Culture with Growth Factor Supplements Improves the Viability and Function of Rat Hepatocytes

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Background: To identify the optimum culture conditions by investigating isolated rat hepatocytes cultured in medium containing different growth factors.

Methods: Hepatocytes were isolated from rats using a two-step perfusion technique and divided into the following four groups cultured in medium containing different growth factors: control, epidermal growth factor (EGF), insulin, and EGF+insulin. The viability of the cultured rat hepatocytes and liver function parameters, including albumin, ammonia, and urea in the culture medium, were measured. Hepatocyte morphology was examined by staining with hematoxylin and eosin, and albumin receptor expression was confirmed by immunofluorescence.

Results: Slightly higher viability was observed in the growth factor groups than in the control group, although without significance (P=0.073). The levels of albumin (P<0.001), ammonia (P<0.001), and urea (P=0.041) differed significantly among the four groups. The functional parameters in the growth factor groups, particularly the EGF+insulin group, were significantly superior to those in the control group. The morphology of the hepatocytes in all growth factor groups was well maintained at 10 days. However, the control group showed deterioration in cell morphology by day 7.

Conclusions: Morphological and functional assessment indicated that the presence of growth factors, particularly EGF+insulin, provided culture conditions superior to those of non-supplemented medium.

Key Words: Culture, Epidermal growth factor, Hepatocytes, Insulin, Rats

INTRODUCTION

Acute liver failure is defined as the rapid development of hepatic cellular dysfunction, specifically coagulopathy and mental status changes, in a patient without known prior liver disease(1). Despite advances in multidisciplinary intensive care management for acute liver failure, the mortality rate is higher than 40%~80% and liver transplantation remains the only definitive treatment(2,3). However, not all patients with acute liver failure are able to receive timely liver transplantation because of an insufficient number of donor organs(4). Although the mortality rate of acute liver failure is high, patients that do recover generally do so without any sequelae(5). Therefore, alternative treatments that can provide at least temporary liver support are necessary until spontaneous recovery or a suitable liver donor becomes available.

Hepatocyte transplantation has been investigated as an alternative treatment for acute liver failure, some inborn metabolic deficiencies, and liver cirrhosis patients(6,7). Hepatocyte transplantation has several advantages over whole-liver
transplantation(8). First, hepatocytes from a single donor may be transplanted into several patients. Second, hepatocyte transplantation is less invasive than whole-liver transplantation and can be performed repeatedly. Third, if cryopreserved or cultured hepatocytes are used, the timing of hepatocyte transplantation is less critical than that of whole-liver transplantation. Fourth, Hepatocyte transplantation may provide support to the liver during regeneration of the native liver, with the aim of avoiding whole-liver transplantation(9).

However, several challenges remain for the clinical application of hepatocyte transplantation(10). The quality of the hepatocytes is key. The techniques used for isolation and culture of hepatocytes, cryopreservation, and the engraftment of transplanted hepatocytes were investigated from the perspective of clinical application. We reviewed the literature on hepatocyte culture methods using hormones and growth factors to evaluate the long-term survival of isolated hepatocytes and their preservation without affecting function(11). Factors affecting survival and preservation include insulin, glucagon, corticosteroids, thyroid hormone, epidermal growth factor (EGF), nerve growth factor, and fibroblast growth factor. The aim of our study was to establish the fundamental basis of hepatocyte transplantation for clinical application. We determined the ideal culture conditions by investigating isolated rat hepatocytes cultured in media containing EGF and/or insulin.

MATERIALS AND METHODS

1. Experimental animals and operation

Male Sprague-Dawley rats weighing 200~250 g were maintained in a controlled environment with continuous 12-hour light/dark illumination cycles, 25°C ambient temperature, and 40% humidity. All experiments were performed after obtaining approval from the Ethics Committee for Animal Experimentation, and the guidelines for animal experimentation were followed under their supervision.

Experimental rats were anesthetized by intraperitoneal injection of Zoletil (Virbac, Carros Cedex, France) and Rompum (Bayer, Toronto, ON, Canada). The abdomen of rats was shaved and disinfected with alcohol, and the rat was immobilized on a surgery table. The liver was exposed by U-shaped incision. One milliliter of freshly prepared heparin solution (0.1-mL heparin [5,000 IU/mL]+ 2.4-mL physiologic saline) was injected into the infrarenal inferior vena cava (IVC) to a final concentration of 200 IU/mL. A 22-gauge intravenous needle was catheterized into the portal vein 5 mm inferior to the liver, and secured by suture. The liver tissue was perfused with perfusion solution. After confirming satisfactory perfusion status, the infrahepatic and then suprahepatic IVC were cut.

2. Two-step perfusion of the rat liver

Hepatocytes were isolated by the two-step perfusion method, as described by Seglen(12). The liver was first flushed with Ca²⁺-free medium (Gibco 17701, Life technology, Grand Island, NY, USA) through the portal vein at 25 mL/
min for 10 minutes. The perfusion medium was then changed to Ca²⁺ collagenase medium (Gibco 17703, Life technology) and the perfusion continued through the same route at the same perfusion rate. The color and consistency of the perfused liver were observed. Upon completion of the perfusion, the liver was disconnected from the perfusion apparatus and the liver was resected (Fig. 1).

3. Purification of rat hepatocytes and determination of cell viability

The resected liver was transferred to a plastic petri dish containing Hank’s balanced salt solution (HBSS; Gibco 14175-095, Life technology). The liver capsule was slit, and the cells loosened from the capsule by gentle agitation in the solution. After filtration through a sterile Perlon filter (100 μm), the resulting cell suspension was pre-incubated at 37°C for 15 minutes in a petri dish with gentle, reciprocating shaking. The petri dish was placed on ice to lower the temperature of the cell suspension to 0°C. The supernatant was gently aspirated and discarded, and the remaining cell suspension was washed with 40-mL HBSS and dispensed into a sterile centrifuge tube. Other cells, connective tissues and contaminants were removed by differential centrifugation at 500 rpm for 5 minutes (three times). The low temperature was maintained during the centrifugation. The quality of the isolated hepatocytes was assessed immediately after purification using the standard trypan blue exclusion method, and the cells were then prepared for primary culture (Fig. 2).

4. Primary hepatocyte culture

Viable cells (1.0×10⁶) were seeded in a 100×15-mm petri dish (Nunclon 150350, Sigma-Aldrich, St. Louis, MO, USA) containing culture medium according to the experimental group. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco 11885-084, Life technology) with 10% fetal bovine serum, penicillin, and streptomycin incubated at 37°C, under a 5% CO₂ atmosphere. The culture medium was exchanged on days 3, 7, 10, and 14.

5. Experimental groups

Experimental groups were classified according to growth factor supplement. A total of six rats were experimented.

Isolated hepatocytes from a rat divided into four groups according to the experimental groups. On day 0 of hepatocyte culture, EGF (SRP3238, Sigma-Aldrich) and insulin (I9278, Sigma-Aldrich) were added according to the experimental group. In the EGF group, 500-ng EGF were added to 10-mL culture medium, and in the insulin group, 0.1-ng insulin was added to 10-mL culture medium. To the EGF+insulin group, identical amounts of EGF and insulin were added to 10-mL culture medium. No growth factor or hormone was added to control medium. EGF and insulin were added each time the culture medium was exchanged; i.e., on days 3, 7, 10, and 14.

1) Control group (n=6): 1 g/L D-glucose DMEM.
2) EGF group (n=6): 1 g/L D-glucose DMEM+EGF.
3) Insulin group (n=6): 1 g/L D-glucose DMEM+insulin.
4) EGF+insulin group (n=6): 1 g/L D-glucose DMEM+EGF+insulin.

6. Evaluation of cultured rat hepatocytes

The cultured rat hepatocytes were morphologically and functionally evaluated at 3, 7, 10, and 14 days.

1) Viability

The viability of the cultured rat hepatocytes was determined using a hemocytometer and the trypan blue exclusion test on days 3, 7, 10, and 14. Cells stained by trypan blue

**Fig. 2.** Evaluation of cell viability by trypan blue exclusion test (×200). Viable cells with an intact plasma membrane exclude dyes such as trypan blue, whereas damaged cells (arrows) become stained, particularly intensively in the nucleus. The viability of isolated hepatocytes in this photograph was about 85%.
were considered non-viable.

Total cell count = volume of cells (mL) × 2 (dilution factor) × mean number of cells (stained cells + unstained cells) × 10^4.

Viability (%) = viable cell count (unstained cell count) / total cell count (stained cell count + unstained cell count) × 100.

2) Cultured hepatocyte function

The concentrations of albumin, ammonia, and urea in the culture medium were measured at 3, 7, 10, and 14 days. The concentration of albumin was measured with the enzyme-linked immunosorbent assay method using affinity-purified rat albumin coating antibody (Bethyl A110-134A, Bethyl Laboratories, Inc., Montgomery, TX, USA), rat reference serum (Bethyl RS10-100, Bethyl Laboratories, Inc.), and horseradish peroxidase-conjugated rat albumin detection antibody (Bethyl A110-134P, Bethyl Laboratories, Inc.). The concentrations of ammonia and urea were measured at 3 days after the addition of 1 mM NH₄Cl when replacing the culture medium. The ammonia concentration was measured using the ammonia assay kit (AA0100, Sigma-Aldrich). The urea concentration was determined by a coupled enzyme reaction, which yields a colorimetric (570 nm) product proportional to the amount of urea present, using a Sigma Urea Assay Kit (MAK006, Sigma-Aldrich).

3) Histologic assessment

The morphology of cultured rat hepatocytes was examined by hematoxylin and eosin staining. The presence of albumin receptors was confirmed by immunofluorescence. The cultured cell suspension was fixed with 10% formalin for 15 minutes at room temperature and incubated for 10 minutes in phosphate-buffered saline containing 0.25% Triton X-100 (PBST) for permeabilization. The cells were incubated with 1% bovine serum albumin in PBST for 30 minutes to block nonspecific binding. Finally, the cells were incubated with anti-albumin antibody (Abcam ab106582, Abcam, Cambridge, UK), and then rabbit anti-chicken immunoglobulin Y H&L secondary antibody (Abcam ab6749).

7. Statistical analysis

All data are expressed as mean ± standard deviation. The experimental results were assessed by one-way analysis of variance with repeated measurements and Bonferroni’s post hoc test. Because its distribution was skewed, the albumin level was transformed into a logarithmic scale. Statistical analysis was performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA). A P < 0.05 was considered to indicate statistical significance.

RESULTS

1. Viability

Although viability increased gradually in all groups over time, the increases in the growth factor groups were slightly greater than that in the control group, albeit not sig-

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<tr>
<th></th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>P-value (vs. control)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>58.3±2.9</td>
<td>71.0±6.5</td>
<td>78.0±4.2</td>
<td>81.0±3.3</td>
<td>0.073</td>
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<tr>
<td>EGF</td>
<td>52.3±6.7</td>
<td>79.4±1.9</td>
<td>87.3±1.2</td>
<td>87.3±1.4</td>
<td>1.000</td>
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<tr>
<td>Insulin</td>
<td>49.1±4.6</td>
<td>80.4±2.1</td>
<td>86.8±0.9</td>
<td>85.6±1.3</td>
<td>1.000</td>
</tr>
<tr>
<td>EGF+Insulin</td>
<td>63.2±0.6</td>
<td>83.3±1.8</td>
<td>88.9±1.4</td>
<td>87.3±1.9</td>
<td>0.086</td>
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Fig. 3. The change of viability according to growth factor supplement in culture medium. Data are average value±SEM. Abbreviation: EGF, epidermal growth factor. *P < 0.05 vs. control on each day.
2. Cultured hepatocyte function test

The concentrations of albumin, ammonia, and urea in the culture medium were measured to assess hepatocyte function (Fig. 4). The albumin levels were significantly different among the groups \( (F[3,20]=8.373, P=0.001) \). Post hoc tests revealed that the albumin level in the control group differed significantly from those in the growth factor groups (\( P=0.013 \) for EGF group, \( P=0.006 \) for insulin group, and \( P<0.001 \) for EGF+insulin group). Also, the albumin level in the EGF+insulin group was significantly higher than those in the EGF (\( P=0.036 \)) and insulin (\( P=0.038 \)) groups. The albumin levels significantly decreased in all groups over the culture period, as shown in Fig. 4A.

The ammonia levels were also significantly different among the groups \( (F[3,20]=2.697, P=0.073) \). Post hoc tests revealed that viability was greater in the EGF+insulin group than the control group, but the difference was only marginally significant (\( P=0.066 \)). However, on day 10, the viability in the EGF+insulin group differed significantly from that in the control group (\( P=0.049 \)). The viabilities in the insulin and EGF+insulin groups on day 7 were significantly greater than on day 3 (\( P<0.001 \)). The viability in all growth factor groups was highest on day 10 and had decreased slightly by day 14. There were no significant differences among the growth factor groups (Fig. 3).

![Figure 4](image_url)
time and peaked on day 3 in the growth factor groups, whereas the albumin level in the control group was significantly lower than in the growth factor groups on day 3 (P=0.002 for EGF, P=0.001 for insulin, P=0.002 for EGF+insulin). In particular, the albumin levels in the EGF+insulin group on days 3, 7, 10, and 14 were significantly different from the control group (P=0.002 for 3 days, P=0.035 for 7 days, P=0.036 for 10 days, and P=0.001 for 14 days).

Ammonia levels differed significantly among the groups (F[3,20]=27.465, P<0.001). The ammonia levels in all groups increased gradually over time, but the ammonia level in the control group was significantly higher than those in the growth factor groups on all days (P<0.001 for all). The ammonia levels in all groups on day 7 were significantly higher than on day 3 (P=0.030 for control, P=0.017 for EGF, P<0.001 for insulin, P=0.003 for EGF+insulin).

There were no significant differences among the growth factor groups.

Urea levels differed significantly among the groups (F[3,20]=3.311, P=0.041). Although the urea levels decreased gradually over time in all groups, the urea level in the EGF+insulin group was significantly different from that in the control group (P=0.043). Urea levels decreased gradually over time in the order EGF+insulin group, insulin group, EGF group, and control group.

3. Histological assessment

Histological examination indicated that the typical shape of hepatocytes; i.e., round nucleus, prominent nucleoli, and polygonal cytoplasm, was maintained in all groups after 3 days in culture. On day 7, although the cytoplasm of the cultured hepatocytes in the control group was transformed into a spindle shape, cellularity was more abundant and the

Fig. 5. Histologic feature of cultured rat hepatocytes (HE stain, ×400): (A) 7 days, (B) 10 days, and (C) 14 days after culture. Abbreviation: EGF, epidermal growth factor.
cell-to-cell interaction stronger in the growth factor groups. On day 10, the transformation of cytoplasm into a spindle shape was exaggerated in the control group, whereas hepatocyte shape was well maintained in the growth factor groups. On day 14, the typical characteristics of hepatocytes could no longer be observed in the control group, and the cytoplasm in the EGF and insulin groups was transformed into a spindle shape. In the EGF+insulin group, the hepatocyte shape was maintained, but the cellularity was scarcer (Fig. 5).

On day 3, immunofluorescence staining for albumin in the cytoplasm was intense in all groups, with stronger staining in the growth factor groups than the control group. However on day 7, only weak staining was detected in all groups, with a greater reduction in the control group. There was no staining by day 10 in all groups (Fig. 6).

**DISCUSSION**

Hepatocyte transplantation has been performed in patients with several liver disease and acute liver failure as an alternative to whole-liver transplantation(13). In 1976, Matas et al.(14) reported that a sustained decrease in plasma bilirubin concentrations occurred in homozygous recessive Gunn rats lacking the enzyme uridine diphosphate glucuronyl transferase following transplantation of hepatocytes from heterozygous non-jaundiced Gunn rats possessing the enzyme. The first hepatocyte transplantation in human subjects was attempted by Mito et al.(15) in 1992, who performed autologous hepatocyte transplantation into the spleen in patients with chronic liver disease and confirmed the stability of hepatocyte transplantation. To date, hepatocyte transplantation has been investigated for three conditions: (1) metabolic liver disease, (2) chronic liver disease, and (3) acute liver failure. Because of the success of experimental hepatocyte transplantation, a number of institutions have attempted its application to the clinical treatment of a variety of hepatic diseases. However, a lack of controlled trials complicates interpretation of the results and the limitations of the technique must be overcome(16). The major limitation is that hepatocytes that have maintained viable liver function must be readily available in time for transplantation. Hence, we investigated the ideal culture conditions using isolated rat hepatocytes cultured in media containing EGF and/or insulin.

Primary hepatocytes, immortalized hepatocytes(17), hepatic progenitor/stem cells(18), and xenogeneic hepatocytes are used for transplantation(19). Of these, primary hepatocytes have been evaluated most extensively, while the others are currently under investigation. The majority of experimental and clinical studies of hepatocyte transplantation have used primary hepatocytes. Our study also examined primary hepatocytes.

Techniques of hepatocyte isolation can be divided into...
mechanical and enzymatic methods. Initial efforts to isolate hepatocytes by mechanical methods had poor success; the majority of cells were damaged and the survival rate was low. Since the introduction of enzymes—such as collagenase and hyaluronidase—as dissociating agents, numerous advances in the isolation of hepatocytes have been made. In 1969, Berry and Friend(20) established the collagenase perfusion method, which enables a uniform distribution in vivo. This method produced a high yield of viable hepatocytes. The technique was further modified by Seglen(12) to become the two-step collagenase-perfusion technique. This technique has been adopted widely and was used in our study.

For successful hepatocyte transplantation, hepatocytes should have a viability >60% with no microbial contamination, and the metabolic functions of hepatocytes should be maintained. A sufficient quantity of hepatocytes should also be obtained for transplantation(21). In our study, we employed the modified Seglen’s method as the hepatocyte isolation technique; the viability of the isolated hepatocytes was >80%.

Considerable research on the isolation and culturing of primary hepatocytes of high quality has been carried out (11,20). In particular, researchers have attempted to identify the ideal culture environment for primary hepatocytes. In 1976, Richman et al.(22) were the first to report a prominent role for EGF in promoting hepatic DNA synthesis by acting in concert with insulin and glucagon. Thereafter, many studies have aimed to identify the essential factors for triggering hepatocyte proliferation. McGowan et al.(23) suggested that EGF in combination with insulin and glucagon alters the morphological characteristics of the cells during the early stages, promoting spreading and aggregation. Numerous growth factors and hormones are involved in hepatocyte regeneration, but more research is required to reveal the exact mechanism: e.g., the role of pancreatic hormones, such as insulin and glucagon, and EGF in the regeneration of hepatocytes. Michalopoulos and DeFrances (24) categorized the factors involved in hepatocyte proliferation as “complete hepatocyte mitogens” and “comitogenic growth factors.” According to this classification, EGF is a complete mitogen, and insulin a comitogen.

In our study, we added EGF and insulin as growth factors and hormones to the culture medium as follows: (1) control group, (2) EGF group, (3) insulin group, and (4) EGF+insulin group, to determine the optimal conditions for hepatocyte culture, in terms of maintaining their function and enabling long-term survival. Hepatocytes in culture medium containing both EGF and insulin maintained their morphology and liver function significantly better than cells in the control group. Although no significant difference existed between the EGF and insulin groups, there was a significant difference between these two groups and the control group. The control group displayed deterioration of cell morphology by day 7 and a loss of the typical hepatocyte shape by day 10. However, the EGF and/or insulin groups maintained the typical hepatocyte shape for up to 10 days. Therefore, addition of growth factors and hormones to the culture medium results in long-term survival of functional hepatocytes.

Our study was limited by the small number of samples, and the basic nature of the methodology. Further studies should identify the optimal conditions for hepatocyte culture and will improve the acquisition and storage of hepatocytes for the clinical application of hepatocyte transplantation.

CONCLUSION

Our results suggested that culture medium containing EGF and insulin was superior to non-supplemented medium in terms of hepatocyte culture. Morphological and functional assessments indicated that the function and morphology of hepatocytes cultured in the presence of EGF and insulin could be maintained for 10 days. Further studies of culture media supplemented with other growth factors and hormones are necessary to identify the optimal culture conditions.

REFERENCES