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BASIC RESEARCH

Hydrodynamics based transfection in normal and fibrotic rats

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Abstract

AIM: Hydrodynamics based transfection (HBT), the injection of a large volume of naked plasmid DNA in a short time is a relatively simple, efficient and safe method for *in vivo* transfection of liver cells. Though used for quite some time, the mechanism of gene transfection has not yet been elucidated.

METHODS: A luciferase encoding plasmid was injected using the hydrodynamics based procedure into normal and thioacetamide-induced fibrotic Sprague Dawley rats. Scanning and transmission electron microscopy images were taken. The consequence of a dual injection of Ringer solution and luciferase pDNA was followed. Halofuginone, an anti collagen type I inhibitor was used to reduce ECM load in fibrotic rats prior to the hydrodynamic injection.

RESULTS: Large endothelial gaps formed as soon as 10' following hydrodynamic injection; these gradually returned to normal 10 d post injection. Hydrodynamic administration of Ringer 10 or 30 m prior to moderate injection of plasmid did not result in efficient transfection suggesting that endothelial gaps by themselves are not sufficient for gene expression. Gene transfection following hydrodynamic injection in thioacetamide induced fibrotic rats was diminished coinciding with the level of fibrosis. Halofuginone, a specific collagen type I inhibitor, alleviated this effect.

CONCLUSION: The hydrodynamic pressure formed following HBT results in the formation of large endothelial gaps. These gaps, though important in the transfer of DNA molecules from the blood to the space of Disse are not enough to provide the appropriate conditions for hepatocyte transfection. Hydrodynamics based injection is applicable in fibrotic rats provided that ECM load is reduced.

Key words: Gene transfection; Fibrosis; *In vivo* transfection; Fibrotic; Hepatic; Endothelial lining; Endothelium; Sinusoidal; Fenestrae; Space of disse; Extracellular matrix

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INTRODUCTION

The ability to introduce foreign genes has paved the way for elucidating the physiological functions of genes and their therapeutic potential in the context of the entire organism[1,2]. A crucial yet unresolved issue in successfully implementing gene delivery as an effective procedure is the methodology by which genes can be introduced. In recent years naked DNA rather than synthetic vectors or viruses were applied to animal models. In doing so, various procedures were used including direct injection to liver vessels or bile duct[3-7].

The introduction of the hydrodynamics based transfection (HBT) by Liu *et al*[2] and Zhang *et al*[1] presented new opportunities in the field owing to its relatively straightforward manner, eliminating the need for surgical procedures. The technique has been widely used ever since, reaching beyond the goals of treating liver diseases. Gene suppression by RNAi or defining regulatory DNA sequences[8,9] are among the topics investigated using this approach. Two parameters influence more than any other the outcome of the hydrodynamic injection, the volume administered and the rate of injection. In the mouse model up to 10% of body weight is administered within 5 s. Under these harsh conditions the output of the heart fails to cope with the heavy load of fluid, leading to excessive blood flow into the hepatic vein circulation and consequent transfection of hepatocytes[10].

Attempts to elucidate the mechanism underlying the HBT have yielded conflicting reports. These reports

include receptor mediated endocytosis[11], non specific entry to hepatocytes through transient small pores caused by high pressure[12] or transient inversion of intrahepatic flow associated with massive endocytosis[10]. Regardless of the mechanism, it is obvious that the sinusoids and associated cells, i.e., sinusoidal endothelial cells, hepatic stellate cells and Kuppfer cells are influenced by the high pressure created by the procedure.

The current study follows the fate of the reporter gene luciferase in fibrotic rats undergoing HBT. Such animals demonstrated lower levels of transfection compared to healthy control rats, yet the outcome of the HBT could be improved by halofuginone, an anti collagen type I inhibitor. We also bring evidence demonstrating that the morphological alterations in the form of fused fenestrae, gaps, inflicted on the membrane of sinusoidal endothelial cells following HBT are either not involved or not sufficient to achieve transfection of liver cells.

MATERIALS AND METHODS

Plasmid

pGL3-Control vector (Promega, Madison, WI) encoding for the luciferase gene was purified using a commercial kit (Qiagen, Valencia, CA). The purity of the plasmid was checked by Nanodrop (Wilmington, DE) and agarose gel electrophoresis.

Hydrodynamic injection and thioacetamide administration

Sprague-Dawely rats (250-280g) were injected via the tail vein with luciferase encoding plasmid using a 22G venflon cannula in a volume equivalent to 5.25% of the body weight and a rate of 5-8 s. The amount of DNA administered throughout this study, 400 μg/injection, was deduced from a preliminary study in which various amounts of naked DNA were used. In all experiments the DNA was dissolved in Ringer solution (117 mmol/L NaCl, 2.5 mmol/L KCl, 2 mmol/L $CaCl_2$, 9.1mmol/L Hepes, 5 mmol/L D-glucose pH 7.4). Prior to injection rats were anesthetized using Ketamin HCl (90 mg/kg) and Acepromazine Maleate PB (2.5 mg/kg). The amount of Acepromazine Maleate PB was reduced to 1 mg/kg in fibrotic rats. Liver fibrosis was induced by injection of 0.2 mg/g thiocetamide (TAA) intraperitoneally twice weekly for the designated time. The level of fibrosis was determined using the Ishak scoring system[13] following staining with Sirius Red. Halofuginone (Collgard, Petach Tikva, Israel) was supplied in the food at a concentration of 5 ppm.

Histochemistry

Liver samples were fixed in 4% paraformaldehyde, dehydrated in gradual ethanol solutions and embedded in paraffin. Five μm thick sections were blocked for endogenous peroxidase and stained with Sirius Red[14].

Luciferase assay

Two hundred mg of liver tissue were homogenized by Polytron homogenizer (Kinematika, Lucerne, Switzerland) in 1ml Reporter lysis buffer (Promega Corp., Madison, WI). Luciferase activity was determined using a commercial assay kit (Bright-Glo Luciferase Assay System, Promega, Madison, WI) according to manufacturer's instructions. Luciferase was expressed as Relative Light Units per mg protein ± standard deviation.

Cell proliferation

BrdU labeling was used to determine cell proliferation. Briefly animals were intraperitoneally injected one and two hours before sacrifice with PBS containing 5-bromo-2-deoxyuridine (Sigma, St. Louis, MO) at a dose of 50 mg/kg body weight. Liver samples were fixed, dehydrated, embedded in paraffin and cut to 5 μm slices. Staining was performed using the BrdU kit (Zymed, South San Francisco, CA).

Electron microscopy

Liver tissue samples were prepared according to standard protocols[15]. Briefly, liver tissue was prefixed, cut to 1 mm^3 samples and stored in 1.5% glutaraldehyde in 0.12 mol/L cacodylate buffer at pH 6.9. Following glutaraldehyde fixation, tissue blocks were postfixed in 1% Osmium tetroxide. Dehydrated tissue blocks were dried with hexamethyldisilazane and subsequently broken in liquid nitrogen, mounted on stubs and sputter coated with a thin layer of 20 nm gold[16]. Scanning electron microscopy (SEM) samples were studied in a Philips XL-20 at 30 kV. Morphometric analysis was performed on randomly acquired digitized SEM images at magnifications × 5000 or × 20 000 as previously described[17]. The UTHSCSA Image Tool 2.0 software was used to trace the number and diameter of liver sinusoidal endothelial gaps. Gaps were discriminated from fenestrae based on their morphology and size as described[15,16,18]. Each experimental variable was repeated three times (3 animals) using 10 images in the periportal and pericentral zone (regions up to 100 μm in diameter) randomly selected and captured at both magnifications. All experiments were repeated three times and data were expressed as mean ± standard deviation of the mean.

Statistical analysis

Statistical analysis was performed with the Mann Whitney two-sided *U*-test for endothelial gap sizes and one way ANOVA for plasmid distribution in the liver lobes. The t-test was used for plasmid expression in the different experimental groups.

RESULTS

Luciferase gene distribution in rat liver lobes following HBT

Given the conflicting reports as to the distribution of naked DNA in the liver following HBT[19-21] we first addressed the above, using the pGL3-Control vector. Following HBT, random segments from each of the four liver lobes were thus picked, minced in Reporter lysis buffer (Promega, Madison, WI) supplemented with Complete Mini Protease Inhibitor (Roche Diagnostics, Mannheim, Germany) and tested for luciferase. Surprisingly, variations in luciferase expression were noted between segments of the same lobe. To avoid false interpretation in all further experiments, liver lobes were chopped into small pieces and a 200 mg representative sample was collected and

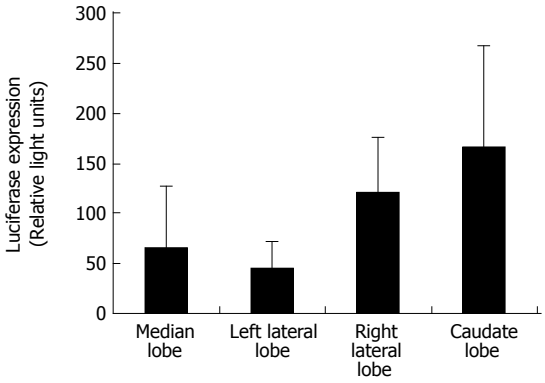

Figure 1 400 μg of pGL3-Control plasmid DNA was injected in 5.25% (volume per animal weight) Ringer to healthy rats by the hydrodynamics based injection (5 to 8 s). 24 h later the liver was excised, the lobes minced and 200 mg samples of each of the four lobes homogenized in reporter lysis buffer and luciferase activity monitored (n = 10).

Table 1 Number of gaps along the sinusoidal endothelial lining after hydrodynamic injections (mean ± SD, n = 30)

	n gaps/10 μm^2 (periportal)	n gaps/10 μm^2 (pericentral)
Control	0.14 ± 0.15	0.18 ± 0.21
10 min	3.04 ± 0.62[a,c]	0.86 ± 0.42[e]
1 h	3.02 ± 0.35[a,c]	0.76 ± 0.42[e]
8 h	3.08 ± 0.64[a,c]	1.39 ± 0.46[e]
24 h	2.98 ± 0.55[a,c]	1.07 ± 0.39[e]
72 h	1.42 ± 0.32[a,c]	0.72 ± 0.27[e]
168 h	0.92 ± 0.33[a,c]	0.49 ± 0.21
240 h	0.45 ± 0.19	0.39 ± 0.20

Scanning electron microscopy based morphometric analysis of gaps per area along the sinusoidal endothelial lining cells. Significance of data was determined using the Mann Whitney two-sided U-test. Significant differences between the number of gaps in the periportal and pericentral zones were noted at all time points indicated ([a]$P \leqslant 0.05$) following hydrodynamic injections. Significant differences between control and time points indicated following hydrodynamic injections were evident at both periportal ([c]$P \leqslant 0.05$) and pericentral ([e]$P \leqslant 0.05$) areas of the liver. A gap is defined as a hole with a diameter size of 300-2000 nm.

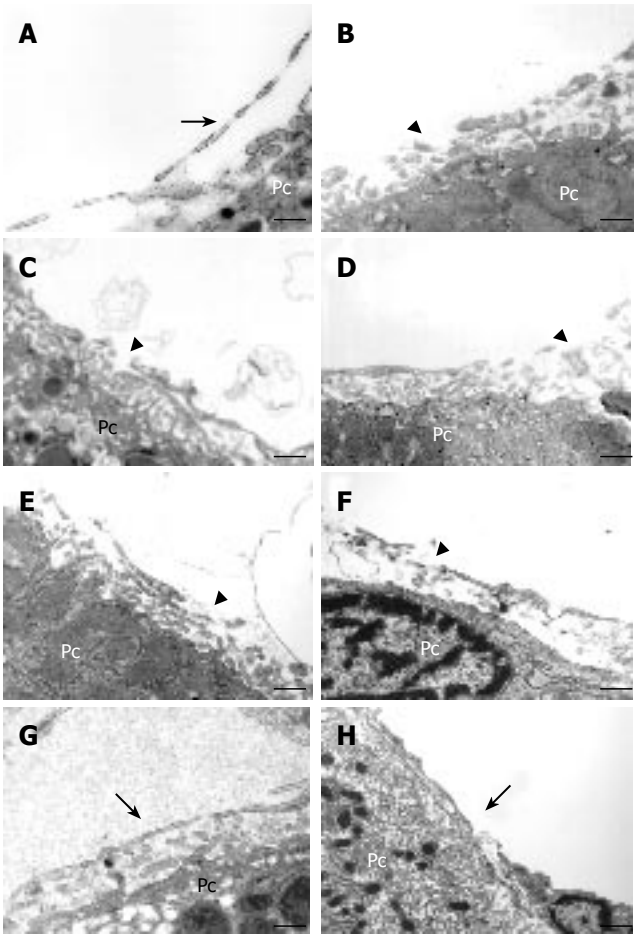

Figure 2 High magnification electron micrographs of liver sinusoids following hydrodynamic injection. **A**: Control image illustrates intact histological relationship between liver sinusoidal endothelial and neighboring liver parenchymal cells (Pc). Note the fenestrated processes (arrow) of endothelial cells; **B**: Ten minutes after hydrodynamic injections, severe damage of the endothelial lining in the form of gaps is noted (arrowhead). Similar features were observed at 1 (**C**), 8 (**D**), 24 (**E**), and 72 (**F**) h, i.e, a disrupted endothelial lining with the presence of large structural rearrangement in the form of gaps (arrowheads). The architecture of hepatocytes remained intact when compared to the control. Between seven (**G**) to ten (**H**) days post injection, the number of gaps decreased significantly. Interestingly, the hepatic sinusoidal endothelial lining is less fenestrated when compared to the control (arrow). Scale bars, 1 μm.

used to give a reliable representation of the expression in the lobe (Figure 1). Comparison between the four lobes showed statistical difference in expression (d.f. = 3, F = 6.78, $P < 0.001$). The left lateral lobe was found by post hoc analysis to express significantly less plasmid ($P < 0.05$).

HBT is associated with the formation of large endothelial gaps

An enlargement in liver fenestrae was reported when high pressure was used in liver perfusion[22]. We have demonstrated an increase in blood flow to the liver following partial hepatectomy which was associated with the formation of "fused" fenestrae (gaps)[23]. The number of these gaps gradually decreased, reaching baseline level 10 d post surgery. To test the effect of hydrodynamic injections on cells occupying the space of Disse, both scanning and transmission electron microscopy were utilized. Prior to HBT injection, normal ultrastucture of liver sinusoids and the surrounding hepatocytes was noted, with patent lumen delineated by endothelial cells with thin processes containing a few fenestrae. These fenestrae ranged in size between 120-140 nm. Microvilli extending from the parenchymal cell surface could be readily seen. Hepatocytes show normal ultrastructure displaying a cytoplasm rich in rough endoplasmatic reticulum, glycogen and multiple membrane bound vesiculo-organelles such as lysosomes, lipid droplets and coated pits. As early as ten minutes after hydrodynamic injections, endothelial-associated changes were noted in the form of fused fenestrae, i.e., gaps, ranging in diameter between 0.3μm and 2 μm. These gaps were more prominent in periportal areas than pericentral areas (Figure 2 and Table 1). Increasing values were noted in subsequent times, i.e., 1 to 24 h post-hydrodynamic injection in both areas. The number of gaps decreased gradually reaching almost pre-hydrodynamic injection levels 240 h post injection (Table 1). Scanning electron microscopy revealed similar results (Figure 3). Control tissue demonstrated intact relationship between sinusoidal endothelial cells and neighboring liver parenchymal cells. Ten minutes after injections the

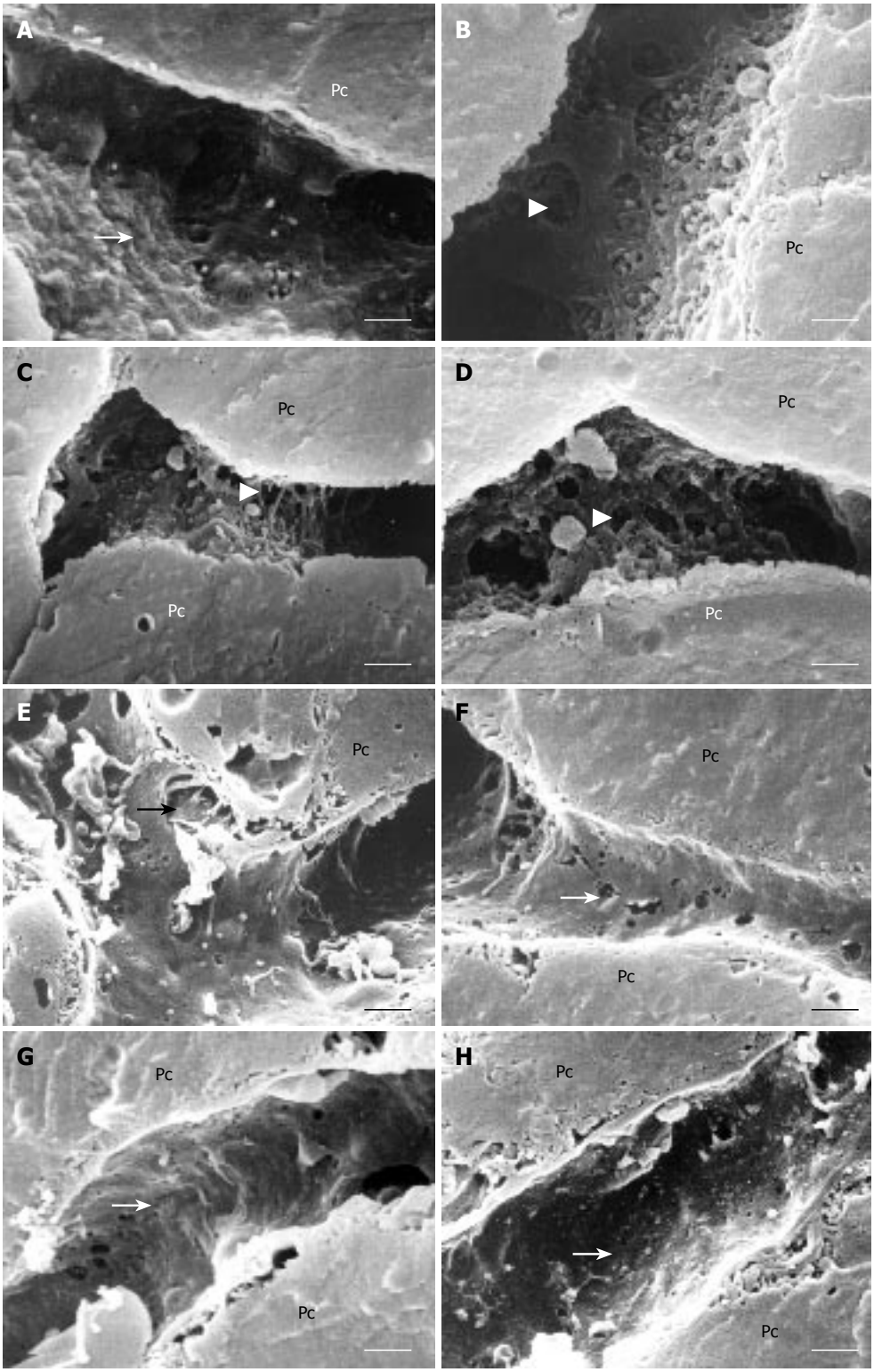

Figure 3 Scanning electron micrographs of liver sinusoids following hydrodynamic injections. **A**: Control liver demonstrates an intact endothelial wall with numerous small pores perforating the endothelial lining (fenestrae) (arrow) and undisrupted bordering parenchymal cells (Pc); **B**: Numerous gaps (arrowhead) are observed as early as ten minutes after hydrodynamic injections and persist at one (**C**) and eight (**D**) hours after injection. From twenty four hours post HBT and onwards the number of gaps (arrowhead) decreases, yet a significant number are still present (**E**, **F**). Between seven (**G**) and ten (**H**) days following HBT, the endothelial lining reveals similar features as described under Figure 2G-H: i.e., less fenestrated appearance. Scale bars, 2 μm.

endothelial lining was disrupted by gaps and microvilli facing toward the sinusoidal lumen. Ten days after HBT liver morphology returned to normal with very few fenestrae.

No increase in BrdU cell labeling was evident in HBT injected rats (data not shown) suggesting that in spite of the morphological damage mentioned above no cell proliferation was associated with our protocol.

Hydrodynamic administration of Ringer solution prior to transfection does not facilitate plasmid transfection

Given the fact that endothelial cell fenestrae undergo major changes resulting in a significant increase in the number of gaps as early as 10 m following HBT we examined whether the induction of gaps by itself enables plasmid DNA transfection. Normal rats were thus injected with Ringer solution in a volume equal to 5.25% of their body weight followed by a moderate injection of 400 μg of pGL3-Control plasmid 10 or 30 m post HBT. Two control groups of rats were injected either hydrodynamically or moderately with the same plasmid. While hydrodynamic administration of the plasmid resulted in a remarkably high level of luciferase expression, hydrodynamic administration of Ringer followed by moderate injection

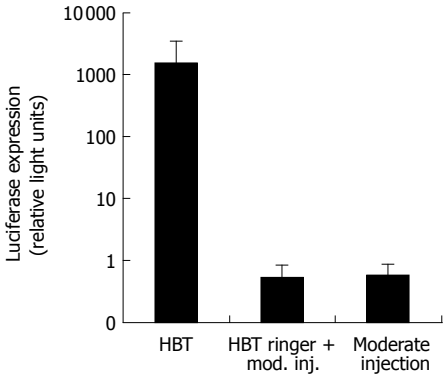

Figure 4 400 μg of pGL3-Control plasmid DNA were injected in the following variations: (1) HBT: 5.25% (volume per animal weight) Ringer solution with plasmid DNA in 5 to 8 s; (2) HBT Ringer + moderate injection: 5.25% (volume per animal weight) Ringer solution in 5 to 8 s followed by 400 μg of plasmid DNA in 500 μL 10 or 30 m later; (3) moderate injection: 500 μL Ringer solution with 400 μg plasmid DNA. Twenty four hours post injection the liver was excised. 200 mg liver sample were then homogenized in reporter buffer and luciferase activity monitored (n = 5).

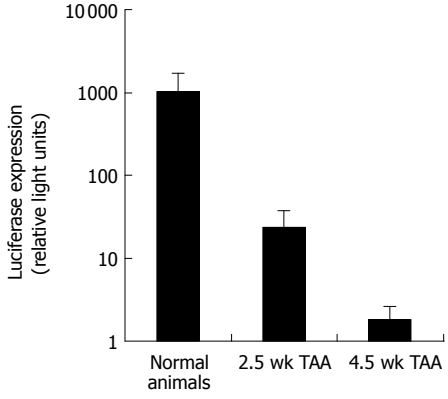

Figure 5 400 μg of pGL3-Control plasmid DNA was injected in 5.25% (volume per animal weight) Ringer in 5-8 s to normal rats, and rats injected intraperitoneally (IP) twice weekly with thioacetamide (TAA) for either 2.5 or 4.5 wk. 24 h later the liver was excised and 200 mg samples of the median lobe were homogenized in reporter lysis buffer and the activity of luciferase monitored (n = 5).

of the plasmid 10 or 30 m after HBT yielded significantly lower expression levels ($P < 0.05$), similar to those of moderate injection ($P > 0.5$ between these two groups) (Figure 4).

Transfection efficiency of HBT in fibrotic rats is reduced

HBT is associated with an increase of hydrodynamic pressure in the liver, leading to an efficient transfection. It follows therefore that "blocking" endothelial sinusoids would lead to gradual decrease in the efficiency of transfection. To test this assumption we followed the fate of pGL3-Control in rats treated with thioacetamide for 2.5 or 4.5 wk. Such treatment results in fibrotic expansion of most portal areas along with the formation of incomplete septa (Ishak scoring 1-2) and portal to portal and portal to central septa including occasional nodules (Ishak scoring 5) respectively. HBT in both fibrotic groups resulted in a significant reduction in the expression of luciferase ($P < 0.000$). Between the two fibrotic groups there was also siginificant difference in expression ($P < 0.005$) (Figure 5). Furthermore, luciferase expression strongly correlated

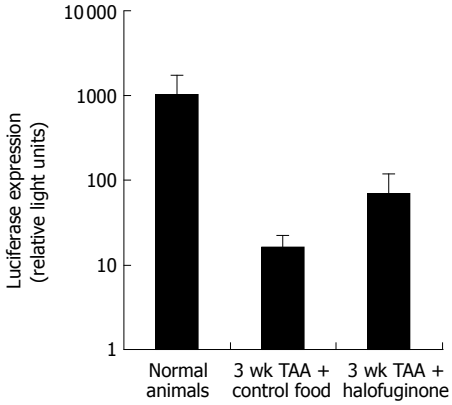

Figure 6 400 μg of pGL3-Control plasmid was injected in 5.25% (volume per animal weight) Ringer in 5-8 s to normal rats, and rats previously injected intraperitoneally twice weekly with thioacetamide (TAA) for 3 wk. Prior to the hydrodynamic injection the rats were fed for 3 wk with either control food or food containing halofuginone at a concentration of 5 ppm. Twenty four hours post HBT the liver was excised and 200 mg samples of the median lobe were homogenized in reporter lysis buffer and the activity of luciferase monitored (n = 5).

inversely with the severity of fibrosis as quantitated by Ishak (r = -0.77). Halofuginone is a specific collagen type I inhibitor. In previous studies[24,25] we have shown its beneficial usage in reducing collagen deposition in fibrotic rats. When given to rats with established fibrosis prior to hydrodynamic injection, an increase in the level of luciferase expression following HBT was evident ($P < 0.05$ between the treated and untreated groups) (Figure 6). Such animals also demonstrated a reduced level of fibrosis as determined by Sirius Red staining.

DISCUSSION

Hydrodynamics based transfection allows efficient delivery of plasmid DNA to the liver. This simple procedure has been used to test the potential benefits of certain genes like the hemophilia Factor IX or for screening novel elements *in vivo*[26-28]. Yet in spite of the intense usage of the methodology, the mechanism underlying the entry and expression of the targeted gene is still unclear. The present study elucidates basic features associated with the technique, which are of fundamental importance when the technique is being implemented. It also demonstrates the potential usage of halofuginone, a collagen type I inhibitor in the event that the HBT technology is used in fibrotic rats. Conflicting reports exist as to the number of cells expressing the desired gene following HBT. Song and colleagues identified close to 40% positively stained cells most of which are hepatocytes[29]. Others[3] reported significantly lower numbers of 10% or less. It is obvious that incorrect sampling of the liver may lead to a wrong conclusion. Our current analysis using samples representing the complete lobe previously cut into small pieces was reliable and reproducible. In that context, it is worth noting that variations in gene expression between the four liver lobes were noted, though these differences were not significant except for the left lateral lobe which showed lower expression ($P < 0.05$). We conducted all experiments using the median lobe which is the largest.

The term "liver sieve" is used to describe the endothelial

cells that line the hepatic sinusoids. These fenestrated endothelial cells which have no basement membrane allow free transfer from the sinusoidal lumen of various substrates including growth factors, hormones, proteases and chylomicrons[15]. Evidence points to the availability of receptors responsible for the removal of soluble molecules via sinusoidal endothelial cells (SEC)[30]. Fenestration of sinusoidal endothelial cells is a dynamic process whose size and density may be affected by various factors ranging from physical factors to soluble substances[31]. The formation of large gaps following an increase in hydrodynamic pressure in the liver has been documented in different set-ups. Fraser and colleagues have shown enlarged fenestrae when high pressure was applied in a liver perfusion model[22]. Wack and colleagues[31] reported increased porosity and fenestration diameters following 70% partial hepatectomy. We have recently been able to demonstrate the formation of large gaps ranging in diameter between 0.3 μm and 2 μm following 70% partial hepatectomy[23]. These gaps are most probably formed by the increased shear stress associated with elevated blood flow following hepatectomy. Though gaps are evident 10 m post hepatectomy, it is likely that these are formed concomitantly with the elevation in blood flow. We now show a similar pattern of gap formation following hydrodynamic injection. In both cases, the increase in the number of gaps was more prominent in periportal areas than pericentral areas. These differences cannot be simply explained by higher pressure formed close to portal zones as increased pressure in the inferior cava vein and therefore in central veins has been described with the hydrodynamic injections[10]. The reported increase in the number of gaps was evident 10 m post HBT, peaking between 8 and 24 h. Gradual recovery was thereafter recorded approaching normal values ten days post injection.

For naked pDNA to be expressed following HBT it needs to overcome the obstacle of the lining endothelial cells, and reach the hepatocytes which constitute the majority of cells transfected under hydrodynamic pressure, penetrate the membrane and arrive in the nucleus. The formation of gaps, sized up to 0.3 nm following HBT seems to facilitate the crossing of DNA from the blood to the space of Disse. Further, such gaps may also help clear pDNA from the blood instantaneously, thereby avoiding exposure to nucleases and rapid degradation[12,32]. The importance of the space of Disse and the state of its ECM components on the expression of naked pDNA using the hydrodynamic technology was proven in rats previously administered with thioacetamide.

HBT in fibrotic rats was less effective compared to healthy control rats. Further, the expression of luciferase correlated with the level of fibrosis. Liver fibrosis is associated with both qualitative and quantitative changes in the composition of hepatic ECM. In the healthy liver the endothelium expresses very little collagen thus minimizing the barrier to substrate diffusion[30,33]. Following injury, the total content of ECM increases 3-5 times and the sub-endothelial type of ECM shifts from normal low density basement membrane like matrix to interstitial type matrix containing fibril forming collagen[34]. These changes have been reported to also be associated with the closure of HSC fenestrae[35]. The low expression of luciferase achieved in fibrotic rats undergoing HBT thus seems to coincide with the deposition of ECM and the blocking of the fenestrae. Given the fact that collagen type I may account for 5%-30% of the deposited ECM components in fibrotic liver, drugs inhibiting collagen synthesis or supporting collagen degradation should be used to help gene expression following HBT. In the present study we used halofuginone to reduce ECM content induced by thioacetamide. Halofuginone is a well known collagen I inhibitor. The drug has been proven effective in various models[36-40] including fibrotic rats using either dimethylnitrosamine or thioacetamide[25,40]. In rats exhibiting moderate levels of fibrosis halofuginone treatment resulted in a decrease in the level of fibrosis. The expression of luciferase in this group was higher than the control group, demonstrating its efficacy.

The mechanism underlying the HBT technology is still unresolved. The formation of endothelial gaps associated with the hydrodymanic pressure developing in the liver following HBT may account for the transfer of pDNA from the blood to the space of Disse, yet it does not explain why liver cells, mainly hepatocytes, are transfected by the same pDNA. Indeed when a moderate injection of pGL3-Control (500 μL) was performed 10 or 30 m following hydrodynamic injection of Ringer solution, the level of luciferase expression was the same as that obtained following moderate injection only. The increase in the number of endothelial gaps as early as 10 m post injection suggests that they are either not involved in the transfection procedure or are not the only event occurring following HBT.

In a separate study[19,41] an interval longer than 10 s between the two injections results in a significant and gradual decrease in the number of cells expressing the desired gene.

Isolated hepatocytes shortly after HBT demonstrate maximal pDNA expression, suggesting that DNA molecules have been taken up soon after injection. While our data on the formation of gaps generally coincide with that reported by others[32] we found no evidence for the formation of membrane pores following HBT. If indeed DNA molecules enter hepatocytes through pores, then a certain concentration of DNA should be achieved within a short time and the number of pores should be sufficient to allow entry. Based on experimental data and theoretical assumptions Budker and colleagues[11] are not in favor of the membrane pore mechanism but rather support the involvement of a receptor mediated process. Regardless of the unresolved mechanism underlying the hydrodynamic injection our study paves the way for using the technology in fibrotic animals as well. The usage of halofuginone in partially resolving some of the accumulated ECM components in the liver should encourage the search for drugs that might be used in future combined therapy.

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