Cellularized Biosynthetic Microhydrogel Polymers for Intravascular Liver Tissue Regeneration Therapy

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Introduction: The liver is the natural microenvironment for hepatocytes transplantation but unfortunately engraftment efficiency is low. Cell-laden microhydrogels made of fibrinogen attached to poly(ethylene glycol) (PEG)-diacrylate side chains, were used as a cell carrier, for intravascular transplantation. This approach may reduce shear stress and immediate immunological pressure after intravascular transplantation and provide bio-matrix for environmental support.

Aims: *In vitro* assessment of HuH-7 viability and function after polymerization within PEGylated fibrinogen-hydrogel. *In vivo* assessment of intraportal transplantation of cell-laden microhydrogels with rat adult parenchymal cells.

Methods: (1) *In vitro* assessment of HuH-7 cell viability and function, after cell-laden hydrogel (hydrogel volume 30 μ L) fabrication, by propidium iodide (PI)/fluorescein diacetate (FDA), and MTT assays, albumin concentration and CYP1A activity. (2) Fabrication of cell-laden microhydrogels and their intraportal transplantation. Engraftment efficiency *in vivo* was evaluated by real-time qPCR of Y chromosome (SRY gene) and histology.

Results: The viability of cells in hydrogels in culture was comparable to viability of not embedded cells during the first 48 h. However, the viability of cells in hydrogels was reduced after 72 h compared with not embedded cells. Activity of CYP1A in hydrogel was comparable to that of not embedded cells (4.33 ± 1 pmole/ μ g DNA/4 h vs. 5.13 ± 1 pmole/ μ g DNA/4 h, respectively). Albumin concentration increased at day 3 in hydrogels to 1.4 ± 0.6 μ g/ 10^4 /24 h and was greater to that of free cells, 0.3 ± 0.1 μ g/ 10^4 /24 h. Cell-laden microhydrogels at a size of 150–150–600 μ m (6×10^6 cells/rat) showed better engraftment efficiency at 21 days post-transplantation, compared with isolated cell transplantation ($54.6\% \pm 5\%$ vs. $1.8\% \pm 1.2\%$, $p < 0.001$). Conclusions: The *in vitro* HuH-7 viability and function after polymerization in PEGylated fibrinogen hydrogel was comparable to cells without the hydrogel. Long-term survival and engraftment efficiency of intravascular transplanted adult hepatocytes is much better in within cell-laden microhydrogels compared with isolated cells. The overall efficiency of the procedure needs to be improved.

Introduction

CURRENTLY, liver transplantation is the most practical treatment for liver failure. However, the need for an alternative therapy is becoming urgent, because the number of donor livers that are available for transplantation is shrinking, due to the fatty liver epidemic, and the increasing rate of chronic liver disease due to hepatitis C.¹ Despite the fact that hepatocyte transplantation is using a similar donor pool, it is still a promising treatment for liver failure because the risk and cost are lower than those of liver transplantation.^{2–4}

Moreover, its efficacy has been demonstrated in patients with acute liver failure and children with inherited or congenital liver disease.² Nevertheless, hepatocyte transplantation is associated with several problems that need to be overcome before it can be used as a substitute therapy. New technologies directing to create other cell sources, like stem cells, and technology for cell collection and cryopreservation are in process but so far did not give significant results.^{5,6} Improving cell engraftment is a must for using adult hepatocytes.⁴ Adult hepatocytes, on the one hand have very limited potential to proliferate, but on the other hand have an immediate ability to

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function given the right environment, and thus they may help reverse acute hepatic failure quickly.^{7,8}

Despite the difficult intravascular access and the failing liver itself, it is still an important organ for cell repopulation, due to the fact that this is the natural environment for the hepatocytes to develop. Transplanted cells in the liver have an immediate access to bile flow and get portal vein blood supply.⁹ This understanding holds at least, as long as the vasculature is not interrupted or damaged during acute liver failure.^{10–12} Based on previous observations, it is likely that the main loci in the liver that allow cell engraftment after cells are transplanted, is the portal venule and not the hepatic sinusoids. The evidence comes from the observation that transplanted cells appear soon after transplantation, grouped very close to the portal tract and remain around the portal tracts for the long run.^{13–16} This perceptive may explain the massive loss of hepatocytes when they are injected into the portal vasculature, because once they enter the hepatic sinusoids they are swept away in the blood stream to the pulmonary and systemic circulation, just to get lost.¹⁷ Other cells can be destroyed by macrophages while getting stacked in the terminal portal venules.¹⁸

Fibrinogen-based hydrogels are cross-linked polymeric networks with a wide range of physical properties that are made of fibrinogen and whose water content is high.^{19,20} Hydrogels can be engineered into stable three-dimensional (3D) constructs that are cell compatible, resemble the cellular microenvironment, and are capable of regulating cell transport and cell–cell interactions in a tissue. The fibrinogen component forms a biological matrix for cell interaction and attachment, which can be gradually degraded by natural enzymes. An additional advantage is the provision of the scaffold, which provides temporary physical and immunological protection to the cells.²¹

We posited that incorporation of hepatocytes in a 3D cell-compatible hydrogel could be used to successfully deliver hepatocytes for hepatic engrafting following their injection into the portal vasculature. We further hypothesized, that the successful delivery of hepatocytes is due to trapping of the construct in the intrahepatic portal radicles, thereby preventing the hepatocytes from entering the hepatic sinusoids and from being swept away in the blood flow. In this report, we inform on the results of an investigation in which we compared the hepatic engraftment of cell-laden microhydrogels and isolated adult hepatocytes in rats following their injection into the portal vasculature.

Materials and Methods

Reagents

All chemicals were purchased from Sigma unless otherwise indicated. The antibody against BrdU was from Labvision, anti-Ki67 was from Abcam, and anti-HepPar-1 was from Dako. The secondary antibody was Histofine® Simple Stain Rat MAX PO (Nechirei, Inc.).

Preparation of a cell-laden polymer hydrogel construct

Poly(ethylene glycol)(PEG)-diacrylate was prepared from linear PEG-OH (10 kDa) and conjugated with bovine fibrinogen to produce liquid PEG-fibrinogen according to a previously described protocol.²² The cell-laden hydrogel

construct was fabricated by gentle mixing of this liquid PEG-fibrinogen with cells (HuH7 or adult hepatocytes, as described herein) and polymerization of the mixture. To allow polymerization the mixture was supplemented with 0.1% (w/v) Irgacure-2959 photoinitiator (Ciba-Specialty-Chemicals) and exposed to UV-light (UV 365 nm, 10–20 mW/cm², 5 min) to form cell-laden hydrogel (Fig. 1A). Cell-laden microhydrogels for *in vivo* study were fabricated from cell-laden hydrogel construct as described further.

In vitro studies using the HuH-7 cell-laden hydrogel constructs

The initial studies on cell viability and function in the cell-laden hydrogel were done *in vitro* using HuH-7 cells,²³ maintained at 37°C in a humidified 5% CO₂ atmosphere, in DMEM under standard conditions. The cell-laden hydrogels (3 × 10⁴ cells per 30 µL hydrogel) were cultured in a 24-well plate (one construct/well). Not embedded cells were seeded at 3 × 10⁴ cells/well.

Cell-laden hydrogel morphology was examined after 0, 24, 48, and 72 h of culture using staining with fluorescein diacetate (FDA). Cell-laden hydrogels were fixed in 10% neutral buffered formalin (NBF) and were smeared onto glass slides for hematoxylin and eosin (H&E) staining.

HuH-7 cell proliferation, viability, and function

Cell proliferation. Bromodeoxyuridine (BrdU) (1 mM) was added to cell-laden hydrogel in culture for 2 h, the hydrogel was embedded in O.C.T. compound (Tissue-Tek®; Sakura Finetek) and snap-frozen in liquid nitrogen. Frozen sections were prepared and immunostained with anti-BrdU antibody.

Cell viability. Cell viability of cell-laden hydrogels and of not embedded cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the FDA/propidium iodide (FDA/PI) live/dead assays.²⁴

The MTT assay. After incubation with MTT (4 h at 37°C), the cell-laden hydrogel was dissolved in 500 µL of a collagenase Type IA solution (0.6 mg/mL in PBS), and lysed in DMSO. The cultured isolated cells were lysed in DMSO as well and the viability was estimated as described previously.²⁴ The resulting viability was expressed as a ratio to not embedded cells under the same culture conditions or as the absolute cell number, determined from a standard curve where cell dilutions were plotted against the absorbance.

The FDA/PI assay. After staining the cells with FDA (2 µg/mL)/PI (2 µg/mL), their viability was determined under a fluorescent inverted microscope (Axiovert135; Zeiss) to which a charge coupled device camera (Roper Scientific) was attached for capturing the images. Since, the cells in the deep layers of the 30 µL hydrogels suffers from low oxygen supply and therefore from low viability at the third day of culture, here, we estimated cell viability only in outer layer (200 µm). The number of live and dead cells in the images was counted in ten nonoverlapping high-power optical fields using ImageJ (v1.43) software (U.S. National Institutes of Health). The resulting viability was expressed as percentage of live cells from total cell number

FIG. 1. Cell-laden hydrogel in culture. **(A)** PEG-fibrinogen plug. **(B)** Fluorescein diacetate (FDA)/propidium iodide (PI) (live/dead) staining of HuH-7 cells in plug (7.5 mg/mL fibrinogen) after a 24-h incubation ($\times 20$). **(C)** Viability of HuH-7 cells plugs of varying fibrinogen concentrations (6.5, 7.5, and 8.5 mg/mL) after 24 h of incubation. The resulting viability was expressed as the ratio between the viability in the hydrogel and the viability of isolated cells under the same conditions. Phase-contrast microscopy showing **(D)** HuH-7 cells in plugs after 24 h ($\times 10$) and **(E)** spheroid formation after 72 h of incubation ($\times 20$). FDA/PI staining of HuH-7 cells in plugs after **(F)** 24 h ($\times 10$) and **(G)** 72 h of incubation ($\times 20$), green live cells (wide arrows) and red dead cells (thin arrow). The dotted line represents the border of outer viable layer. **(H)** H&E staining and **(I)** BrdU staining of spheroids after 72 h incubation of HuH-7 cells in plugs ($\times 20$). The black arrow indicates DNA in spheroids. $*p < 0.05$. H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea

Specific assays of liver cell function

Cytochrome P450 (CYP)1A activity. It was tested using a previously described protocol.²⁵ Resorufin fluorescence was detected at 535-nm excitation and 581-nm emission in a FLUOstar Fluorescence microplate reader (BMG labtech). CYP1A activity was measured by comparing the fluorescence of each sample using a standard curve of resorufin.

Albumin secretion assay. The cells and the cell-laden hydrogels were cultured in serum-free and phenol-free DMEM, the medium in each well was collected daily and albumin concentration was determined by enzyme-linked

immunosorbent assay (ELISA). Albumin secretion was expressed as $\mu\text{g}/10^4$ live cells/24 h.

In vivo studies

Animals. Lewis rats (180–230 g) (Harlan Laboratories) were used for the isolation of adult hepatocytes (males) and as recipients for cell transplantation (females). The rats were kept at a controlled temperature under a 12-h light–dark cycle, and were fed a standard rat chow and had access to water *ad libitum*. The study was reviewed and approved by the Technion's Animal Care and Use Committee and the animals were kept and the experiments were performed in

strict accordance with committee's criteria for the care and use of laboratory animals. All surgical procedures were done under general anesthesia using either an isoflurane-oxygen mixture or an intramuscular injection of a mixture of ketamine (90 mg/kg)/xylazine (10 mg/kg).

Preparation of cell-laden microhydrogels for intravenous transplantations

The male's hepatocytes were obtained by perfusing the livers of the rats *in situ* with collagenase H (Roche) via the portal vein according to previously described protocols.^{26,27}

The viability of the hepatocytes was determined by the trypan blue dye exclusion test (at least 85% viability). After polymerization the cell-laden hydrogels were sliced (150 μ m slice width) at three different angles into cell-laden microhydrogels using a McIlwainTMTissue Chopper (Mickle Laboratory Engineering Co.) in the presence of 200 μ L DMEM. The average size of each cell-laden microhydrogel was 150 $\times$ 150 $\times$ 600 μ m and about 15,000 cell-laden microhydrogels were produced from 250 μ L cell hydrogel construct (about 15% loss). The viability of cells during the preparation of the cell-laden microhydrogels was measured after each step using the FDA/PI assay. The efficiency of cell-laden microhydrogel fabrication was determined from the ratio of the number of cells in microhydrogels and the number of cells that had detached from the cell-laden microhydrogels. To facilitate the count of cells in cell-laden microhydrogels, PEGilated fibrinogen was conjugated to fluorescent dye, rhodamin.

In vivo direct transplantation of HuH-7 cell-laden hydrogel into the liver, spleen, and subcutis

Once anesthetized, the liver was exposed through a 2-cm midline abdominal incision. Hydrogel constructs were transplanted directly into the liver and the spleen parenchyma (0.5×10^6 cells/0.4 mL), and the subcutis (0.25×10^6 cells/0.25 mL) of the rats. The rats were treated daily with cyclosporin (10 mg/kg) after the surgery, and were sacrificed 24, 48, or 72 h after transplantation. Samples of the hydrogel construct remnants and the adjacent tissues were harvested and stained with FDA/PI (to see whether there were any live cells in the remnants) or were fixed in 10% NBF before their embedding in paraffin for H&E staining.

In vivo experiments with hepatocyte-laden microhydrogels

Female Lewis rats (180–230 g) (Harlan Laboratories) were recipients of the cell-laden microhydrogels or isolated (free) hepatocytes. A partial (34%) hepatectomy (PHP) was performed prior to the transplantation.²⁸ The rats were treated daily with solvasol (50 mg/kg, s.q.) and buprenorphine (0.03 mg/kg, s.q.) as required.

To direct the transplanted cells to the right and caudate lobes of the liver, the portal vein was clamped proximal to its entry into the median and lateral lobes of the liver. Hepatocytes (15×10^3 cell-laden microhydrogels that contained 6×10^6 cells or isolated cells) were then infused intraportally using a 23G needle. Immediately after completion of the surgery, all rats were subcutaneously injected with 4 mL of a 5% dextrose solution. The rats were sacrificed 0, 24, 48,

168 h, 14, or 21 days after the surgery and the livers were harvested for further examination. Samples of the organs were then either fixed in 4% NBF and embedded in paraffin for histological examination or frozen in liquid nitrogen for DNA analysis.

Post-transplant studies to detect hydrogel or isolated transplanted cells in the liver lobes

Histology and immunostaining procedures. After H&E staining, the mitotic index for each liver was estimated by counting the number of mitotic figures (condensed chromosomes) in hepatocytes in ten nonoverlapping high-power optical fields ($\times 40$) per slide. The number of hepatocytes that were undergoing mitosis was expressed as a percentage of the number of mitotic hepatocytes per 100 hepatocytes.

Cells were immunostained for HepPar1, a hepatocyte marker, and cell proliferation was estimated by Ki67 immunostaining. For immunohistostaining, 4-mm thick paraffin sections were stained using anti-Ki67 (Abcam) and anti-HepPar1 (Dako) as primary antibodies and Histofine[®] Simple Stain Rat MAX PO (Nechirei, Inc.) as a secondary antibody. The sections were examined under a light microscope.

Real-time qPCR of SRY gene. DNA was purified from the low liver lobes using the Wizard SV Genomic kit (Promega). qPCR was done using specific primers for the rat SRY gene and the rat AmelX as the reference gene (Table 1) in a thermal cycler Rotor-Gene 6000 (Corbett Robotics). The relative expression of the SRY gene in the host livers were calculated from the *SRY/AmelX* amplification signals. Regression curves for both genes were obtained from serial dilutions (0–100%) of male rat cells. The reaction efficiency was found to be 90–110% and was determined using these regression curves, where slope was between -3.1 and -3.6 .

Statistical analysis

A computerized software package (Instat 3+, Reading University, UK) was used to analyze the data. The study parameters of each group were compared using nonparametric statistics (the Kruskal–Wallis test, followed by the Mann–Whitney U test). Data are presented as the mean $\pm$ standard error of the mean (SEM) and statistical significance was set at 5%. The results of the *in vitro* experiments are the mean of four repetitions from

TABLE 1. PRIMERS AND CONDITIONS USED FOR QUANTITATIVE REAL-TIME-POLYMERASE CHAIN REACTION

Primers	The cycling conditions
<i>SRY</i>	95°C 15 min 40 cycles
F 5-AGCTGGGATATCAGTGGAAA-3	95°C 10 s 53°C 15 s
R 5-TGCAGCAGGTTGTACAGTTT-3	95°C 20 min
<i>AmelX</i>	95°C 15 min 40cycles:
F 5-TGCCTGTGCCAAATACACAT-3	95°C 10 s 56°C 15 s
R 5-AGAGAGGCTGCTGTTCAAGC-3	95°C 20 min

four different experiments. Four to five rats were used for each *in vivo* experimental group at each time point.

Results

In vitro studies of HuH-7 cell-laden hydrogel

Cell viability in the hydrogels of varying fibrinogen concentrations. Because cell viability in a hydrogel construct depends on the construct's composition,²⁹ we selected fibrinogen concentration of 7.5 mg/mL for the cell construct since its resultant cell viability was similar to that of the not embedded HuH-7 cells ($84\% \pm 6\%$) (Fig. 1A–C). When the fibrinogen concentration was 6.5 mg/mL, the structure of the construct was loose in that it did not retain the cells whose viability was almost the same as that of the not embedded HuH-7 cells (Fig. 1C).

Morphology of cells in hydrogel. HuH-7 cells (3×10^4 cells) were assembled into $30 \mu\text{L}$ cell-laden hydrogel and then maintained in DMEM. Multicellular spheroids (cell aggregates, $21 \pm 3 \mu\text{m}$ ϕ) could be already seen after 24 h and their diameters progressively increased over the next 72 h up to $66 \pm 5 \mu\text{m}$ (Fig. 1D–G). A typical spheroid is shown by H&E-stained constructs and increased proliferation of spheroid is demonstrated by BrdU staining (Fig. 1H, I).

Cell viability *in vitro*. Twenty-four hours after cell-laden hydrogel preparation, the cell viability in constructs was not different from that of not embedded cells, as measured by the MTT assay (Fig. 2A). However, proliferation of the HuH-7 cells in the hydrogel was nonsignificantly inhibited at 48 h, and the number of not embedded cells was almost threefold higher ($p < 0.001$) than those in the cell-laden hydrogel after 72 h. When the cell-laden hydrogel was stained with FDA/PI, live cells were predominantly seen in the periphery of the constructs and dead cells were predominantly seen in their centers (Fig. 1G). The viability of the cells in the constructs by the FDA/PI method was $80\% \pm 13\%$ after 24, 48, and 72 h culture (Fig. 1F, G).

The rate of detached HuH-7 cells from the constructs was 0–2% after 24 h, 0–10% after 48 h, and 0–15% after 72 h.

Cell function. The CYP1A activity of cell-laden hydrogels was comparable to that of not embedded cells. Although the CYP1A activity of all cultures was the highest after 48 h of culture, the activity of cell-laden hydrogels was significantly lower ($p < 0.05$) than that of the not embedded cells (8.58 ± 1.98 vs. 15.38 ± 0.57 pmole resorufin/ μg DNA/4 h) (Fig. 2B). There was no significant difference in albumin secretion between cell-laden hydrogels and not embedded cells at any time point (Fig. 2C). Although albumin production by the cell-laden hydrogels was greater than that of the not embedded cells, this increase was not statistically significant.

Viability of HuH-7 cell-laden hydrogels directly injected into tissues. HuH-7 cell-laden hydrogels were directly transplanted into the liver (three rats) (Fig. 3A), spleen (three rats) (Fig. 3B), and subcutis (three rats). The rats were sacrificed 24, 48, or 72 h later. Remnants of the cell-laden hydrogels were found at all transplantation sites. H&E stained sections revealed degeneration of the hydrogels and a normal microarchitecture of the tissue that surrounded

FIG. 2. Comparison of viability and function of HuH-7 cells in hydrogel and as free (isolated) cells. (A) Cell viability as determined by MTT assay. (B) Activity of CYP1A as determined by ethoxoresorufin O-deethylation assay. (C) Albumin synthesis determined by ELISA. The results are the average of four repetitions from four different experiments. The results are presented as mean $\pm$ standard error of the mean (SEM). The study parameters of each group were compared using nonparametric statistics (the Kruskal–Wallis test, followed by the Mann–Whitney U test). * $p < 0.05$.

the construct (Fig. 3C, D). Moreover, mitotic figures were shown in transplanted constructs. (Fig. 3C, D) Examination of the FDA/PI-stained sections of the remnants of the cell-laden hydrogels revealed the presence of live cells inside the constructs. By 72 h most of these live cells were in the hydrogel's periphery (Fig. 3E, F). There was a minimal inflammatory reaction around the injected cell-laden hydrogel due to the inert property of the hydrogel and the nondirect contact of the cells with the recipient tissue.³⁰

In vivo: Transplantation of adult hepatocyte-laden microhydrogels into the portal vein

Cell viability. Cell-laden microhydrogel constructs ($150 \times 150 \times 600 \mu\text{m}$) containing adult male hepatocytes (6×10^6 adult rat hepatocytes in about 15×10^3 microhydrogels) were transplanted by the intraportal route into female rats after 34% partial hepatectomy. The preparation of the cell-laden microhydrogels included three steps, (1) incorporation into the hydrogel construct, (2) slicing the cell-laden hydrogels to

FIG. 3. Cell-laden hydrogels transplanted directly into tissues, 72 h after the transplantation. Remnants of the hydrogel in the (A) liver and (B) spleen. Inset: Section showing hydrogel remnant in the spleen. Sections of (C) liver and (D) spleen hydrogel remnants, (H&E, $\times 40$), (Hy = hydrogel). Arrows point to mitotic nuclei. Hydrogel remnant in subcutis (E) H&E staining and (F) FDA/PI (live/dead) staining, ($\times 20$). Color images available online at www.liebertpub.com/tea

make microhydrogels, and (3) injection through 23G needle. The viability of the cells was tested after each step and was significantly reduced after the first step, from 90.0% to 70–75%, ($p < 0.05$). In contrast, cell viability was not influenced by slicing the cell-laden hydrogel constructs into cell-laden microhydrogels or by the passage of the cell-laden microhydrogels through a 23G needle (data not shown). The overall efficiency of cell-laden microhydrogel assembly (cell percentage within cell-laden microhydrogels vs. outside cells) was 60%.

H&E staining and immunohistochemistry after intravascular transplantation

Examination of the H&E-stained sections of the recipient liver lobes revealed the presence of clusters of viable he-

patocytes in the portal vein radicles of all rats after transplantation of isolated cells and cell-laden microhydrogels, immediately after completion of surgery and 24 h later. The hepatocytes that were transplanted as free cells disappeared from portal vein radicles by 72 h. However, in cell-laden microhydrogels, clusters of hepatocytes were still presented 72 h after their transplantation, but only single hepatocytes were observed 1 week after the surgery (Fig. 4A–C). Staining of these cells with the HepPar1 antibody confirmed that the cells in the hydrogel were hepatocytes (Fig. 4D). Twenty-four hours after their infusion, these hepatocytes in cell-laden microhydrogels did not proliferate, as no positive Ki67 staining was seen (data not shown). Ki67-positive endothelial cells were seen around and within the cell-laden microhydrogel, 72 h after transplantation (Fig. 4E). Seventy-two hours after cell-laden microhydrogels transplantation,

FIG. 4. Intravascular cell laden microhydrogels stained for H&E, HepPar-1 and Ki-67 after the intravascular transplantation. (A–D) show healthy hepatocytes in the hydrogel. (A) 24 h, (B) 72 h, and (C) 1 week after transplantation (H&E, $\times 40$, bar = 50 μm). (D) Hepatocyte staining with HepPar1 antibody, 24 h after transplantation ($\times 40$, bar = 50 μm). (E) Liver tissue staining with Ki-67 antibody, 72 h after transplantation ($\times 40$, bar = 50 μm), showing that proliferation occurs only in endothelial cells. Arrowheads indicate hepatocytes in portal radicles. The thin arrow indicates Ki67-labeled endothelial cells. Color images available online at www.liebertpub.com/tea

the mitotic indices of recipient livers were significantly higher than after transplantation of isolated cells ($2.1\% \pm 0.4\%$ vs. $0.6\% \pm 0.2\%$, $p < 0.05$) (Fig. 5).

Engraftment efficiency. The results of the qPCR for SRY gene revealed the presence of transplanted male cells in the transplanted liver lobes of the female rats up to 21 days after cell transplantation. Yet, the presence of transplanted hepatocytes (isolated or in cell-laden microhydrogels) declined extensively over time. The efficiency of transplantation was calculated as percentage of the number of hepatocytes present in the liver immediately after transplantation. While the number of hepatocytes after transplantation of isolated cells declined continuously, the number of hepatocytes in microhydrogels stabilized and was impressively higher at 21 days ($54.6\% \pm 5.3\%$ vs. $1.8\% \pm 1.2\%$, $p < 0.001$) (Fig. 6).

Discussion

In this study, we reported that cell-laden microhydrogels derived from a hepatocyte-laden hydrogel construct can be used to deliver viable adult hepatocytes into the liver by the intraportal route. Using this route of administration, we showed that the long-term efficiency of engraftment of hepatocytes within microhydrogels was substantially higher than that of isolated hepatocytes. We found that HuH-7 cells

FIG. 5. Hepatocyte mitosis in liver parenchyma following the transplantation of isolated cells or cell-laden microhydrogels. Cell mitosis was estimated by counting the number of mitotic figures (condensed chromosomes) in hepatocytes in ten nonoverlapping high-power optical fields ($\times 40$) per slide. The number of hepatocytes that were undergoing mitosis was expressed as a percentage of the number of mitotic hepatocytes per 100 hepatocytes. The results are presented as mean $\pm$ standard error of the mean (SEM). The study parameters of each group were compared using nonparametric statistics (the Kruskal–Wallis test, followed by the Mann–Whitney U test). * $p < 0.05$, $n = 4$ –5.

FIG. 6. The results of the qPCR for SRY gene showing the presence of transplanted male cells in the transplanted liver lobes of the female rats up to 21 days after cell transplantation. qPCR was done using specific primers for the rat SRY gene and the rat AmelX as the reference gene. Comparison was done between transplantation of isolated cells and cell-laden microhydrogels. The results were expressed as the percentage of transplanted cells determined immediately after transplantation. The results are presented as mean $\pm$ standard error of the mean (SEM). The study parameters of each group were compared using nonparametric statistics (the Kruskal–Wallis test, followed by the Mann–Whitney U test). * $p < 0.05$, $n = 4-5$.

retain their viability and function and even proliferate in a polymer hydrogel construct, and this finding prompted us to use a cell-laden microhydrogel construct for our cell transplant studies in partially hepatectomized rats. It has been previously reported that the efficiency of hepatocyte engraftment is low and short lived, because there is a continuous rapid loss of transplanted hepatocytes when isolated hepatocytes are infused.^{13,31–33} Also in human studies, the efficiency of cell transplantation is low, and this low efficiency is a source of concern as this is a major reason for the slow implementation of this method into clinical practice.³⁴ Though the immediate rate of cell delivery, in this study, was better with the isolated cell procedure, yet the number of engrafted cells expressed as percentage of the original 24 h cell engraftment by the polymer technique was significantly better over 3 weeks. Satisfactorily, hepatocytes could survive within the polymer construct in the blood stream for as long as 1 week from transplantation, proving the ability of the hydrogels to protect the cells from shear stress and other injuries on the long way to get engrafted in the liver lobule.³⁵ Although we have made up a tool for intravenous delivery of hepatocytes to the liver, it is still in its early stages of development. One of the problems that still need to be overcome is the low efficiency of cell-laden microhydrogels fabrication. Moreover, due to the complexity of the *in vivo* experiments, with overall low cell viability, we were not able to show the advantage of the microhydrogels in every experiment, yet it was very clear after 3 weeks.

Hydrogel polymer constructs have been reported to be good biological scaffolds when cells are injected directly into tissues.²¹ The liver in the setup of acute liver failure is

not the ideal target for direct injections due to coagulation disorders and parenchyma destruction; yet, the polymer material allow to inject semisolid hydrogel constructs and to complete polymerization in the tissue. This method may help to introduce cells into tissue and allow them to take the shape of the tissue, thus permitting the filling of cystic spaces and sites after tumor resection or ablation. The major drawback of this method is the distance of cells from oxygen and nutrient source supply limiting the ability of cells to survive, as also shown in our study.^{36,37} With the *in vivo* HuH-7 studies we showed that only the cells in the periphery of the cell-laden hydrogel could survive for more than 72 h.

In our vision, the vascular bed due to its restricted size would not be the only site for cell transplantation. Due to the importance of this site whereby cells are placed directly to their natural microenvironment we are suggesting an approach to overcome some of the difficulties seen in transplanting isolated cells. So the drawbacks include an overall inefficient cell polymer preparation and low volume of the liver vascular bed. The advantages include the ability to introduce higher amount of cells within polymers that are protected from shear stress and direct immunological reaction. In this technique we prevent the rapid passage of transplanted cells that get lost through the liver sinusoids.

The same cell-laden microhydrogel can then be used for both intravascular and intratissue injection for the treatment of liver failure. This is in line with our concept that there will not be just one modality in the same patient for the treatment of hepatic failure. The patient with acute hepatic failure is a candidate for cell transplantation into different parts of the body whereby the liver is the preferred target for cell repopulation and organ regeneration and the spleen, directly connected to the liver is the second target.^{38,39}

The results of the *in vitro* and the *in vivo* experiments confirm that cells in a cell-laden hydrogel benefit from the advantages of the polymer.²¹ One of these benefits is the formation of spheroids, as their formation protects the cells from shear stress and immune-mediated recognition after their transplantation.²² Another advantage of a 3D hepatocyte's culture and spheroids is cell to cell contacts and their impact on cell activities, which are of importance for regeneration therapy. This 3D culture of hepatocytes is well documented in the literature and might explain the high level of albumin synthesis in the *in vitro* approach (Fig. 2C).⁴⁰ Concomitantly, essential nutrients and oxygen can enter the constructs to ensure cell viability and survival after their transplantation.

We speculate that once transplanted hepatocytes are in the sinusoids they will go all the way through, and those that remain and have the potential to engraft are those stacked in the portal radicles.^{31,35} This explains in our view, the very low transplantation efficiency in various experiments, on the one hand, but on the other hand target the portal vein radicles as the site needs to be manipulated rather than the sinusoidal bed. It is this site that probably allows redistribution of blood after cell transplantation to reduce portal hypertension and to allow transplanted cell aggregates to be included in the liver tissue.⁴¹ With this kind of polymer material we have the advantage to control the size, shape, cell number, and cell type for transplantation.²¹ The microhydrogels can protect groups of hepatocytes from shear

stress, immune damage, and prevent them from passing through the sinusoids. These particles are stacked in the portal radicle allowing eventually for a group of cells to engraft into the host liver.

In the present work we did not measure portal pressure but obviously we do know that particles stacked in the portal venules would cause portal hypertension. Interestingly the increased pressure is transient as was studied with albumin macroaggregates.⁴¹

Following the transplantation of the hepatocyte-laden microhydrogels, we found a high mitotic index in the livers 72 h after cell-laden microhydrogel transplantation compared with transplantation of isolated cells. We attribute this high mitotic index to occlusion of the portal vein, which in turn was a stimulus for liver regeneration. So, we believe that this can be another advantage of these micropolymer as this would be another way for priming the liver for cell^{4,34}

In this study we did not show yet better cell engraftment efficiency as we have lost hepatocytes in the cumbersome process of producing cell-laden microhydrogels. We therefore need to refine these methods in the future and to allow for better efficiency along the above concept.

Conclusion

In this study, we offer a novel approach for intravascular hepatocytes cell transplantation. This method is better than transplantation of isolated cells probably due to the fact that cells are not flushed through the sinusoids to get lost in the systemic circulation and are also protected from shear stress. Yet, the method is not efficient due to technical problems in the polymer cell construct preparation.

Disclosure Statement

No competing financial interests exist.

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