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The protective effect of transplanted liver cells into the mesentery on the rescue of acute liver failure after massive hepatectomy

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Liver cell transplantation into an extra-hepatic site

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Abstract

Postoperative liver failure is one of the most critical complications following extensive hepatectomy. Although allohepatocyte transplantation is an attractive therapy for posthepatectomy liver failure, transplanted cells via the portal veins typically form portal vein embolization. The embolization by transplanted cells would be lethal in patients who have undergone massive hepatectomy. Thus, transplant surgeons need to select extra-hepatic sites as a transplant site to prevent portal vein embolization. We aimed to investigate the mechanism of how liver cells transplanted into the mesentery protect recipient rats from acute liver failure after massive hepatectomy. We induced posthepatectomy liver failure via 90% hepatectomy in rats. Liver cells harvested from rat livers were transplanted into hepatectomized rats' mesenteries. Twenty percent of the harvested cells, which consisted of hepatocytes and non-parenchymal cells, were transplanted into each recipient. The survival rate improved significantly in the liver cell transplantation group compared to the control group 7 days after hepatectomy (69% vs. 7%). Histological findings of the transplantation site, in vivo imaging system study findings, quantitative polymerase chain reaction assays of the transplanted cells, and serum albumin measurements of transplanted Nagase analbuminemic rats showed rapid deterioration of viable transplanted cells. Although viable transplanted cells deteriorated in the transplanted site, histological findings and an adenosine-5'-triphosphate assay showed that the transplanted cells had a protective effect on the remnant livers. These results indicated that the paracrine factor of transplanted liver cells had an effect on the remnant livers. The same protective effects were observed in the hepatocytes transplantation group, not in the liver non-parenchymal cells transplantation group. Therefore, this effect on the remnant liver was mainly due to the hepatocytes of the

transplanted liver cells. We demonstrated that transplanted liver cells protect the remnant liver from severe damage after massive hepatectomy.

Keywords:

Liver cell transplantation, acute liver failure after massive hepatectomy, mesenteric cell transplantation, paracrine factor of transplanted liver cells, remnant liver, liver non-parenchymal cells

Introduction

Although orthotopic liver transplantation has been one of the most effective therapies for lethal liver failure, a chronic donor shortage requires alternative therapies, including hepatocyte transplantation (6). Some clinical trials for congenital enzyme deficiency have demonstrated that hepatocyte transplantation is useful for improving liver function, and it can be a temporal bridge for orthotopic liver transplantation (11,12,42). Postoperative liver failure is one of the most critical complications following extensive hepatectomy for treating hepatobiliary cancer (34). Patients typically present with massive ascites, progressive hyperbilirubinemia, coagulopathy, encephalopathy, and eventually death (29).

Several previous studies have investigated the possibility of hepatocyte transplantation for resolving acute liver failure after massive hepatectomy, which requires much more functional hepatocytes to be transplanted (1,2,24,47). A large amount of transplanted cells via portal veins, including the spleen, typically form portal vein embolization (16,19,22,25,39), which leads to functional deterioration (4).

However, several extra-hepatic sites, including the intra-peritoneal cavity, pancreas, subcutaneous cavity, kidney capsule, and mesentery were evaluated as suitable liver cell

transplantation sites to avoid portal vein embolization (21,23,32,41). Some researchers have reported on the recovery of a 90% hepatectomized rat model by hepatocyte transplantation in a peritoneal cavity, which allows a large amount of functional hepatocytes to be transplanted (9,10,13,17,36). Indeed, extra-hepatic sites that allow a large amount of transplanted cells with poor vascularity should lead to the poor maintenance of transplanted cells. Therefore, it should be elucidated how ectopic liver cell transplantation works to protect acute liver failure after massive hepatectomy, as this remains unclear.

In the present study, we aimed to investigate the mechanism of how liver cells transplanted into the mesentery prevent acute liver failure after massive hepatectomy.

Materials & Methods

Animals

F344 rats, Sprague-Dawley (SD) rats, and Nagase analbuminemic rats (NARs) were purchased from SLC. Transgenic green fluorescent protein (GFP)/F344 rats (48) were kindly provided by Ochiya (Section for Studies on Metastasis, National Cancer Center Research Institute). Rats used in the experiments weighed between 170 and 300 g, with the exception of the NARs. Animals were housed at Kyoto University. Rats were fed a standard diet and tap water *ad libitum*. All the animal experimental procedures were performed according to the Animal Protection Guidelines of Kyoto University and were approved by the Animal Research Committee of Kyoto University.

Cell isolation

Graft liver cells were obtained from male or female F344 wild-type rats or transgenic

rats carrying the *GFP* gene, which helped distinguish graft hepatocytes from original residual cells. Adult liver cells were isolated using a modified two-step collagenase perfusion technique, as described previously by our laboratory (3).

Briefly, under general anesthesia with isoflurane (Wako Pure Chemical Industries), donor rats underwent laparotomy, and their portal veins were cannulated. The livers were then preperfused with HBSS-EGTA solution, followed by perfusion with a collagenase solution containing 0.3% dispase II (Sanko Junyaku Co., Ltd.), 0.3% collagenase type II (Gibco), 150 mmol/L NaCl, 5.4 mmol/L KCl, 0.34 mmol/L NaHPO₄, 0.1 mmol/L MgSO₄, 5.0 mmol/L CaCl₂, 4.2 mmol/L NaHCO₃, 5.6 mmol/L glucose, 10 mmol/L HEPES, 0.01% deoxyribonuclease, and 0.005% trypsin inhibitor (all of the chemical reagents previously described were purchased from Wako Pure Chemical Industries, Ltd.). Then the livers were excised, minced, and immediately filtered through 100-N polypropylene mesh. In the isolated liver cells, suspension, hepatocytes, and non-parenchymal cells (NPCs) were observed using light microscope, showing that 20% of all the isolated liver cells contained approximately 6×10^7 hepatocytes and 1×10^7 NPCs. Liver cell homogenates were prepared by sonication for 10 min on ice (The Handy Sonic UR-20P, Tomy Seiko Co., Ltd.), and the sonicated samples were examined by light microscopy to confirm the loss of cell integrity. Cell homogenates from 20% of all the isolated liver cells were transplanted into each recipient rat in the homogenate group. The suspension was mixed with collagen gel (Nitta Gelatin Inc.) just before transplantation.

In addition, hepatocytes and NPCs were divided as follows. The suspension was centrifuged three times at $50 \times g$ for 4 min, the hepatocytes were pelleted, and the NPCs were separated into the supernatant. The hepatocytes were isolated from the pellets. To

isolate the NPCs, the supernatant was collected and centrifuged at $150 \times g$ for 4 min (31). After quantifying the hepatocytes or NPCs, they were suspended in HBSS-EGTA solution and mixed with collagen gel just before transplantation.

Cell transplantation

Male F344 wild-type rats and NARs were used as recipients. First, posthepatectomy liver failure was induced by 90% hepatectomy, which was modified from the Higgins-Anderson operation. Rats received isoflurane anesthesia to begin the procedure. After laparotomy, the left and median lobes (70% partial hepatectomy), and right lobes (20%) were removed, with the remaining caudate lobes (10%).

Just after hepatectomy, transplanted cells were suspended in a collagen gel mixture (HBSS-EGTA) and directly injected into each recipient's mesenteric fat. Equivalent amounts of the collagen gel mixture without liver cells were injected into the mesentery of the control rats. We evaluated the transplanted cells immediately posttransplantation using a transilluminator.

Survival study

Thirty-nine male F344 wild-type rats were divided into three groups. The liver cell transplantation group ($n = 13$) received 20% of all the isolated liver cells from the donor GFP rat liver mixed with the collagen gel. The liver cell homogenate group ($n = 13$) received cell homogenates from 20% of all the isolated liver cells mixed with the collagen gel. The control group ($n = 13$) was injected with collagen without liver cells.

To evaluate differences between receiving hepatocyte (HEP) alone and non-parenchymal cell (NPC) alone, 16 male F344 wild-type rats were divided into two

groups (each group, $n = 8$). In the HEP group, 1×10^7 hepatocytes were transplanted, and in the NPC group, 1×10^7 NPCs were transplanted. Survival probability was calculated using the Kaplan-Meier method, and it was analyzed using the log-rank test.

Biochemical analysis of the liver cell transplantation model

Peripheral blood samples were obtained by cutting the live animal's tail under general anesthesia. Serum ammonia levels were determined using the Test Wako kit (Wako Pure Chemical Industries) (transplantation [Tx]: $n = 4$, control [Cont]: $n = 4$), and the total bilirubin levels were determined using the QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems) (Tx: $n = 3$, Cont: $n = 3$), according to the manufacturer's protocol. Statistical significance between these groups was evaluated by repeated-measures analysis of variance (ANOVA) followed by Holm-Sidak's test.

Histological, periodic acid-Schiff stain, and immunohistological analyses of the liver cell transplantation model

After the rats were deeply anesthetized with isoflurane, the mesenteries or remnant livers were harvested followed by cervical dislocation. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H/E). Engraftment of the transplanted liver cells in the mesentery was analyzed at 8, 24, and 72 h posttransplantation, and the remnant liver was evaluated at 8 and 24 h posttransplantation.

For immunohistochemical analysis, a Target Retrieval Solution (Dako) was used for antigen retrieval, and then a nonspecific binding was blocked with 0.1% Triton-X (Sigma-Aldrich) dissolved in phosphate-buffered saline. The sections were incubated

overnight at 4°C with primary antibodies as follows: rabbit polyclonal anti-GFP antibody (1:200; Molecular Probes Inc.) and sheep polyclonal anti-human albumin antibody (1:100, Abcam). After washing, the stained sections were incubated with Alexa 488-conjugated donkey anti-rabbit immunoglobulin (Ig)G and Alexa 555-conjugated donkey anti-sheep IgG for 2 h at room temperature. After washing, the stained sections were covered with Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories).

Periodic acid-Schiff (PAS) stain was performed using the PAS Staining Kit (Muto Pure Chemicals), according to the manufacturer's protocol.

Qualitative and quantitative analysis of the transplanted cells' existence

The expression of GFP in live rats of the liver cell transplantation group was measured using an in vivo imaging system (IVIS) (Xenogen IVIS Spectrum System) at 0, 8, and 16 h posttransplantation ($n = 3$). *Regenerative Medicine Journal*

Additionally, we compared the GFP expression at the transplantation sites between the hepatocyte transplantation group (HEP group) and the liver cell transplantation group (liver cell group), which consisted of hepatocytes and liver non-parenchymal cells. In the liver cell group ($n = 3$), rats received 6×10^7 hepatocytes from GFP rats with 1×10^7 NPC liver cells from wild-type rats that were GFP negative. In the HEP group ($n = 3$), rats received 6×10^7 hepatocytes from GFP rats. We compared the GFP expression between the groups at 0, 4, and 8 h posttransplantation and used repeated-measures ANOVA followed by Holm-Sidak's test.

Quantitative Polymerase Chain Reaction

Total RNA was extracted from the liver cells just before transplantation and from the transplanted liver cells in the mesentery at 8, 24, and 72 h after transplantation using the PureLink RNA Mini Kit (Invitrogen) (n = 3/each time point). The ReverTra Ace (Toyobo) was used according to the manufacturer's protocol to reverse transcribe 1 µg of total RNA into complement DNA. We performed quantitative polymerase chain reaction (PCR) assays with the SYBR-green PCR Master Mix (Applied Biosystems) using the ABI 7500 system (Applied Biosystems). Following primers were used for albumin: 5'- CGTTCTGGTTCGATACACC-3' and 5'- GAAGTCACCCATCACCGTC-3'; for glucose-6-phosphatase (G6Pase): 5'- GACCTCCTGTGGACTTTGGA -3' and 5'- AGGCTTGGGTGCTCCTAGT -3'; for cytochrome 3A1 (CYP3A1) : 5'- GGAAATTCGATGTGGAGTGC -3' and 5'- AGGTTTGCCTTTCTCTTGCC -3'; for cytochrome 2E1 (CYP2E1) : 5'- ACTTCTACCTGCTGAGCAC -3' and 5'- TTCAGGTCTCATGAACGGG -3'; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Journal: 5'-GGCACAGTCAAGGCTGAGAATG -3' and 5'-ATGGTGGTGAAGACGCCAGTA -3' (49). The quantification of the given genes was expressed as the relative messenger (m)-RNA level compared after normalization to GAPDH. Statistical significance between these groups was evaluated using Student's t-test.

Functional analysis of the transplanted hepatocytes in the liver cell transplantation model

To evaluate the function of transplanted hepatocytes in the liver cell transplantation model, 20% of all the liver cells isolated from male SD rats were transplanted into the mesenteries of 6- or 7-wk-old male NARs (weight, 150–210 g), the original strain of

which is an SD rat, and the serum albumin level of peripheral blood was subsequently measured. In the control group, collagen gel was injected into the mesentery fats. To measure albumin, blood samples were harvested every 4 h until 24 h posttransplantation, and they were evaluated using the rat albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories), according to the manufacturer's protocol. Statistical significance between these groups (Tx: n = 3, Cont: n = 4) was evaluated by repeated-measures analysis of variance (ANOVA) followed by Holm-Sidak's test.

Adenosine-5'-triphosphate assay of the remnant liver

The remnant livers were collected 24 h after 90% hepatectomy and were stored at -80°C. The levels of adenosine-5'-triphosphate (ATP) in the remnant livers were measured using a firefly bioluminescence assay kit (AMERIC-ATP kit; Wako Industries), according to the manufacturer's instructions. The luciferase activity was measured using a plate reader (ARVO X5; Perkin Elmer Inc.). Statistical significance between these groups was evaluated using Student's t-test (Tx: n = 3, Cont: n = 3) (HEP: n = 3, NPC: n = 3).

Proliferation analysis of the hepatocytes in the remnant livers

To detect cells synthesizing deoxyribonucleic acid, proliferating cell nuclear antigen (PCNA) expression in the liver sections was determined immunohistochemically. The deparaffinized sections were incubated in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Sections were incubated at 4°C for 16 h with mouse anti-PCNA antibody (1:100; Santa Cruz) and diluted at 1:100. After the primary antibody had been washed off, the samples were incubated for 60 min with horseradish

peroxidase-conjugated anti-mouse IgG (Envision plus Kit; Dako) and for 1 min with 3,3'-diaminobenzidine substrates (Dako). Counterstaining was performed using hematoxylin. After staining, positive cells from 10 randomly selected fields were counted using a light microscope with a 20× objective lens. Statistical significance between these groups was evaluated by Student's t-test (Tx: n = 3, Cont: n = 3).

Removal study of the transplanted cells

To determine how transplanted cells have a role in functional support in the remnant liver of massive hepatectomy, liver damage was evaluated in the new model of transplantation.

Briefly, transplanted cells were surgically removed from the transplant sites 8 h after transplantation (remove group). Twenty-four h after transplantation, the serum ammonia levels and histological findings were analyzed with (n = 3) and without (n = 4) the removal of transplanted cells (Figure 3G–K).

Cytokine array

To detect factors that were produced from transplanted cells, we comprehensively analyzed cytokines, growth factors, and other proteins in the portal vein blood, which connected the remnant liver with the transplanted site. It is supposed that cytokine or growth factors produced from transplanted cells are distributed from mesenteric fats to the portal vein. Blood samples in the portal vein were obtained from each group (Tx: n = 3, Cont: n = 3) of rats at 4 h after hepatectomy, and they were centrifuged for 20 min at 5,000 rpm. The serum was collected and stored at -20°C. Then the serum was diluted at approximately 25-fold and analyzed for specific proteins using the Rat Cytokine

Antibody Array - Membrane (Abcam), according to the manufacturer's instructions. Quantitation of the detected spots was performed using Quantity One Software 4.4.1 (BioRad Laboratories).

Statistical analysis

Data are expressed as mean \pm standard deviation. We performed statistical analyses using GraphPad Prism for Windows, version 6.0 (GraphPad Software). Statistical significance was defined as $p < 0.05$.

Results

Liver cell transplantation recovery in acute liver failure models

The probability of survival was significantly higher in the transplantation group than in the control group (69.2% vs. 7.7%, $p = 0.0004$), and the homogenate group (69.2% vs. 23.1%, $p = 0.0086$). However, the probability of survival was not significantly higher in the homogenate group than in the control group (Figure 1B). These results indicated that survival required liver cell transplantation, not the liver cell homogenate. All animals that survived longer than 7 days survived for the duration of the experiments (>100 days).

Serum total bilirubin levels increased in the control group after 90% hepatectomy, which was significantly different from the relative lack of increase in the transplantation group ($p < 0.005$) (Figure 1C). Serum ammonia levels were also lower in the transplantation group than in the control group ($p < 0.005$) after 90% hepatectomy (Figure 1D). These results indicated that liver cell transplantation improved the post-hepatectomy survival rate and prevented acute liver failure.

Assessment of the transplanted cells

The transplanted cells were successfully engrafted into the recipients' mesenteries, which was confirmed by histological and immunohistological analyses at 8, 24, and 72 h posttransplantation. The living cells with nuclei gradually decreased over time. To monitor donor-derived cells, the mesentery tissue sections were analyzed for the presence of GFP and albumin (Figure 2A–I). The morphological characteristics of the GFP-positive cells were identical as the donor hepatocytes, positively stained for albumin, and palely stained for PAS. The GFP and albumin expression deteriorated in time-dependent manner until 72 h after transplantation. Furthermore, the number of PAS-positive cells and the strength of PAS staining gradually decreased over time.

To assess any time-dependent changes in the transplanted cells across the entire transplanted site, we measured the GFP expression across the entire transplantation site of live rats with IVIS (Figure 2J–L). Within 8 h after transplantation, the GFP expression had reduced to 10% of the initially measured activity. During the next 8 h, the expression reduced to 10%. By 24 h after transplantation, the GFP expression was indistinguishable from the intestinal autofluorescence around the transplanted sites. Reverse transcription-PCR showed that the mRNA levels of CYP2E1 and CYP3A1 at 24 and 72 h after transplantation were significantly lower than those before transplantation (Figure 2M–P). mRNA levels of albumin at 8, 24, and 72 h after transplantation were significantly lower than those before transplantation. Although there was no significant difference at 8, 24, and 72 h after transplantation, mRNA levels of G6Pase rapidly decreased over time. These results indicated that the hepatic function of transplanted liver cells had also rapidly deteriorated.

Serum albumin measurement of NARs

To evaluate the function of transplanted hepatocytes, we transplanted normal hepatocytes into the NARs. Just after transplantation, the rat albumin level markedly increased in the transplanted NARs' serum and peaked at 16 h after transplantation (Figure 2Q). Thus, the transplanted cells produced albumin in a time-dependent manner until 16 h after transplantation. However, the NARs' serum albumin levels subsequently decreased ($p < 0.05$). This result also indicated the rapid deterioration of viable transplanted liver cells.

Remnant liver evaluation

H/E staining showed severe damage to the remnant liver structure. In the control group of 90% hepatectomy, diffuse hepatocyte vacuolar degeneration of hepatocytes was observed in the remnant liver at 24 h after hepatectomy, whereas the hepatocytes and liver structure of the remnant liver were maintained in the transplantation group (Figure 3A,B). The ATP levels of the remnant liver tissues were significantly lower in the control group than in the transplantation group ($p < 0.05$) (Figure 3C).

The number of PCNA-positive cells in the remnant liver 8 h after 90% hepatectomy was much higher in the transplantation group ($p < 0.05$) (Figure 3D–F) than in the control group.

Removal study of the transplanted cells

The serum ammonia level and histological findings of remnant livers were compared with and without removal of the transplanted cells (Figure 3G) identified on IVIS

(Figure 3H). There was no significant difference in the serum ammonia level between groups, indicating that the remnant liver are responsible for maintaining low ammonia levels >8 h after transplantation ($p = 0.5659$) (Figure 3I). In addition, histological findings showed a protective effect of the remnant liver, which occurred due to cell transplantation and was maintained despite the removal of transplanted cells 8 h after transplantation (Figure 3J,K). These results indicated that the existence of transplanted cells within 8 h after transplantation was enough to protect the remnant liver from severe damage due to massive hepatectomy. Furthermore, these findings suggested that intra-mesenteric transplanted liver cells play essential roles during the early period after massive hepatectomy by protecting the remnant liver from liver damage via a remote effect such as secreting cytokines.

Detection of the paracrine factor for protecting the remnant liver

To investigate factors working to protect the remnant liver, we performed a comprehensive analysis of cytokines and growth factors in the portal vein that flowed from the transplanted site to the remnant liver. No specific factor was significantly elevated in the transplantation group (Figure 4).

The role of hepatocytes and non-parenchymal cells in liver cell transplantation

To investigate whether hepatocytes or NPCs played a more significant role in protecting remnant livers and promoting hepatic regeneration, liver cells harvested from the donor liver tissue were divided into hepatocytes and NPCs, and each was transplanted.

H/E staining showed that severe liver damage occurred in the remnant liver of the

NPC transplanted group, which was the same as in the control group (non-transplanted group). However, the remnant liver of the HEP group was protected from severe liver damage due to massive hepatectomy ($n = 3/\text{group}$) (Figure 5A,B). ATP levels of the remnant liver tissue 24 h after 90% hepatectomy were significantly higher in the HEP group than in the NPC group (Figure 5C) ($p < 0.05$).

To assess the influence of transplanted hepatocytes and NPCs on improving survival, the Kaplan-Meier curves for 7-day survival after 90% hepatectomy were calculated and compared between the HEP and NPC groups (Figure 5D). The HEP group had a higher probability of survival than the NPC group with no significant difference (62.5% vs. 25.0%, $p = 0.2811$).

In addition, we compared the GFP expression at the transplantation sites between the liver cell and HEP groups to determine whether the NPCs supported transplanted hepatocytes. Although GFP expression decreased in both groups, the rate of decline of GFP expression was slightly lower in the total liver cell group than in the HEP group, with no significant difference ($p = 0.1128$) (Figure 5E).

Discussion

In present study, we demonstrated that liver cell transplantation into the mesentery recovered and prevented acute liver failure after massive hepatectomy.

Lee et al. reported that the small intestinal mesentery was a better transplantation site than the subcutaneous space in terms of engraftment of the donor hepatocytes with delivery devices (26). However, in the present study, histological findings of the transplantation site, quantitative PCR assays of the transplanted cells, and the IVIS study findings showed the rapid deterioration of viable transplanted liver cells, which

would be caused by the inappropriate oxygen delivery of minimal vascularization (30,40), or the attack of native granulocytes (33), or the transplantation in present study without the delivery devices as used in the Lee's study. Furthermore, albumin is a protein with an extremely long half-life in the body (about 3 wk). As long as the hepatocytes survive, the serum albumin levels do not decrease. The decreasing serum albumin levels of the NARs indicated the rapid deterioration of viable transplanted liver cells.

Nevertheless, the overall mortality due to acute liver failure significantly improved in the transplantation group. Thus, it is reasonable to suppose that remnant liver function was maintained and improved by transplantation. H/E staining of the remnant liver showed swelling and vacuolar degeneration of the hepatocytes in the control group same findings as previously described (20,34), whereas in the transplantation group, there were almost normal findings. In addition, the ATP levels in the remnant liver tissue were significantly improved in the transplantation group. Energy supply is critical for cell survival and proliferation. In particular, ATP is required to supply energy for maintaining cell function (8,14,35,44). Reportedly, ATP decreases markedly after partial hepatectomy (14,15,35,45), and decreased ATP production in small-for-size liver grafts has been associated with increased hepatocyte injury and a higher mortality (35,50,51).

Acute liver failure after massive hepatectomy is also characterized by suppressed hepatocyte proliferation (18,27). We observed a significantly higher PCNA index at 8 h after 90% hepatectomy in the transplantation group than in the control. These findings suggested that the liver cells transplanted in the mesentery prevent the remnant liver from liver damage due to massive hepatectomy.

Furthermore, the findings of the removal study on the transplanted cells indicated that

the existence of liver cells in the mesentery until 8 h after transplantation is sufficient for protecting the remnant liver. Therefore, intra-mesenteric transplanted liver cells play essential roles in the early period after massive hepatectomy by indirectly supporting the remnant liver.

The liver cells transplanted as donor graft in the present study contained hepatocytes and NPCs. Hepatocytes and NPCs secrete multiple cytokines, growth factors, and other molecules for example hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor- β 1 (TGF- β 1), interleukin -6 (IL-6) and some chemokines (5,37), and they promote liver regeneration in the injured liver (28,38,43). We hypothesized that any of the several cytokines produced by transplanted hepatocytes and the NPCs either protected the remnant livers or promoted remnant liver regeneration. In addition, it is supposed that these paracrine factors secreted by transplanted cells were absorbed through mesenteric microvessels into the portal vein (21). Concerning the portal blood flow draining into the liver, we speculated that the mesentery was a better site than the other extra-hepatic sites in terms of the effectiveness of the paracrine effect of donor liver cells. Although we comprehensively analyzed cytokines in portal vein blood including HGF and TGF- β 1 (data not shown), no specific factors significantly elevated in the transplantation group could be detected. However, the same protection effects represented by histological findings and an elevation in the ATP levels were observed in the hepatocyte transplantation group, as in the liver cell transplantation group previously described. Nevertheless, damage to the remnant liver was not prevented by isolated NPCs transplantation in the same manner as in non-transplantation. Therefore, the protective effect on the remnant liver was mainly because of hepatocytes among the liver cells transplanted in the mesentery. Although it

would be supposed that several non-significant factors have multiplier effect on the protection of remnant liver, further experiments that investigate factors released by transplanted hepatocytes are required. Additionally, the survival rate was slightly higher in the homogenate group than in the control group, without significant difference between these two groups. This result indicated that the liver cells homogenate, which may contain many factors for example albumin, glucose cytokines, growth factors etc., could not prevent liver failure in the liver cell transplantation group. Although the reason of this result is not yet determined, several possibilities could be raised as follows: those liver protective factors would be chemically destroyed in the process of cell homogenate, or their biological activity would be deteriorated in the mixture of cell homogenate.

Some reports have described that the liver NPCs support hepatocyte survival and maintain hepatic function (7,46,52). We investigated whether NPCs support hepatocytes at transplanted sites by monitoring GFP fluorescence, which represents viable transplanted cells (Figure 5E). Improvement in the GFP deterioration in the liver cell group compared to the hepatocyte group indicates that NPC tends to support engraftment of the transplanted cells.

In conclusion, the present study described the effect of the intra-mesenteric transplantation of liver cells for acute liver failure after massive hepatectomy, and the protective effect of transplanting liver cells to improve survival. Namely, although transplanted liver cells hardly worked as functional liver cells in the very early period after transplantation, they protect the remnant liver from severe damage after massive hepatectomy, which would have a major effect on survival via protection of the remnant liver. Although these findings may provide the opportunity to facilitate liver

cells from the resected liver tissue of autograft to prevent liver failure after massive hepatectomy, for example in cases of perihilar cholangiocellular carcinoma, several issues need resolved. For example, the contamination of cancer cells must be prevented. In addition, further experiments should be planned for diseased recipients with chronic liver failure, metabolic liver failure, and liver cirrhosis to utilize this technique in the clinical setting in the foreseeable future.

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Conflict of interest

The authors of this manuscript have no conflicts of interest to disclose as described by *Cell Transplantation*.

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Figure legends

Figure 1: Green fluorescent protein-positive liver cell transplantation into the mesentery after massive hepatectomy. (A) Under an ultra-violet transilluminator, the transplanted green fluorescent protein positive cells (encircled by a red arrow head) were confirmed in the mesentery. (B) Kaplan-Meier curves for survival in the control (dotted line), homogenate (broken line), and transplantation (full line) groups after 90% hepatectomy (n = 13/group). (C) The serum total bilirubin level (n = 3/group) and (D) serum ammonia level (n = 4/group) after 90% hepatectomy with (transplantation [Tx], full line) and without (control, dotted line) liver cell transplantation. P-values represent the result of repeated-measures analysis of variance. P-values from the post hoc tests comparing the Tx to the control group at each time point are represented by asterisks (C, D).** $p < 0.01$, *** $p < 0.001$

Figure 2: Viability of the transplanted liver cells after intra-mesenteric transplantation. Histological (A–C), immunohistological (D–F), and periodic acid-Schiff (PAS) analyses (G–I) of the transplanted cells in the mesentery. Specimens were obtained at 8 (A, D, G), 24 (B, E, H), and 72 h posttransplantation (C, F, I). Green fluorescence represents green fluorescent protein (GFP), and red fluorescence indicates rat albumin. Blue fluorescence indicates 4', 6-diamidino-2-phenylindole staining. The scale bar represents 50 μm . (J–L) GFP expressions of the transplanted site of live rats. (J, K) Viable transplanted cells, represented by the GFP signal intensity, are detected by the in vivo imaging system as high (red) to low (blue). (L) The signal counts of GFP after transplantation (n = 3).

(M–P) Quantitative polymerase chain reaction analysis for hepatocyte-specific messenger (m)-RNA of transplanted cells in the mesentery. The mRNA of the transplanted cells in the mesentery at 8, 24, and 72 h after transplantation (n = 3/each time point) compared to that before transplantation, using a set at a value of 1 (n = 3).

(Q) Functional analysis of transplanted hepatocytes in the liver cell transplantation model. Serum albumin levels of the Nagase analbuminemic rats in the control (control, dotted line) and transplantation (Tx, full line) groups after liver cell transplantation (Tx: n = 3, control: n = 4). P-values in the figure represent the results of the repeated-measures analysis of variance. P-values from the post hoc tests comparing the Tx and control groups at each time point are indicated by asterisks. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. DAPI, 4', 6-diamidino-2-phenylindole; H&E, hematoxylin and eosin.

Figure 3: Damage to the remnant liver tissue after massive hepatectomy.

(A, B) Hematoxylin and eosin (H/E) staining of the remnant liver sections (n = 5/group).

(C) Adenosine-5'-triphosphate (ATP) assay (n = 3/group) of the remnant liver at 24 h after 90% hepatectomy with (Tx) and without (control) liver cell transplantation.

(D, E) Representative photographs of the rat livers showing the proliferating cell nuclear antigen (PCNA) expression and (F) the PCNA index (n = 3/group) at 8 h after 90% hepatectomy with (Tx) and without (control) liver cell transplantation.

(G) Experimental protocol for the removal study of transplanted cells. At 8 h after transplantation, the transplanted cells were removed. At 24 h after transplantation, the

serum ammonia level and remnant liver tissues were evaluated with (remove) and without (Tx) removing the transplanted liver cells. (H) In vivo image showing green fluorescent protein of the transplanted liver cells before and after removing the transplanted cells. It was confirmed that the transplanted cells were removed. (I) Serum ammonia measurement (remove: $n = 3$, Tx: $n = 4$). (J, K) H/E staining of the remnant liver tissues in the remove and Tx groups. * $p < 0.05$. The scale bar represents 50 μm .

Figure 4: Proteome levels of the cytokines, growth factors, and other proteins in the serum of portal vein blood. Data at 4 h after transplantation in the transplantation (Tx) and control groups ($n = 3/\text{group}$). CINC, cytokine-induced neutrophil chemoattractant; CNTF, ciliary neurotrophic factor; GMCSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LIX, lipopolysaccharide-induced CXC chemokine; MCP, monocyte chemoattractant protein; MIP, macrophage-inflammatory protein; NFG, nerve growth factor; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Figure 5: Intra-mesenteric cell transplantation of isolated hepatocytes and non-parenchymal cells for massive hepatectomy. (A, B) Hematoxylin and eosin staining of the remnant liver sections at 24 h after 90% hepatectomy in the hepatocyte (HEP) (A) and non-parenchymal cell (NPC) (B) groups. The scale bar represents 50 μm ($n = 3/\text{group}$). (C) The adenosine-5'-triphosphate measurement ($n = 3/\text{group}$). * $p < 0.05$.

(D) Kaplan-Meier curves for 7-day survival in the HEP group (dashed line) and NPC group (dotted line) (n = 8). (E) Green fluorescent protein expressions of the transplanted site in the liver cell group (n = 4) and the HEP group (n = 3). Tx, transplantation.

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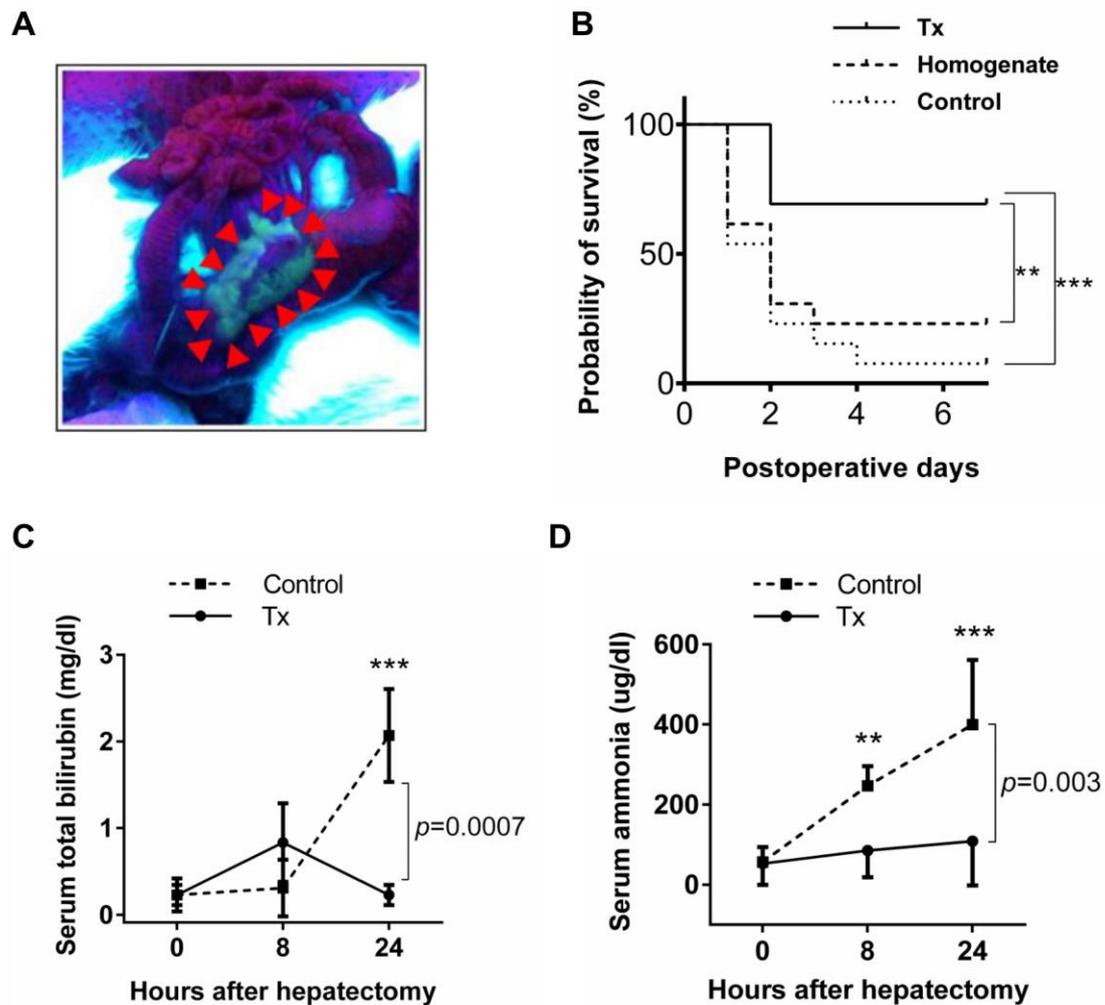


Figure 1

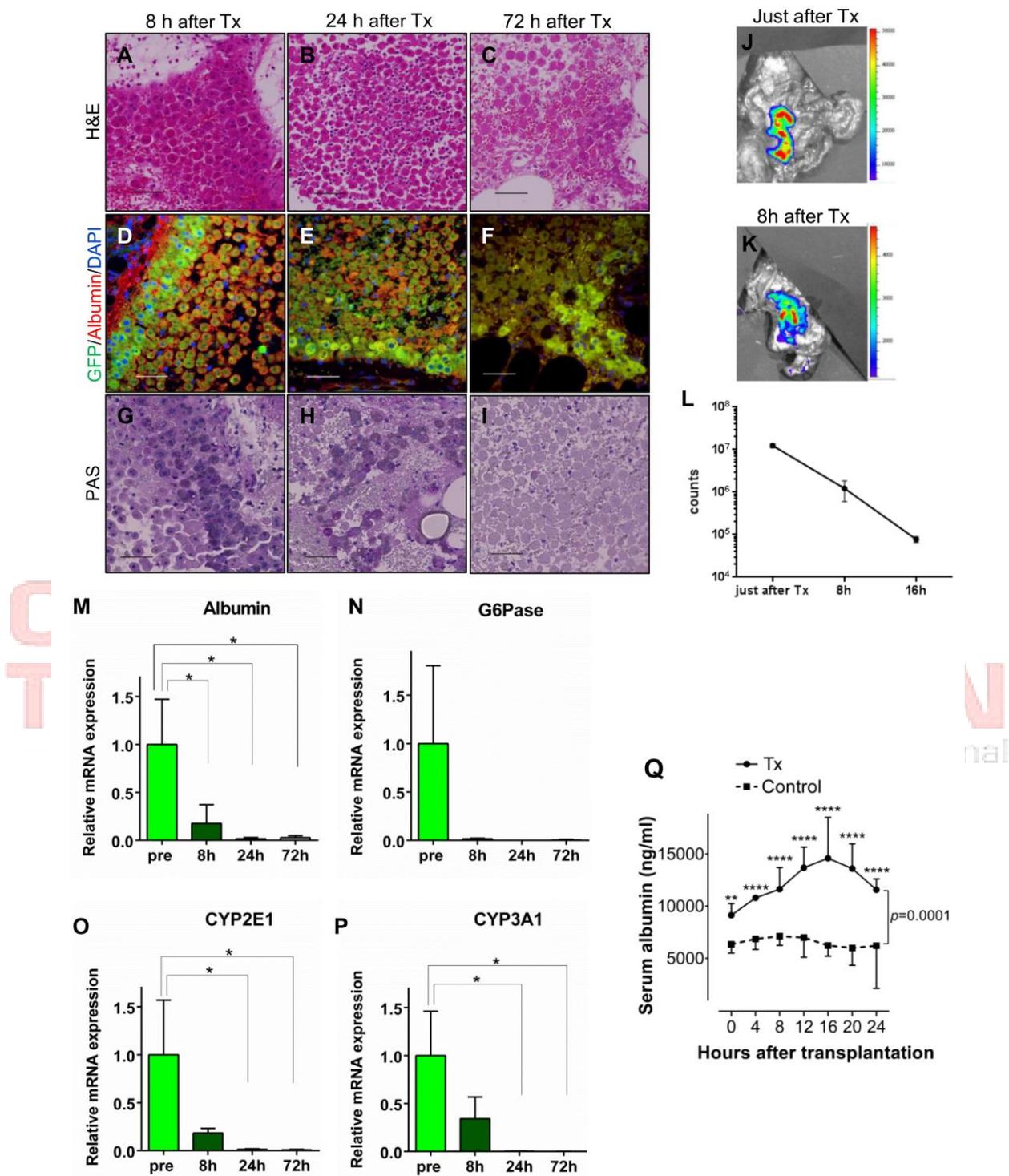


Figure 2

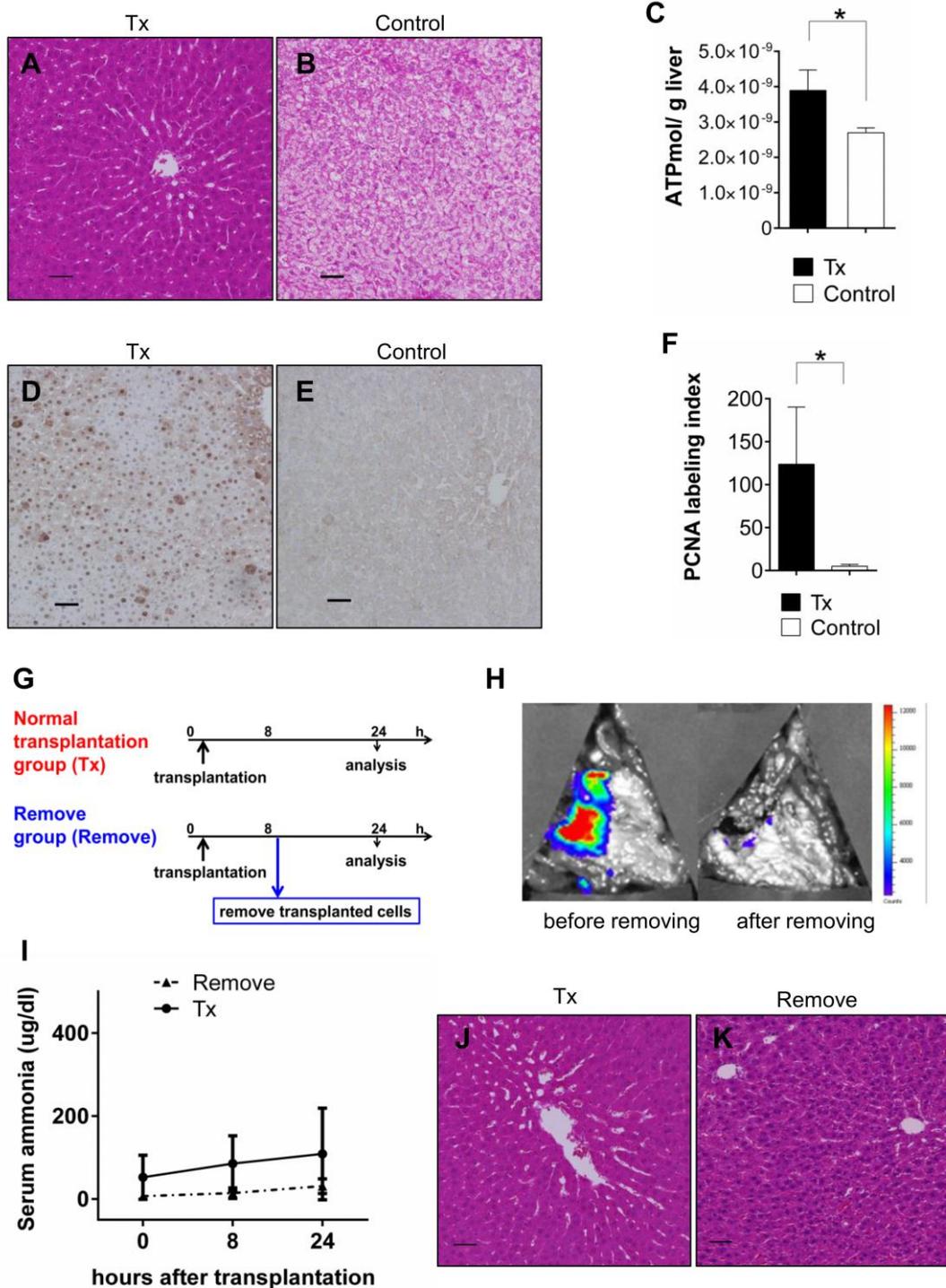


Figure 3

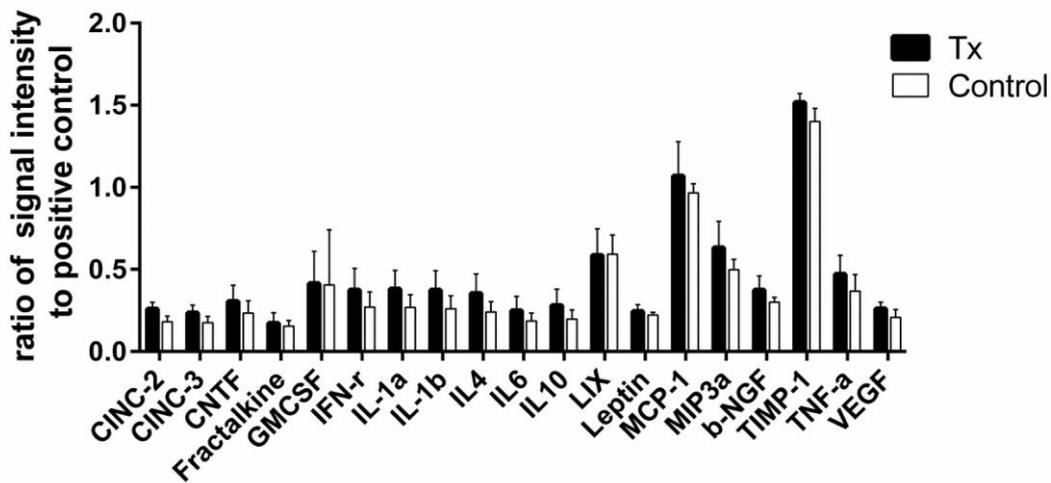


Figure 4

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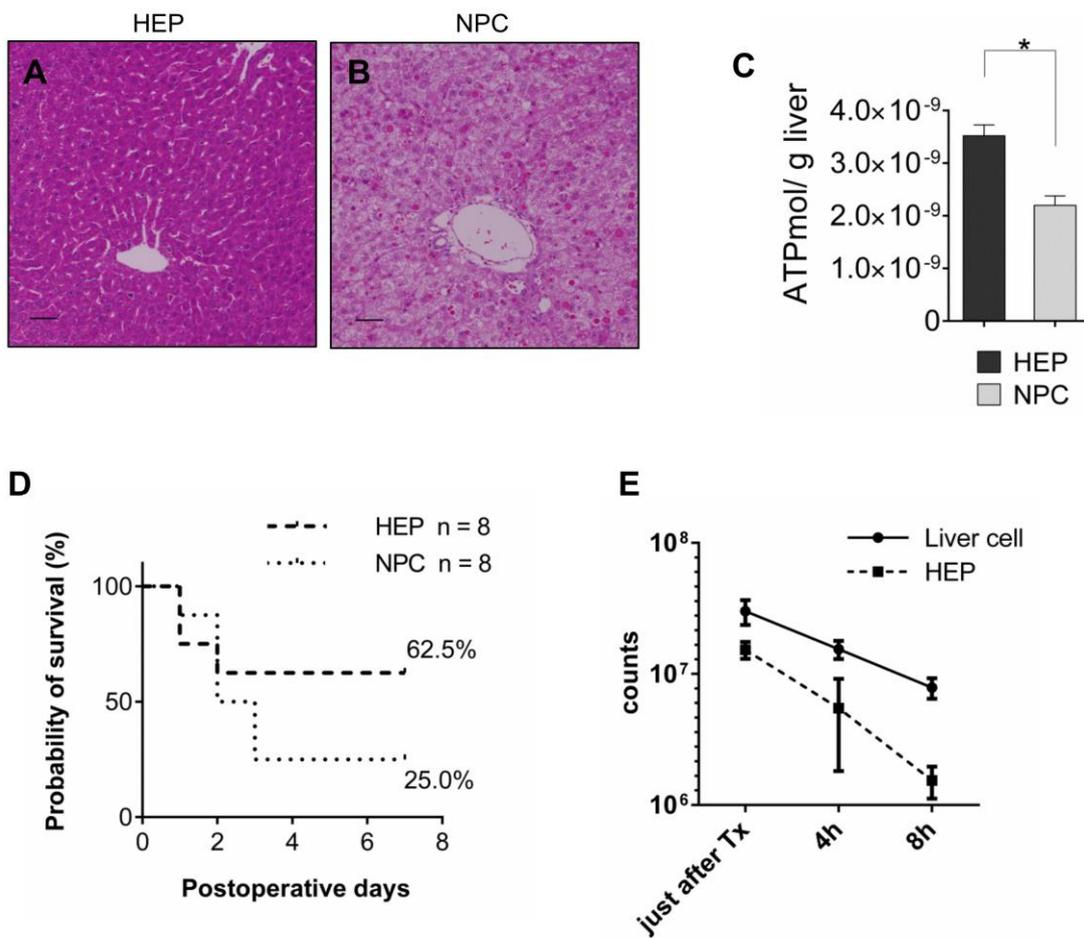


Figure 5