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Intraperitoneal Transplantation of Hepatocytes Embedded in Thermoreversible Gelation Polymer (Mebiol Gel) in Acute Liver Failure Rat Model

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Orthotopic liver transplantation (OLT) is the only treatment that improves the survival rate in patients with acute liver failure (ALF). Hepatocytes are anchorage dependent and require a substratum for their long-term survival. Various substrates are being used to improve the function and survival of these hepatocytes, but cannot be used clinically as they are animal derived. This has been overcome by thermoreversible gelation polymer (TGP). In the present study, rat hepatocytes embedded in TGP, were transplanted intraperitoneally in acute liver failure rat model. Efficacy of the transplanted cells was studied by assessing biochemical parameters and histopathology at different time points. The ALF condition reverted to normal 15 days after cell transplantation. The transplanted animals survived 100%. Therefore this study suggests that the cells in TGP provide substratum to the transplanted cells for long term survival and could successfully provide liver support in severe ALF.

Keywords: Hepatocytes, Acute Liver Failure, Transplantation, Thermoreversible Gelation Polymers, Histopathology

Introduction

Fulminant Hepatic Failure is defined as the development of hepatic encephalopathy and profound coagulopathy within 8 weeks of the onset of symptoms in patients without a preexisting liver disease. The various causes of this devastation include drug-induced liver injury from other medications, hepatitis A, B and E, ingestion of various hepatotoxins and metabolic disorders. There is rapid development of cerebral edema and multiorgan failure within days to weeks of clinical presentation. Liver transplantation is the only viable therapeutic option for the treatment of acute liver failure. In the last two decades, research has focused on the development of alternative or supportive measures to deal with acute liver failure; one of the most studied is hepatocyte transplantation (HTx). The potential advantages of cell transplantation include a simpler, safer, less costly procedure. Liver cells can be infused into ectopic sites (other than liver) such as peritoneum, portal vein or spleen. HTx has shown promising results to support acute liver failure condition in animal models (1-7) as well as humans (8).

Various substrates and scaffolds have been used to increase the survival of the transplanted cells. The use of micro-carriers has been well demonstrated in...
Intraperitoneal transplantation of micro-carrier attached hepatocytes showed long-term survival of the transplanted cells (9). In our earlier study, light and electron microscopy results revealed that the cells were viable and morphologically intact after several days of transplantation. Micro encapsulation technique has been used successfully in various types of cells for providing a substratum for long-term survival and immuno-protection to immobilize transplanted cells. The intra-peritoneal transplantation of microencapsulated hepatocytes using algicnic acid and poly-l-lysine has been previously done in acute liver failure rabbit model where the animals that received encapsulated hepatocytes showed better survival rate and the capsules were intact even 60 days after transplantation with 75% viable cells (10). The results of this study suggested that micro-encapsulation is effective in maintaining functional capacity of the transplanted hepatocytes and preventing their immune destruction. Other than encapsulation, various other extra cellular matrixes made of glycoproteins, proteoglycans have been used to improve the viability and functional capacity of cells. The major limiting factor to grow these cells using these substrates for subsequent clinical use is that these substrates are derived from animal sources (11). Hence, there is a need for synthetic and biocompatible matrices for growing hepatocytes.

The use of biological materials as a substratum is associated with the risks of transmission of certain diseases such as Human Immunodeficiency Virus, Hepatitis B and C, bacterial and fungal infections. Therefore, development of non-toxic biodegradable synthetic polymers is important. In addition, many of the substrates lack the mechanical properties that allow easy handling and suturing, as well as prolonged endurance after transplantation (12). The use of biosynthetic materials as stromal substitutes to support liver cell growth would overcome some of the problems related to the use of biological substrates. These could be custom fabricated to suit each condition and could provide a ready supply of material for clinical use and avoiding the shortcomings, such as poor mechanical strength and risk of immunologic rejection. In this context, developing synthetic polymers, which are non-toxic and biodegradable with adequate tensile strength, is important.

A thermoreversible gel (TGP), Mebiol is a copolymer composed of thermoresponsive polymer blocks [poly (Nisopropylacrylamide-co-n-butyl methacrylate) (poly NIPAAm-co-BMA)] and hydrophilic polymer blocks [polyethylene glycol (PEG)]. This polymer block is hydrophilic at temperatures below 20°C and hydrophobic at temperatures above 20°C forming cross-linking points and homogenous threedimensional (3-D) network of Mebiol. The 3-D cultured cells or tissues can be easily recovered from the TGP at temperatures lower than 20°C, TGP has been used in wound dressing (16), microcapsules for islets (17), electrophoretic gel for DNA separation (18), and three-dimensional culture matrixes for various cells like cancer cells (19), human mesenchymal stem cells (20), continuous cell lines (21), and corneal limbal stem cells (22).

We investigated the role of TGP as a transport vehicle for the transplantation of hepatocytes in an acute liver failure model and showed the long-term survival of hepatocytes embedded in TGP in acute liver failure rat model.

Materials and Methods

Chemicals and reagents

Dulbeccos Modified Eagles Medium (DMEM), Type IV Collagenase, Ammonium chloride, D-Galactosamine (D-Gal) (Sigma Co., USA), 100 µg/ml Streptomycin and 100 IU/L Penicillin (Himedia Co.), Mebiol TGP (Nichi, Japan).

TGP

Under a clean-air laminar hood workbench, a flask containing 1 gm of sterile, TGP was opened and 10 ml of DMEM/Ham-F12 medium was added. The gel got dissolved in the medium at 4 to 8°C in 3 days.

Experimental animals

Wistar rats, 225 to 250 grams of body mass, were maintained on 12-hour light dark cycles & fed ad lib with standard rat chow. All animal procedures were approved by institutional animal care and ethical committee.

Group I (n=15) (Gr-I) received intra-peritoneal injection of 2 ml normal saline alone.

Group II (n=15) (Gr-II) served as positive control, which received D- Galactosamine only.

Group III (n=15) (Gr-III) received intraperitoneal transplantation of 60 million hepatocytes/kg body weight.

Group IV (n=15) (Gr-IV) hosted intraperitoneal transplantation of 60 million hepatocytes/ kg body weight embedded in TGP in 1:2 ratio.

Group V (n=15) (Gr-V) received D-Galactosmine and TGP only.
**Cell isolation**
Cells were isolated from the livers of normal rats by collagenase digestion method. Cell viability was measured by Trypan blue dye exclusion staining.

**Development of acute liver failure model**
Rats were injected with D-Gal at the concentration of 875 mg/kg body weight in 5% dextrose.

**Intraperitoneal injection of hepatocytes**
After 24 hrs of D-Gal injection: In Gr-III isolated hepatocytes were injected into the peritoneal cavities of rats at 60 million/kg body weight in 500 µl PBS using 23 G syringe. In Gr-IV animals, isolated hepatocytes were mixed with TGP in the ratio of 1:2 and were placed into intraperitoneal cavity by making a small incision on left paracostal side under aseptic conditions. In Gr-V, after 24 hours of D-Gal injection, only TGP was transplanted into the peritoneal cavity.

**Follow up**
The animals were followed up for 30 days. The efficacy of the cells was assessed by coagulation parameters, quantifying liver function parameters and by studying histopathological changes.

**Sample collection**
Blood samples were drawn from the orbit of the eye & collected in plain tubes and anticoagulant containing tubes, spun and stored at -20°C. Liver samples were collected by sacrificing animals at various time points. Samples were fixed in 4% buffered formalin.

**Liver function test**
A standard battery of Liver Function Test was performed using semi autoanalyzer. The tests were as follows: Alanine amino transferase (ALT), aspartate amino transferase (AST), bilirubin and albumin.

**Coagulation parameter**
The prothrombin time (PT) was assessed before and after D-Galactosamine injection. The PT was measured using Liquiplastin.

**Histopathology**
The fixed liver samples were processed to thin section of 4-8 µm and were stained with hematoxylin/ eosin stain. A pathologist, who was unaware of the protocol and groups, assessed the prepared and coded sections.

**Results**

**Number and viability of isolated hepatocytes**
A mean of approximately 250±5.3 x10⁶ of hepatocytes were harvested from each donor, with a mean viability of 90±3.4%.

**Survival**
There was 100% survival of animals in Gr-I which did not receive Galactosamine injection. All the rats in groups II and V, which received D-Gal alone and D-Gal along with TGP, died within 48 hrs. The animals in groups III and IV, which received hepatocytes alone and hepatocyte embedded in TGP showed 100% survival.

**Prothrombin time**
Prothrombin time was assessed to detect changes in liver injury by injecting D-Gal. In these tests, there was a marked significant increase in PT in all four groups (II, III, IV, V) as compared to Gr-I before transplantation.

PT started decreasing 24 hrs after cell transplantation in both groups. It came back to normal after 7 days of HTx (Fig. 1). The animals in Gr-III, which received hepatocytes only and in Gr-IV which received hepatocytes and TGP mixed in 1:2 ratio showed comparable results.
Alanine amino transferase

Alanine amino transferase activity which rose after D-Gal injection reverted back to normal within 30 days of HTx of cells in both groups (Gr-III & Gr-IV). The ALT started decreasing 24 hours after cell Tx in all these animals (Fig. 2).

Histopathology

Histopathology of samples 24 hrs after D-Gal injury showed massive necrosis of hepatocytes in the liver (Fig. 4) as compared to the liver of normal rats, where hepatocytes were arranged as cords around the portal veins (Fig. 5). The transplantation of cells in Gr-III & Gr-IV animals showed that the histopathology was reverting back to normal, that is, after liver injury the sections showed mild necrosis on day 15 of HTx (Fig. 6) which became normal on day 30 (Fig. 7) and there was cord formation of hepatocytes around portal veins. The histological study revealed the presence of viable hepatocytes after 30 days of transplantation (Fig. 8).

Figure 2. Shows the alanine amino transferase at different time interval, shows the ALT in various groups of animals before and after HTx. The ALT level was increased in all the groups when compared with the control Gr-I. After 24 hours of cell transplantation in groups III and IV the ALT level started decreasing and came to normal within 30 days. The animals in groups II and V, which received only D-Gal and D-Gal along with TGP alone respectively, died within 48 hours of treatment.

Figure 3. Shows the Serum bilirubin in various groups of animals before and after HTx. The Serum bilirubin level was increased in all groups when compared with the control, Gr-I. After 24 hours of cell transplantation in groups III and IV the Serum bilirubin level started decreasing and came to normal within 15 days. The animals in groups II and V, which received only D-Gal and D-Gal along with TGP alone respectively, died within 48 hours of treatment.

Figure 4. The histopathology of the liver of a normal rat (group Gr-I). The hepatocytes are arranged in cords.

Figure 5. Histopathology of liver after 24-48 hrs of D-Gal induced liver injury. There is massive necrosis of the hepatocytes.

Figure 6. The histopathology of liver 15 days after cell transplantation showing diffuse extensive necrosis.
Discussion

The treatment modalities of hepatic failure have been limited for a long time due to the metabolic complexities of the liver (23). Liver transplantation is the only option in patients suffering from various liver diseases. The major limitation is the shortage of donor organ (24). Isolated hepatocyte transplantation is emerging as an appealing method for the treatment of ALF because of its technical simplicity. The potential of HTx for supporting an acute liver failure by providing metabolic support was suggested by different groups of workers (7, 25). The function and differentiation of the liver cells are influenced by the three-dimensional organ architecture. The use of polymeric matrices permits the three-dimensional formation of a neo-tissue. The culturing of hepatocytes on three-dimensional matrices showed increased function and survival of the cultured cells. The use of microcarriers, microencapsulation and extracellular matrices has been well demonstrated in animal models (9, 10). The problem with using extracellular matrices is that they are derived from the animal source and there is lot of chance for contamination with various pathogens and animal proteins. These limitations were overcome by using several biocompatible polymers like Thermo Gelation Polymer (13). This study demonstrates that TGP can be used as a substratum for the hepatocytes. In this present study, hepatocytes immobilized in TGP survived for more than 30 days following intraperitoneal transplantation in acute liver failure animal model. The survival rate of animals in Gr-II was 0% indicating that the experimental induction of liver injury was optimal. All animals in groups III and IV, which received only cells or cells with TGP respectively, survived. All the animals in Gr-II, which received only D-Galactosamine injection, died within 48 hrs of injection. Survival rates in Gr-III and Gr-IV was 100 %, which indicates that the hepatocytes were able to support the acute liver failure compared to control. Furthermore, the biochemical abnormalities developed following acute liver injury reverted back to normal in groups III and IV showing the role of the transplanted cells. The histopathological studies following the liver injury and after transplantation for up to one month of follow up also showed initial hepatic necrosis which was recovered after transplantation of hepatocytes in groups III and IV. This indicates that the transplanted hepatocytes have contributed to functional activity of the liver. Prothrombin time is an important and sensitive indicator of liver damage. In our study, cell therapy was given at 24 hours of D-Gal injection when PT value was >60 seconds. The data showed that there was a significant decrease in PT after 24-48 hrs of transplantation, which reverted back to normal in groups III and IV. Similar observations were made with other liver function tests such as serum bilirubin and ALT. The rationale behind studying these liver specific markers is to assess the status of the liver before and after cell transplantation in order to gain a further insight into the fact whether these transplanted cells were extending any support towards the reversal of the liver failure. No mononuclear cells infiltrations were found in and around and no fibrotic reactions were observed in the retrieved TGP. 70-80% of cells remained viable even after 30 days of transplantation indicating long term survival of hepatocytes when TGP was used. Hence, this study indicates that TGP is a good substratum and provides longevity to the immobilized cells.

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