.10% hyaluronidase the isolated liver cells are viable and the form and function correspond to liver cells *in situ* with the presence of cytoplasmic vacuolization in a low number of cells and loss of potassium that are the only signs of cell lesion [25]. In another study in which the hepatocytes were isolated only by collagenase they showed a viability of 53% [26].

An effective change in our method was the insignificant reduction of collagenase concentration in 100 ml of buffer with approximately 400U to diminish, even insignificantly, the cytotoxic effect of collagenase on hepatocytes and the addition of solution of Dispase I enzyme which is not so cytotoxic at a concentration of 1U/ml.

In the hepatocytes grown medium were added supplements necessary for effective cellular respiration, since hepatocytes are highly specialized cells with intense biochemi-

cal activity requiring high energy consumption for protein, lipid and carbohydrate metabolism. These are: Na, selenium, transferin, bovine albumin, dexamethasone, aminoplasmol, hepatic amino acid complex, growth factors for hepatocytes and fibroblasts. It is important to note that the nutrient medium used for hepatocyte cultivation was changed every 2 days. During the medium changing within the first 4 hours, it should be taken into account that the hepatocytes are fragile and can easily be damaged by direct contact, so they are only pipetted from the side of the well.

Also is very important the supply of oxygen solutions, for which the oxygenator hose was introduced during the infusion first in Buffer I, and then in Buffer II solutions. It was programmed at 0.5 L/min. After strict following the listed steps, the viability of the hepatocytes is 94.7 ± 0.9%, compared to cell viability of 10% to 85%, which was obtained for use only for the detachment of hepatocytes from collagenase.

Conclusions

1. The high hepatocyte viability in dynamics is a priority and the sustained hepatocyte growth is determined by adding supplements necessary for effective cellular metabolism in the cell culture medium used.

2. The hepatocyte isolation method by liver perfusion with the combination of collagenase-dispase is feasible for obtaining a large amount of functional hepatocytes intended for the recellularization *in vitro* of decellularized liver scaffolds.

3. The yield and viability of hepatic cells could be increased by enzymatic digestion of liver tissue using combination of collagenase/dispase solution due to the less cytotoxic effect.

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