EFFECTIVENESS AND SAFETY OF HYDRODYNAMIC GENE DELIVERY IN ANIMALS WITH FIBROTIC LIVER

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Hydrodynamic gene delivery (HGD) has emerged as an effective and safe method for transfecting liver hepatocytes *in vivo*, and has potential for gene therapy of liver fibrosis. The objective of this study was to evaluate the effectiveness and safety of HGD using CCl_4 induced fibrotic liver in rats as a model. I demonstrated that there is a progressive reduction of efficiency of HGD in rats with increasing severity of liver fibrosis. Using a reporter plasmid containing luciferase gene, we showed over 2,000-fold decrease in luciferase activity in the liver with advanced fibrosis compared to that of control animals. Reduction in reporter gene expression in fibrotic liver was correlated to lower copy number of plasmid DNA and less amount of luciferase mRNA in the liver. Microscopy analysis revealed significant accumulation of collagen fibers in the boundary of liver lobules and thickened hepatic sinusoidal endothelium. The morphological changes in fibrotic liver are associated with restriction of flow-through across the liver of DNA solution hydrodynamically injected and are responsible for the reduced gene delivery efficiency of the hydrodynamic procedure. Results from electrocardiogram and serum biochemistry show no difference between the control and fibrotic animals undergone HGD. These results suggest that the HGD is a safe method for gene transfer in animals with liver fibrosis but the effectiveness of gene delivery decreases with increase of severity of fibrosis. Future work should focus on adjustment of injection parameters (DNA dose, injection volume, injection speed) for optimal gene delivery to fibrotic liver.

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1.0 INTRODUCTION

Liver fibrosis, or cirrhosis, featured by excessive accumulation of extracellular collagen fibers are characteristics of chronic liver diseases that affect millions of people worldwide.[1-4] The accumulated matrix proteins distort the hepatic architecture and eventually result in liver failure if the fibrogenic process is not controlled properly. Anti-fibrotic gene therapy has been demonstrated to be helpful in controlling fibrogenesis,[2, 5-9] but the effective and safe gene delivery remains as a critical issue for therapeutic success.[3, 6] The prevailing viral and non-viral vectors for hepatic gene transfer are less than ideal due to concerns of low delivery efficiency of nonviral vectors, and rapid inactivation, immunogenicity, insertional mutagenesis, and high preparation cost of viral vectors.[10-12] These issues significantly limit the applicability of these methods for gene therapy of liver fibrosis. There is a significant need for a new method for intrahepatic gene delivery.

In recent years, hydrodynamic gene delivery (HGD) has emerged as an effective and safe method for intrahepatic gene delivery in rodents and large animals as well.[11, 13] HGD relies on a rapid intravascular injection of large volume of DNA solution into the tail vein of rodents or hepatic vein in large animals to achieve enhanced permeability of liver sinusoidal endothelium and plasma membrane of hepatocytes. Although seemingly harsh, the HGD is proven to be safe and has been widely employed for gene therapy studies (for recent reviews, see[10-11]). Results from repeated HGD into mice showed no difference in gene delivery efficiency, no immune response to plasmid DNA, and no inactivation of gene expression by preexisting immunity that is seen in viral vector-mediated gene delivery.[11, 13-14] The marked effectiveness, safety and simplicity present the HGD as a promising method for gene delivery to fibrotic liver.

Despite a large number of studies in the literature documenting the effectiveness and safety of HGD in healthy animals,[11] little is known about its effectiveness and safety in animals with liver fibrosis. In this study, we systematically evaluated the effect of the severity of liver fibrosis on

delivery efficiency and safety of HGD in fibrotic rats. We demonstrated that the HGD is less effective in fibrotic liver although being safe. Results from mechanistic studies suggest that the reduced efficiency is due to the enriched extracellular matrix that blocks the expansion of liver sinusoids. These results suggest that future efforts for improving HGD effectiveness in fibrotic liver be directed to increasing gene delivery efficiency by adjusting hydrodynamic parameters including plasmid concentration, injection volume, and injection speed.

2.0 MATERIALS AND METHODS

2.1 Plasmids, reagents and animals

The pCMV-Luc reporter plasmid was constructed by inserting firefly luciferase cDNA between the Hind III and Xba I restriction sites within the multiple cloning site of pcDNA3 backbone (Invitrogen, USA), and propagated in the *E. coli* DH5α. The plasmid was purified using the CsCl-ethidium bromide gradient centrifugation[15] and stored in TE buffer (10 mM Tris HCl, 1 mM ethylene diaminetetraacetic acid (EDTA), pH 8.0) at -20^{o}C until use. The quality and quantity of the purified plasmid DNA was evaluated by absorbency at 260 and 280 nm and gel electrophoresis on 1% agrose gel in Tris/Borate/EDTA buffer. CCl_4 and olive oil were purchased from Sigma-Aldrich. Male Sprague Dawley rats weighing 50-70 g were purchased from Charles River and used to establish liver fibrosis model.

2.2 Establishment of liver fibrosis

Rats with free access to standard chow and water were injected intraperitoneally with 0.2 ml/100 g sterile CCl_4 in a 1:1 ratio (v/v) with olive oil twice weekly for 2 weeks, followed by the same dosing interval with reduced dose at 0.1 ml/100 g for 2 to 6 weeks. Olive oil was used instead of CCl_4 for the treatment of control animals.

2.3 Examination of severity of fibrosis and HGD effectiveness in fibrotic liver

On the 6th day after the last CCl_4 injection, the control and CCl_4 treated rats were randomly selected, i.p. anesthetized using 2,2,2-tribromoethanol (TBE; concentration, 0.016 g/ml in 0.9% saline; dose, 1.25 ml/100g body weight), and injected from tail vein with 7.5% body weight of saline containing pCMV-Luc plasmid (10 μg/ml) within 7-10 sec (n=3-5), by using a 21-gauge winged infusion set (Terumo, Japan). Nine hours after injection, the liver was harvested and subjected to examination of fibrosis severity and HGD effectiveness. The fibrosis severity was examined by Masson's Trichrome and H&E staining. The right lateral lobe of each animal was removed 9 hrs after plasmid injection and immersed in 10% formalin for 1 week. Tissue

embedding, sectioning (5 μm), and Masson's Trichrome and H&E staining were performed by staff members in the Research Histology Lab of the University of Pittsburgh Department of Pathology. The stained sections were then observed and photographed using a regular light microscope. The average luciferase activity of left lateral lobe, left medial lobe and right lateral lobe was used to determine HGD effectiveness. For luciferase assay, around 200 mg of liver sample were collected and thoroughly homogenized using a tissue Tearor for 30 s at its maximum speed, and the tissue homogenates were centrifuged for 10 min at 13,000×g at 4°C. The supernatant was further diluted tenfold using HEPES buffer and 10 μl of supernatant was mixed with 100 μl of luciferase substrates, and the luciferase activity was measured in a luminometer (AutoLumant LB 953, EG&G, Salem, MA) for 10 s. Protein concentration of the supernatant was determined by using Coomassie Blue assay. Luciferase level was expressed as the relative light unit per mg of extracted proteins.

2.4 Determination of luciferase mRNA level and the copy number of plasmid in liver samples

For the determination of luciferase mRNA level, the liver total RNA was extracted using Trizol reagent (Invitrogen) and the reverse transcription was performed using SuperScript III first-strand cDNA synthesis kit (Invitrogen) with oligo$(dT)_{20}$ to initiate reverse transcription. The primers for the real-time PCR were cLuc-F: 5'-GCCTGAAGTCTCTGATTAAGT-3'; cLuc-R: 5'-ACACCTGCGTCGAAGT-3'. The quantities of luciferase mRNA transcripts per μg of total RNA in control and CCl_4-treated rats were derived from the standard curve established by q-PCR using serially diluted known amount of pCMV-Luc plasmid. For determination of plasmid copy number, liver DNA was extracted by using the DNeasy Blood and Tissue Kit (Qiagen). The primers for real-time PCR were derived from neomycin resistance gene in plasmid backbone as Neomycin-F: 5'-TGCTCCTGCCGAGAAAGTAT-3'; Neomycin-R: 5'-GCTCTTCGTCCAGATCATCC-3'. The copy number of plasmid DNA per μg of total DNA in control and CCl_4-treated rats were extrapolated from a standard curve. Real-time PCR experiments were performed using StepOnePlus systems (Applied Biosystems) with the following parameters: an initial denaturation at 95ºC for 10 min, followed by 40 cycles at 94ºC for 15 sec and 60ºC for 1 min. SyBrGreen (Applied Biosystems) was used to indicate target gene amplification and the specificity of amplification was examined by automated melting curve

analysis.

2.5 Measurement of IVC and PV pressure during hydrodynamic injection

The measurement of intravascular pressure was performed as described by Suda *et al.*[16] with modifications. An abdominal incision was made in TBE-anesthetized animals and the internal organs in the peritoneal cavity were exposed. A pressure detector was inserted into the catheter at the IVC or PV through a three-way connector and connected to a transducer for the real-time measurement of the pressure change during the hydrodynamic injection of saline.

2.6 Scanning and transmission electron microscopy

For both scanning and transmission microscopy (SEM and TEM), PBS containing 2.5% glutaraldehyde was hydrodynamically injected through the IVC into the anesthetized animals, and the liver was removed immediately after injection and immersed in the same fixative overnight for SEM, or immersed for 3 days for TEM. Small samples of the liver were processed for TEM as described by Stolz *et al.*[17] For SEM, sections from fixed liver samples were further washed with PBS and processed with osmium tetroxide and ethanol according to Suda *et al.*[16] The processed sections were mounted onto aluminum stubs, sputter-coated with 3.5 nm gold/palladium and viewed under a JEOL JSM-6330F scanning electron microscope, and photographed by using the interactive Quartz PCI image system.

2.7 Electrocardiogram and serum biochemistry assay

ECG on the CCl_4-treated rats was monitored using the BIOPAC 100C ECG detection system (Santa Barbara, CA) as described by Zhou *et al.*[14] The Monopol needle electrodes (EL452, 15 mm TP) were inserted subcutaneously into the chest area of an anesthetized rat. The ECG waves were recorded and processed using the Acqcknowledge software before, during, and after hydrodynamic injection. For serum biochemistry assay, serum samples were collected from the control and CCl_4-treated rats by clipping the tail before and 9 hr after hydrodynamic injection and subjected to the automated analysis using an IDEXX VetTest Chemistry Analyzer (Westbrook, ME).

2.8 Statistical methods

Differences were statistically evaluated by Student's t-test between two groups. A p-value of 0.05 is considered as statistically significant, and 0.01 as very significant.

3.0 RESULTS

3.1 Establishment of liver fibrosis in rats

Liver fibrosis was established by weekly intraperitoneal injection of CCl_4 according to a previously published procedure.[18] Figure 1 shows a time dependent, progressive accumulation of extracellular matrix proteins in the liver. In control animals treated with carrier solution (olive oil) for 8 weeks (Figure 1A), no collagen fibers are visible by Masson's trichrome staining compared to an enhanced level of collagen fibers (blue bands) at the edge of the liver lobules 4 weeks after CCl_4 treatment (Figure 1B). In the CCl_4 treated rats for 8 weeks, a network of fibrotic tissue resulting from extensive portal-portal bridging and occasional portal-central bridging are evident (Figure 1C). These results suggest that moderate and advanced liver fibrosis was successfully developed in CCl_4-treated rats. Figures 1D-1F are H&E staining of liver samples from control (Figure 1D), CCl_4 treated for 4 weeks (Figure 1E), or 8 weeks (Figure 1F), exhibiting no visible damage at cellular level, but with enriched matrix protein near the portal site of CCl_4 treated animal for 8 weeks (Figure 1F).

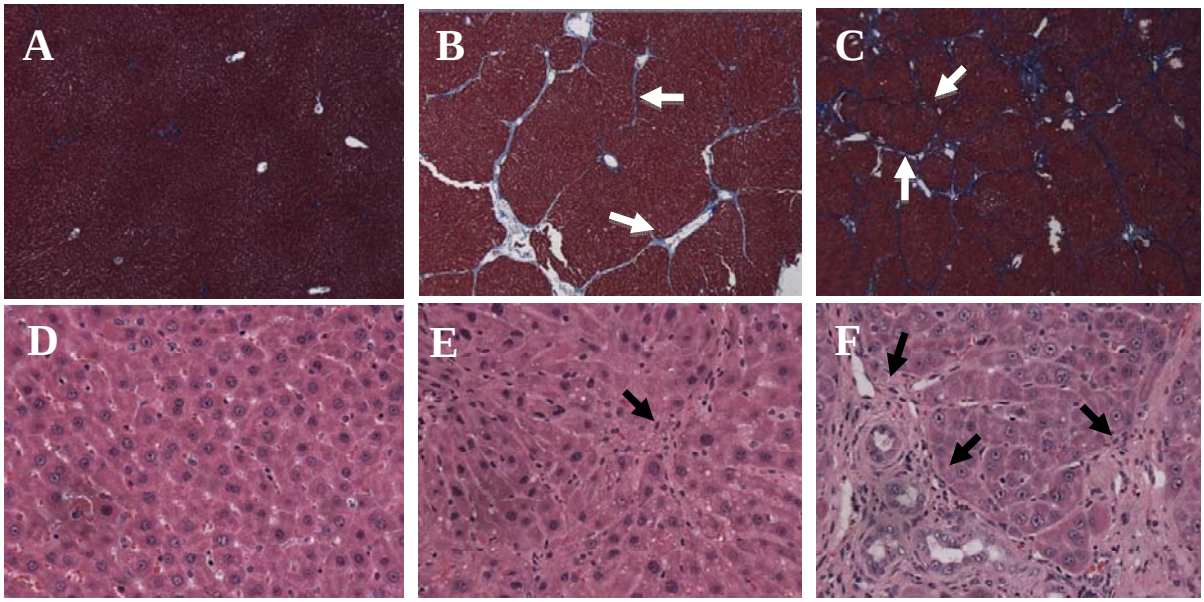

Figure 1. Histochemistry of the Liver. Liver sections from rats treated with either olive oil or CCl_4 were Masson's trichrome stained for collagen (A, B, C), or H&E (D, E, F) for cellular structure. A and D represent the structures of control animals treated with olive oil for 8 weeks. B and E represent the structures of animals treated with CCl_4 for 4 weeks. C and E represent structure of animals treated with CCl_4 for 8 weeks. White arrows point collagen distribution in the liver. Magnification, 25 x. Black arrows indicate monocyte infiltration. Magnification, x 400.

3.2 Effect of liver fibrosis on efficiency of hydrodynamic gene delivery

A standard HGD from the tail vein was performed using 7.5% body weight as injection volume and DNA concentration of 10 μg/ml. Animals were sacrificed 9 hrs after hydrodynamic injection of pCMV-Luc and the liver samples were collected. Figure 2A shows luciferase activity at 3.5 x 10^8 RLU/mg of proteins in olive oil treated control animals compared to 1.0 x 10^7 RLU/mg in animals treated with CCl_4 for 4 weeks. In the 8-week treated groups, the luciferase activity was 1.4 x 10^9 RLU/mg for control, and 6.0 x 10^5 for CCl_4-treated animals. The level of luciferase gene expression in CCl_4 treated animals is 35 folds lower than that of control animals in CCl_4 treated animals for 4 weeks, and 2,320 folds lower in animals treated with CCl_4 for 8 weeks.

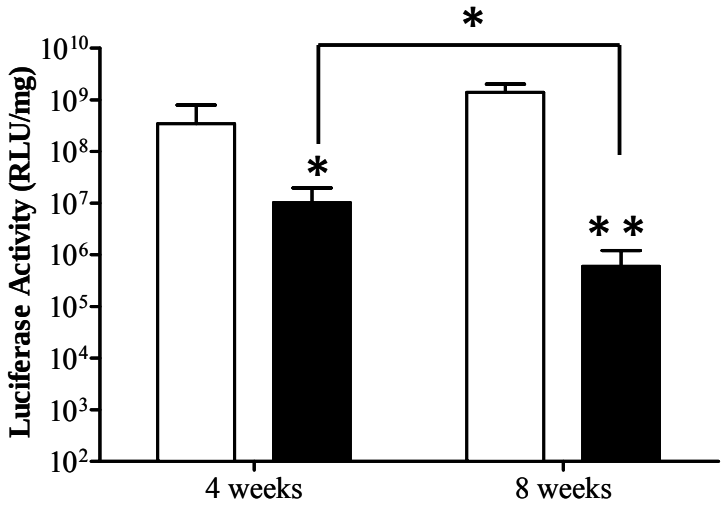

Figure 2. Level of Luciferase Gene Expression in Rat Liver. Animals were

hydrodynamically transfected with pCMV-Luc plasmid DNA (10 μg/ml) and sacrificed 9 hr later. Liver samples were collected and luciferase activity determined using luciferase assay. Open columns represent level of luciferase activity in liver of control animals treated with olive oil, and the solid columns represent that of animals treated with CCl_4. Standard bars represent SD of the average value from 3-5 animals. *, $p<0.05$; **, $p<0.01$.

To examine whether the reduction of luciferase gene expression in fibrotic liver is correlated to the amount of plasmid delivered to the liver cells, we performed quantitative PCR with liver samples collected from control and CCl_4 treated (8 weeks) animals 9 hr post HGD. Table 1 summarizes the copy number of pCMV-Luc plasmids in 1 μg of extracted DNA from the liver. There are approximately 1.84×10^6 copies/ μg of extracted DNA in control animal compared to 1.54×10^4 copies/μg of DNA in fibrotic liver, an approximately 120-fold reduction. In addition, real-time q-PCR was performed to examine whether the reduction in luciferase expression is correlated to the mRNA level. Compared to that of control liver, luciferase mRNA per μg of total RNA in CCl_4 treated rats (8 weeks) decreased by 572 folds. A rough calculation of transcription index (total mRNA divided by the copy number of pCMV-Luc plasmid) reveals 5-fold reduction in fibrotic liver, suggesting that in addition to a decrease in delivering plasmid DNA to cells in the fibrotic liver, the intracellular environment of fibrotic liver may also play a role in determining the overall level of reporter gene expression.

Table 1. **Relative Amount of Plasmid DNA and Luciferase mRNA in the Liver of Hydrodynamically Transfected Animals.***

Liver Sample	Number of pCMV-Luc plasmid in 1 μg of total DNA	Luciferase mRNA in 1 μg of total RNA	Transcription Index (mRNA/plasmid DNA)
Control (olive oil, 8 wks)	$1.84 \times 10^6 \pm 8.81 \times 10^5$	$9.63 \times 10^6 \pm 1.16 \times 10^6$	5.23
Fibrotic liver (CCl_4, 8 wks)	$1.54 \times 10^4 \pm 1.15 \times 10^4$	$1.68 \times 10^4 \pm 1.54 \times 10^4$	1.10

*Animals were hydrodynamically transfected with pCMV-Luc plasmid DNA (10 μg/ml) and sacrificed 9 hr later. Total DNA or RNA were extracted from the liver and used for determination of copy number of pCMV-Luc plasmids by q-PCR and for

estimation of total amount of luciferase mRNA by q-RT-PCR.

3.3 Effect of liver fibrosis on HGD-induced structural changes

Previous reports showed that HGD induces structural change in the liver, including enlargement of the endothelial fenestrae and stretching of liver lobules,[16, 19] which are believed to be the critical elements of HGD. To examine whether the structural impact of the procedure was weakened on fibrotic liver, we performed hydrodynamic injection of the fixative solution directly into the anesthetized rats with or without liver fibrosis. The liver samples were collected and subjected to scanning and transmission electron microscopy (SEM and TEM). Figure 3 shows sinusoidal structure of the liver from control animals receiving a slow infusion (Figure 3A) or hydrodynamic injection (Figure 3B), respectively. Enlarged fenestrae (Figure 3B) are readily visible in hydrodynamically injected control animal. However, in animals with liver fibrosis (Figures 3C and 3D), no obvious changes in sinusoids are observed with (Figure 3D) or without (Figure 3C) hydrodynamic injection. Compared to that of control animals (Figure 3A), liver sinusoids in fibrotic liver is significantly less fenestrated with no or smaller pores (Figure 3C).

Figure 3E is an image of TEM from the liver of a control animal infused slowly with the same volume of fixative solution. The thin layer of endothelium, the space of Disse, the microvilla of the hepatocytes, and the fenestrae are seen. Upon hydrodynamic injection, different structures were seen in the liver of control animal (Figure 3F). The sinusoidal endothelium became discontinuous due to enlargement of fenestrae (Figure 3F), and the space of Disse became expanded (Figure 3F). Vesicle-like structures are seen in the hepatocytes surrounding the sinusoids of control animal after hydrodynamic injection. In contrast, the space of Disse in fibrotic liver appears smaller (Figure 3G), and the sinusoids were not affected by the hydrodynamic injection in CCl_4-treated animals (Figures 3G, 3H), although moderately enlarged fenestrae could be occasionally observed (Figure 3H). The SEM and TEM data suggest that the hydrodynamic procedure that could result in efficient plasmid delivery in healthy liver was less capable of inducing necessary structural changes in fibrotic liver.

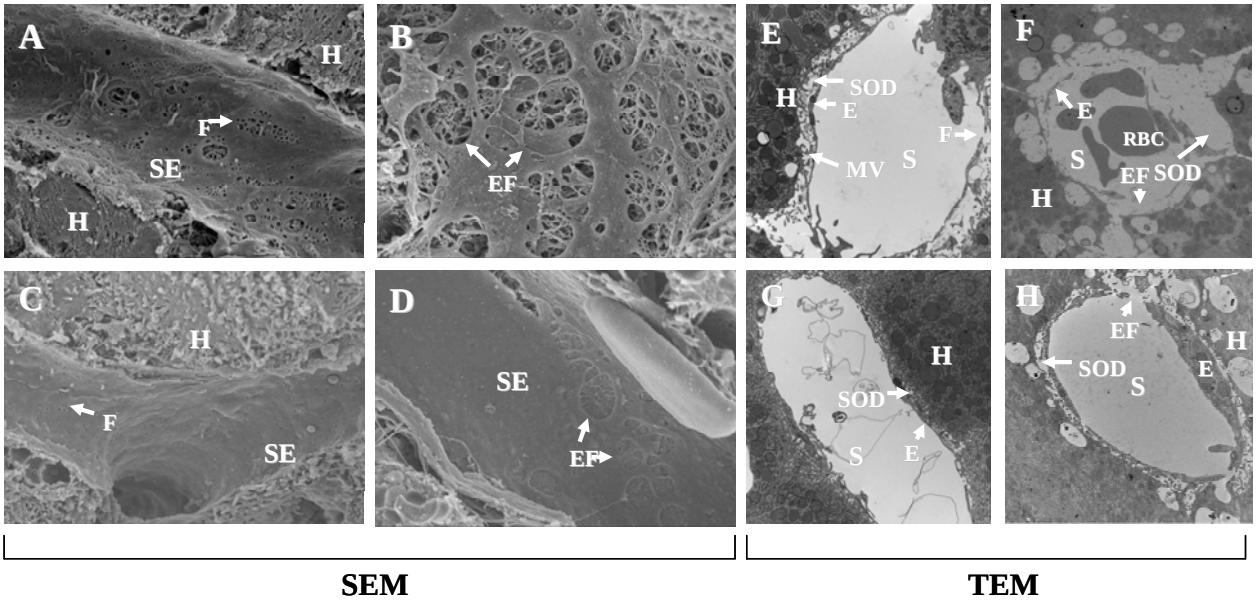

Figure 3. Electron Microscopic Images of the Liver. CCl_4 treated (8 weeks) animals were either slowly infused or hydrodynamically injected into the inferior vena cava with 2.5% glutaraldehyde in PBS in a volume equals to 10% body weight. Liver samples were collected, fixed and sectioned for SEM (A-D) and TEM (E-H) examination. A, C, E and F are representative structures of animals receiving slow infusion, and B, D, F, G are structures of hydrodynamically injected animals. H, hepatocyte; F, fenestrae; SE, sealed endothelium; EF, enlarged fenestrae; S, sinusoid; SOD, space of Disse; RBC, red blood cell; MV, microvilla. Magnification, x 10,000.

3.4 Hepatocytes were less permeabilized by hydrodynamic injection in CCl_4-treated liver.

HGD generates transient defects on hepatocyte membrane which was reflected by elevation of serum concentration of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).[11, 13-14] The release of liver specific enzymes from hepatocytes is an indicator for intracellular gene transfer into hepatocytes. Serum concentration of ALT and AST was determined 9 hrs after hydrodynamic injection. Figure 4 shows significant increase of ALT and AST induced by hydrodynamic injection in control animals, compared to very limited increase in CCl_4-treated animals, suggesting a reduced impact of hydrodynamic procedure on peameabilizing hepatocytes

in fibrotic liver.

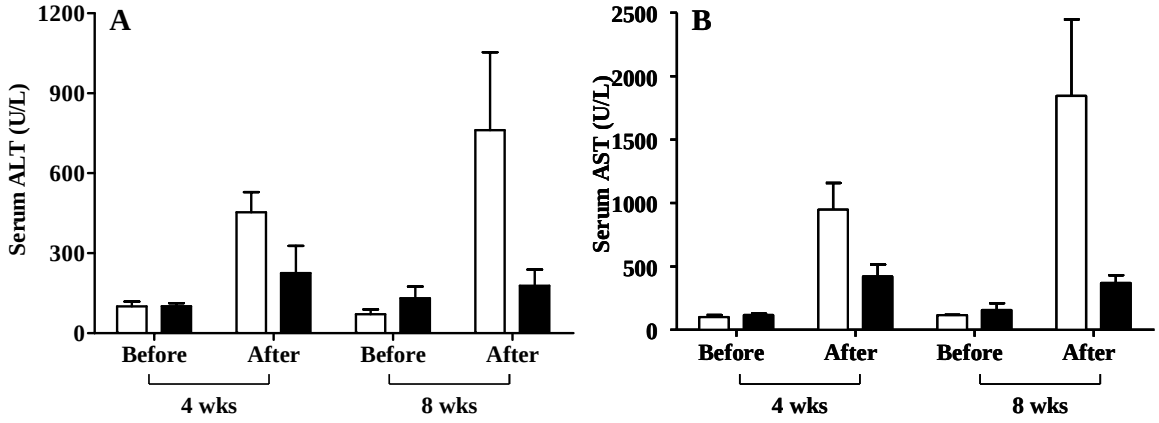

Figure 4. Effect of Hydrodynamic Injection on the Serum Concentration of ALT and AST. Serum samples from hydrodynamically treated animals were collected 9 hr post injection and ALT and AST concentration determined. A and B represent serum level of ALT and AST in olive oil treated control animals (open bar) or CCl_4 treated animals (solid bar), respectively.

3.5 Fibrotic liver is highly resistant to the flow-through of hydrodynamically injected solution across the liver.

Additional efforts were made to understand different response between the normal and fibrotic liver to HGD. Previous reports showed that an elevated vascular pressure across the entire liver is essential for HGD and the pressure generated by a tail vein injection is the same in inferior vena cava (IVC) and portal vein (PV).[16, 19] To examine whether the fibrotic liver has different pressure profile compared to control animals, I measured the vascular pressure in IVC and PV upon hydrodynamic injection to the tail vein. As expected, hydrodynamic injection induced moderate increase in IVC pressure (20 mmHg) in control liver which was the same as that of PV pressure in control animal (Figure 5), reflecting an unrestricted vasculature that allows pass-through of injected solution across the entire liver.[16] However, the IVC pressure in fibrotic liver upon hydrodynamic injection was around 400 mmHg and the PV pressure was not elevated to the same degree (~20 mmHg). These results suggest that the fibrotic liver is highly resistant to retrograde flow across the liver of the hydrodynamically injected solution.

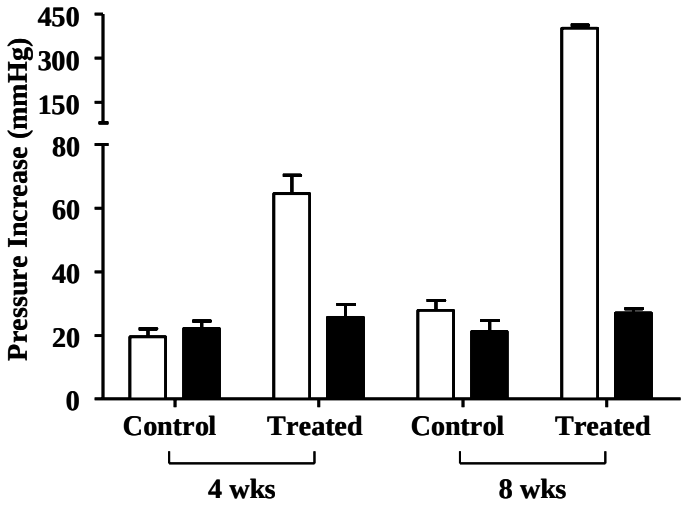

Figure 5. Effect of Hydrodynamic Injection on Vascular Pressure of the Inferior Vena Cava and Portal Vein. Pressure transducer was inserted into the inferior vena cava or portal vein before hydrodynamic injection was performed with needle insertion at the lower end of inferior vena cava. The peak pressure was recorded and plotted. Open bars represent pressure in inferior vena cava and solid bars represent the pressure in the portal vein. The value represent the average $\pm$ SD, n=3.

3.6 Hydrodynamic gene delivery is safe in animals with fibrotic liver.

The profound increase in IVC pressure during hydrodynamic injection in CCl_4-treated rats raised safety concern of the HGD in animals with liver fibrosis. Careful examination of all internal organs including the liver, kidney, heart, lung, and spleen was done for all animals used in the study and no liver rupture or organ damage was seen in any of the animals. No internal bleeding or other abnormality of the internal organs were noticed when the animals were sacrificed for liver harvesting. No obvious necrosis of the parenchyma cells were observed in the CCl_4-treated livers 9 hrs after hydrodynamic injection.

Electrocardiogram (ECG) was performed to examine whether the systemic hydrodynamic injection through tail vein would affect the heart function in animals with fibrotic liver. Compared to ECG before the injection, the heart rate in CCl_4-treated rats started to decrease immediately after the hydrodynamic injection (Figure 6). The P, Q, R, S, T waves reflecting the

atrium and ventricle activity showed irregularity a few minutes after HGD, presumably due to temporary inability to pump out the injected DNA solution from the IVC. Nevertheless, these waves returned to normal 8 min later (Figure 6). Overall, the cardiac disturbance by hydrodynamic injection in CCl_4-treated rats was not severer than that observed in healthy rats.[14]

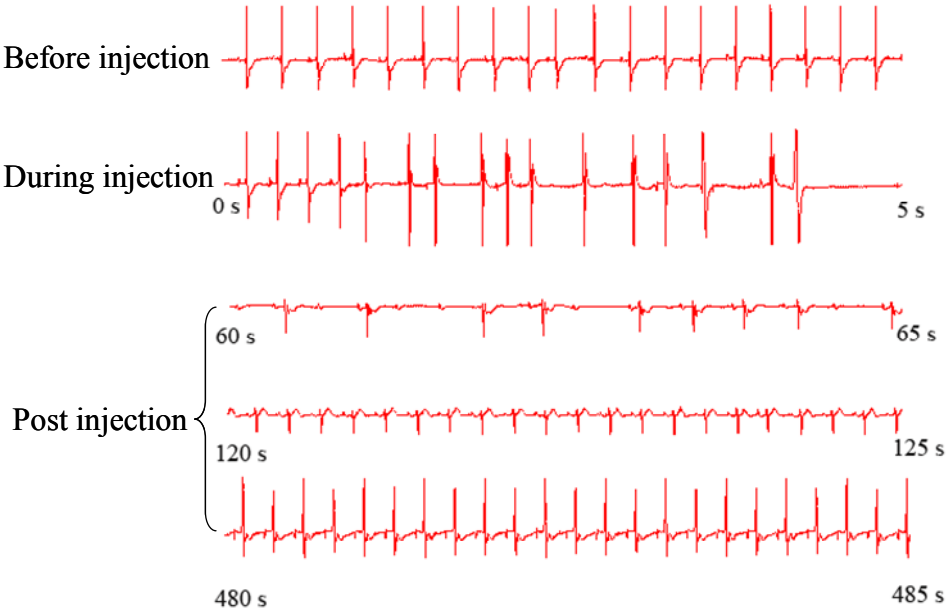

Figure 6. Impact of Hydrodynamic Injection on Cardiac Function of CCl_4-treated Rats. Electrocardiogram was recorded during hydrodynamic tail vein injection in an anesthetized rat which had been treated with CCl_4 for 8 weeks. The ECG spectra before, during and post hydrodynamic injection are shown in 5-sec segments.

The concentrations of serum components were measured before and 9 hrs after hydrodynamic injection, and the results were summarized in Table 2. With the exception of a slight elevation of ALT and AST post injection (Figure 4), the concentrations of other serum components are very similar before and after 9 hr after hydrodynamic injection. In addition, the concentrations of the serum components in CCl_4-treated rats were comparable to those in control animals, suggesting that the HGD procedure did not induce severer organ and blood disturbance in animals with liver fibrosis.

Table 2. Effect of Hydrodynamic Injection on Serum Biochemistry*

Serum Components	Serum Concentration							
	Control (olive oil, 4 wks)		Fibrotic (CCl_4 4 wks)		Control (olive oil, 8 wks)		Fibrotic (CCl_4 8 wks)	
	Before	After	Before	After	Before	After	Before	After
Total Protein (g/dL)	5.7±1.5	4.9±0.5	5.9±0.4	5.4±0.2	7.5±0.2	5.2±0.5	6.4±0.6	5.1±0.4
Albumin (g/dL)	3.7±0.7	3.0±0.3	3.8±0.2	3.3±0.0	4.4±0.2	3.3± 0.2	3.9±0.5	3.0±0.2
Globulin (g/dL)	2.0±0.8	2.1±0.1	2.1±0.2	2.1±0.2	3.1 ± 0.0	1.9 ± 0.3	2.5 ± 0.1	2.1 ± 0.2
Gamma-GT (U/L)	<3	<3	<3	<3	<3	<3	<3	<3
Alkaline Phosphatase (U/L)	267.0±36.8	226.7±86.4	253.7±22.3	240.3±14.5	133.5±21.9	169.7±31.6	217.7±122.5	275.5±20.5
Total Bilirubin (mg/dL)	0.15±0.07	0.10±0.00	0.10±0.00	0.10±0.00	0.20±0.10	0.10±0.06	0.30±0.35	0.27±0.06
BUN (mg/dL)	20.0±12.7	18.0±7.1	27.0±0.0	17.7±3.2	23.5±0.7	17.3±3.1	25.0±2.0	18.0±2.7
Creatinine (mg/dL)	0.50±0.00	0.65±0.07	0.80±0.28	0.57±0.06	0.55±0.21	0.57±0.15	0.73±0.06	0.60±0.00
Glucose (mg/dL)	231.0±77.8	127.5±54.5	173.5±10.6	147.7±40.5	121.0±0.0	186.5±46.0	117.7±32.7	138.0±10.2
Na^+ (mmol/L)	141.5±0.7	153.0±5.7	141.0±0.0	142.0±4.6	140.0±1.8	140.0±1.4	143.3±1.5	145.0±1.4
K^+ (mmol/L)	5.9±2.0	6.2±0.3	6.7±0.4	6.9±2.9	6.9±0.3	5.1±0.2	6.0±0.4	9.4±2.4
Cl^- (mmol/L)	103.0±2.8	106.0±7.1	101.0±0.0	102.7±2.3	97.0±1.6	105.0±0.0	100±1.7	106.5±0.7
Ca^{2+} (mmol/L)	9.5±0.0	9.7±0.1	9.5±0.2	9.5±2.1	10.1±0.7	9.3±0.7	10.1±0.4	10.9±0.8
PO_4^{3-} (mmol/L)	9.6±1.2	13.3±2.2	10.1±0.0	11.7±6.1	8.8±0.9	9.3±3.9	8.0±0.7	9.1±3.0
HCO_3^- (mmol/L)	24.0±1.4	14.5±2.1	24.5±0.7	20.0±6.1	21.0±0.0	26.0±0.0	21.7±4.0	25.0±5.3

*****Sera were obtained from rats before and 9hr after hydrodynamic injection of plasmid solution and subjected to automated analysis for the concentration of serum components. Abbreviations: Gamma-GT, gamma-glutamic aminotransferase; BUN, blood urea nitrogen; Na^+, sodium ion; K^+, potassium ion; Cl^-, chloride ion; Ca^{2+}, calcium ion; PO_4^{3-}, phosphate ion; HCO_3^-, bicarbonate ion.

4.0 DISCUSSION

The present work shows that HGD, although safe, is less effective in gene delivery to fibrotic liver as demonstrated by lower level of reporter gene expression (Figure 1), reduced copy number of plasmid DNA and lower amount of mRNA (Table 1). The microscopic studies suggest that the reduced efficiency of HGD in fibrotic liver is caused by accumulated collagen fibers at lobular boundary that limits the expansion of hepatic lobule (Figure 1) and permeabilization of plasma membrane of hepatocytes (Figure 4). Thickened endothelium and reduced liver fenestrae in highly fibrotic liver (CCl_4 treated for 8 weeks) (Figure 3) also play important role in preventing the expansion and enlargement of fenestrae that, otherwise, provides the access of plasmid DNA to space of Disse and the parenchyma cells.

Evidently, the effectiveness of HGD appears reversibly proportional to the severity of liver fibrosis (Figure 2). For animals treated with CCl_4 for 4 weeks, the reduction in luciferase gene expression was 35 about folds lower than that of control animals compared to greater than 2,000 folds of the fibrotic liver in advanced stage (CCl_4 treated for 8 weeks). While the progressive effect of the liver fibrosis on the efficiency of HGD suggests that it is more desirable to apply HGD as earlier as possible during fibrogenesis, these results also suggest that there are cells in fibrotic liver that are still hydrodynamically transfectable because some reporter gene expression was detected. Effort in identifying the unique nature of these cells or liver structure in favor of HGD in fibrotic liver may yield new information toward developing new strategies for improved efficiency of HGD. In addition, strategies that would reduce the level of collagen fibers in the liver would be helpful in achieving higher effectiveness of HGD.

The transcription index (total mRNA/copy number of plasmid) is an interesting parameter because it indicates efficacy of gene delivery and transcription in the liver. Our results (Table 1) indicate that the transcription index decreased about 5 folds in fibrotic liver, suggesting that the condition in normal liver must offer cells advantage for gene expression or cells in fibrotic liver

disadvantage for gene expression. Considering that fibrosis is a pathological condition of the liver and associated with severe inflammation, the lower transcription index seen in fibrotic liver could be the result of down regulation of reporter gene expression by higher concentration of proinflammatory factors such as IFN-γ,[20-21] which are over expressed in the injured liver as part of the defending and repairing mechanism.[22] A prudent strategy to enhance gene expression in fibrotic liver cells is to include into the plasmid of the elements that would avoid the inflammation-mediated down regulation, or/and the elements that would enhance gene expression by pathological factors.

Effectiveness of HGD to liver is dependent on the fenestrated vasculature and the elasticity of the liver. This is because the expansion and permeabilization of the blood vasculature and the plasma membrane of the hepatocytes are consequence of the procedure and essential for plasmid DNA to reach the space of Disse and enter the hepatocytes.[16, 19] Microscopy study shows (Figures 1 and 3) obvious morphological change of the sinusoids and lobular structure in the fibrotic liver with significant decrease of fenestrae in both density and diameter, and accumulation of protein fibers at the boundary of each lobule, making the sinusoids more resistant to hydrodynamic pressure and less elastic for expansion. Also, the morphological changes were associated with resistance to the flow-through of injected DNA solution across the liver (Figure 5) and a decrease in gene delivery efficiency. The fact that effective gene transfer to skeletal muscle has been achieved in pigs using a computer–controlled injection device[23] suggests that it may be possible to improve gene delivery efficiency to fibrotic liver by increasing the injection speed and injection volume. Alternatively, localized injection into the hepatic vein employing the technique of image-guided hydrodynamic gene delivery[24] could enhance gene delivery efficiency.

Despite the reduced effectiveness, the HGD procedure appeared to be safe in animals with fibrotic liver. No obvious cell death in the CCl_4-treated liver was observed after HGD (Figures 1D-1F) and the changes in ECG and serum composition after hydrodynamic injection were similar between control and CCl_4-treated animals. An acute overload of large volume of DNA solution in animals appears very transient and did not cause additional cardiac irregularity in fibrotic rats compared to that of control animals.

5.0 SUMMARY AND CONCLUSION

In summary, as evidenced by excessive accumulation of extracellular matrix and morphological change of the sinusoids and hepatic lobules, we have shown in this study that progressive liver fibrosis can be developed by weekly injection of CCl_4. The accumulation of protein fibers induced structural and morphological change of the liver and is directly responsible for reduced efficacy of HGD. As far as the application of HGD to gene therapy of liver fibrosis is concerned, it is more desirable to start the treatment as early as possible when the liver remains its normal structure. Alternatively, more research is needed to explore the possibility of either removing the protein fibers or employing higher hydrodynamic pressure to achieve higher gene delivery efficiency. In this respect, we believe that additional research could result in new information based on which a new and more effective method of gene delivery can be developed for gene therapy on a devastating disease that affects millions of people each year.

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