

Delivering siRNA Compounds During HOPE to Modulate Organ Function: A Proof-of-concept Study in a Rat Liver Transplant Model

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Background. Apoptosis contributes to the severity of ischemia-reperfusion injury (IRI), limiting the use of extended criteria donors in liver transplantation (LT). Machine perfusion has been proposed as a platform to administer specific therapies to improve graft function. Alternatively, the inhibition of genes associated with apoptosis during machine perfusion could alleviate IRI post-LT. The aim of the study was to investigate whether inhibition of an apoptosis-associated gene (FAS) using a small interfering RNA (siRNA) approach could alleviate IRI in a rat LT model. **Methods.** In 2 different experimental protocols, FASsiRNA (500 µg) was administered to rat donors 2 h before organ procurement, followed by 22 h of static cold storage, (SCS) or was added to the perfusate during 1 h of ex situ hypothermic oxygenated perfusion (HOPE) to livers previously preserved for 4 h in SCS. **Results.** Transaminase levels were significantly lower in the SCS-FASsiRNA group at 24 h post-LT. Proinflammatory cytokines (interleukin-2, C-X-C motif chemokine 10, tumor necrosis factor alpha, and interferon gamma) were significantly decreased in the SCS-FASsiRNA group, whereas the interleukin-10 anti-inflammatory cytokine was significantly increased in the HOPE-FASsiRNA group. Liver absorption of FASsiRNA after HOPE session was demonstrated by confocal microscopy; however, no statistically significant differences on the apoptotic index, necrosis levels, and FAS protein transcription between treated and untreated groups were observed. **Conclusions.** FAS inhibition through siRNA therapy decreases the severity of IRI after LT in a SCS protocol; however the association of siRNA therapy with a HOPE perfusion model is very challenging. Future studies using better designed siRNA compounds and appropriate doses are required to prove the siRNA therapy effectiveness during liver HOPE liver perfusion.

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INTRODUCTION

Organ shortage is a limiting factor for the broader application of liver transplantation (LT) worldwide. This situation leads liver transplant surgeons to increasingly use organs from donors who are obese, elderly, deceased after circulatory death, or the so-called extended criteria donors or use organs that have accrued long static cold ischemia times (CITs); however, these organs are more susceptible to ischemia-reperfusion injury (IRI) consequences, including primary nonfunction and nonanastomotic biliary strictures.¹ Dynamic preservation by machine perfusion (MP) is a promising approach for optimizing and evaluating extended criteria donor graft function.^{2,3} MP has also been proposed as a delivery platform for liver-targeted therapies in the form of defatting cocktails, bone marrow stem cells, and gene modulation, such as RNA interference (RNAi), to reduce IRI.⁴⁻⁸ Our previous studies showed hepatic small interfering RNA (siRNA) absorption during ex situ MP⁸ and transduction of green fluorescent protein via Adeno-Associate Virus 8 administration during hypothermic oxygenated perfusion (HOPE).⁹ In this current proof-of-concept study, we aimed to demonstrate for the first time in a rodent LT model the feasibility of delivering siRNA to transplantable liver grafts during the pretransplant HOPE period to minimize posttransplant IRI.

MATERIALS AND METHODS

Rat Liver Preservation and Transplantation

Animal experiments were conducted at the University of Massachusetts, Worcester, Massachusetts, and at the Catholic University of Louvain, Brussels, Belgium. Both institutional regulatory bodies for animal research have approved the current study protocol. Male Lewis rats weighing 200 to 250 g (Charles River Laboratories, Boston, MA, and Lyon, France), used as donors and recipients, were housed under standard conditions according to the guidelines of each institution. Two different and complementary preservation protocols were used in this study. In the static cold storage (SCS) protocol, a single dose of 38 nmol (500 µg) siRNA diluted in 1 mL of PBS was administered into the penile vein by rapid hydrodynamic injection^{10,11} 2 h before organ procurement in the treated group, whereas the control group received PBS only. Livers were procured and static cold stored in Belzer cold storage preservation solution (Bridge to Life) for 22 h before implantation (Figure 1A). In the HOPE protocol, livers were procured and initially placed in static cold stored for 22 h, then connected to MP for 1 h of HOPE using Belzer MPS machine preservation solution (Bridge to Life). In the treated group, 38 nmol (500 µg) of siRNA was diluted in the perfusate when MP commenced (Figure 1B and C), whereas nothing was added to

the perfusate in the untreated group. At the end of HOPE, all livers were rinsed with 10 mL of cold (4 °C) saline. Unfortunately, with 22 h SCS+1 h HOPE design protocol, all recipients (FASsiRNA treated and untreated) did not recover well post-LT and died before 24 h post-postprocedure, which prevented us from studying the effect of siRNA therapy during HOPE. Therefore, we switched to an established study design of 4 h SCS+1 h HOPE.^{12,13} With this protocol, all recipients survived and were in good conditions at 24 h post-LT like the recipients of the SCS protocol. Transplants were performed using a rearterialized model, as previously described.¹⁴ Isoflurane (Abbot) was used for anesthesia in all operations. In addition to the screening experiments, the present study analyzed samples collected from 36 liver recipients, divided in 6 groups, 4 in the SCS protocol and 2 in the HOPE. There were 6 transplants per group; euthanasia was performed at 24 and 72 h after the transplantation for the groups of the SCS groups and at 24 h in the HOPE groups (Table 1).

FASsiRNA Screening

Two different sequences of FAS-targeting siRNA (sequence 1 [FASsiRNA-1]: modified at 3' with Cy3 5'-ACACG GACAGGAAACACUAdTdT-Cy3-3' [sense], 5'-UAGG UUUCUGUCCGUGUdTdT-3' [antisense]; sequence 2 [FASsiRNA-2]: 5'-GUGCAAGUGCAAACCAGAACdTdT-Cy3-3' [sense] and 5'-GUCUGGUUUGCACUU GCACdTdT-3' [antisense]; Dharmacon) were used in preliminary transplants employing the SCS protocol (22 h) to choose the most effective sequence to decrease posttransplant transaminase levels at 24 h post-LT. Both FASsiRNA sequences have already showed in vitro and in vivo FAS inhibition in previous studies; sequence 1 showed a protective effect in an acute liver failure model,¹¹ and sequence 2 was able to alleviate the post-LT IRI in a rodent model.¹⁵ Additional transplants were performed using livers from donors treated with FASsiRNA-1 plus 100 µL of Invivofectamine 3000 (ThermoFisher) as a nanoparticle to carry siRNA compounds and to protect them against endonucleases, with the aim of increasing hepatic siRNA uptake.

Serum Biochemistry and Cytokines Analyses

Serum samples were obtained from blood samples collected from the tip of the tail at each time point and centrifuged at 3000 g for 15 min. Aspartate aminotransferase and alanine aminotransferase plasma activities were measured by automated analyses using the Piccolo Chemistry Analyzer. Serum cytokine levels were quantified using the RAT Cytokine/Chemokine Magnetic Bead Panel 96-Well Plate Assay MILLIPLEX MAP, according to the manufacturer's recommendations (Millipore Corporation, Billerica, MA).

Liver Tissue Sampling

Following euthanasia, 2 large thin slices (3–5 mm), one from the right side (right median and superior right lobes) and the other from the left side of the liver (left lateral lobe)¹⁶ (Figure S1A, SDC, <http://links.lww.com/TP/C435>), were collected and fixed in 4% paraformaldehyde solution and then embedded in paraffin. Small pieces of liver tissue were immediately frozen in liquid nitrogen and stored at –80 °C. Additional liver fragments were collected and maintained in RNAlater (Ambion) for 24 h at 4 °C and stored at –80 °C.

Supplemental Visual Abstract; <http://links.lww.com/TP/C436>.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

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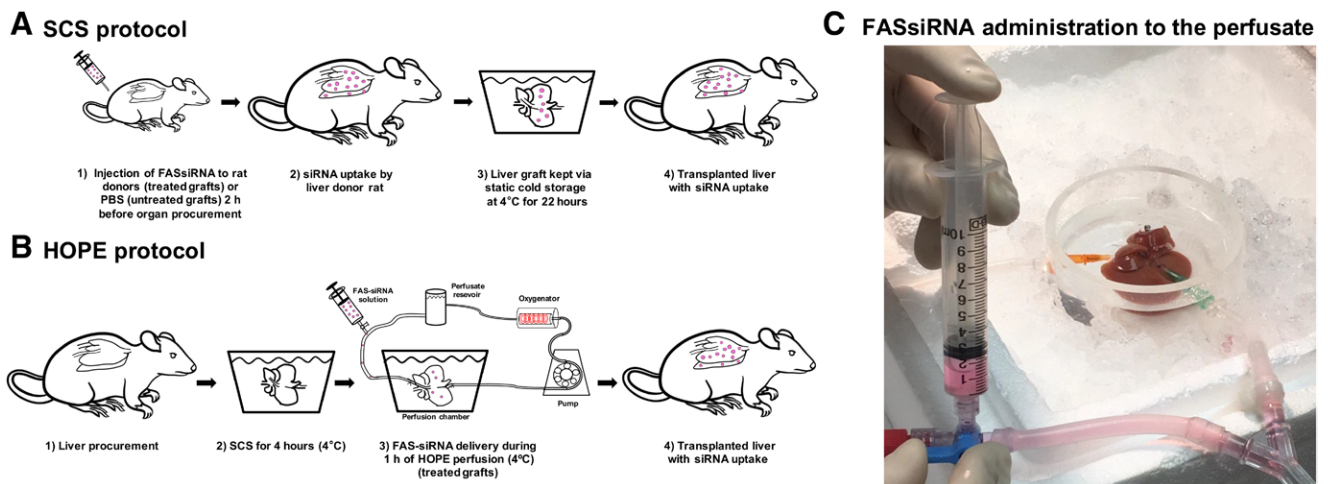


FIGURE 1. Design of the experimental protocols. A, SCS protocol. In the SCS protocol, 38 nmol (500 µg) of FASsiRNA was administered through the penile vein to rat liver donors 2 h before organ procurement in the treated group, whereas the rats of the untreated group received 1 mL of PBS. The liver grafts were kept for 22 h in a container with Belzer cold storage (CS) preservation solution, Bridge for Life, at 4 °C until liver implantation. Euthanasia was performed at 24 and 72 h later. B, HOPE protocol. In a second protocol, livers were procured and kept for 4 h in a container with CS preservation solution at 4 °C, then placed in a customized perfusion chamber and perfused using the Belzer Machine Perfusion Solution, Bridge to Life (MPS) machine perfusion solution, through the portal vein on a continuous close-loop circuit by roller pump for 1 h, maintaining the portal pressure at 6 to 8 mmHg. Oxygen was delivered at a rate of 1.5 L/min by a customized oxygenator at 100%, and temperature was kept at 4 to 6 °C using a cooling water bath. The 38 nmol (500 mg) of FASsiRNA was added to the perfusate of the treated livers at the beginning of the perfusion session. C, Demonstration of the administration of FASsiRNA solution to the HOPE perfusion system. Nothing was added to the perfusate of the untreated livers. Euthanasia was performed 24 h later. HOPE, hypothermic oxygenated machine perfusion; SCS, static cold storage; siRNA, small interfering RNA.

Confocal Microscopy

Paraffin-embedded liver sections were rehydrated in a standard fashion in xylene, followed by serial 100% ethanol and water baths. The liver parenchyma was stained with DAPI (Vector Shield) and mounted. Slides were imaged on a Nikon A1 confocal microscope at $\times 40$ magnification, and images were edited using ImageJ software (NIH).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Analyses

Liver sections were deparaffinized and subjected to blocking of endogenous peroxidase activity, antigen retrieval, and blocking of nonspecific antigen-binding sites. The samples were then fixed with 4% formaldehyde and stained with the DeadEnd Fluorometric terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) system kit (Promega #G3250, FITC revelation) according to the manufacturer's instructions. Nuclei were stained with Hoechst33342. The fluorescent slides were digitized on a Panoramic 250 FlashIII (3DHitech) scanner and analyzed using the Author 2017.2 software (Visiopharm, Hørsholm, Denmark). Stained and unstained cells were

counted, and the results were expressed as cell numbers/mm² of tissue and the percentage of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive (TUNEL⁺) cells per tissue section.

Western Blot Analyses

Liver tissue proteins were extracted, normalized, and resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After migration, proteins were transferred to nitrocellulose membranes and incubated overnight at 4 °C with antibodies against FAS (0.1 µg/mL) and vinculin (0.1 µg/mL; Thermo Fisher Scientific). Immunoreactivity was visualized using electrochemiluminescence, and bands were quantified by densitometry.

Liver Histology

The Suzuki score was used to assess hepatocyte necrosis, sinusoidal congestion, and cytoplasmic congestion in 2 hematoxylin and eosin-stained liver samples per animal (Figure S1B, SDC, <http://links.lww.com/TP/C435>).¹⁷ For a more representative reading of liver injury, necrosis and neutrophil infiltration were also assessed in 10 microscope fields at $\times 20$ magnification in the same samples. A liver pathologist (M.K.), blinded to the study design, performed all the histological evaluations.

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics (version 25, Armonk, NY), and data were plotted using GraphPad Prism software (version 5.0, San Diego, CA). When appropriate, continuous variables were analyzed by an unpaired *t* test or the Mann-Whitney *U* test for comparisons between 2 groups and by analysis of variance for comparisons between >2 groups. Pearson's chi-square test was used to analyze the categorical variables. Results

TABLE 1.
Protocols and study group analyzed in the current study

Protocols	Groups	No. of animal	Post-LT euthanasia	
			24 h	72 h
SCS	SCS (22 h) + PBS	11	6	5
	SCS (22 h) + FASsiRNA	12	6	6
HOPE	HOPE (4 h SCS + 1 h HOPE)	6	6	—
	HOPE (4 h SCS + 1 h HOPE) + FASsiRNA	6	6	—

HOPE, hypothermic oxygenated perfusion; LT, liver transplantation; PBS, phosphate buffered saline; SCS, static cold storage; siRNA, small interfering RNA.

were reported as mean and standard error means and considered statistically significant if *P* values were inferior or equal to 0.05.

RESULTS

FASsiRNA Reduces Transaminases Levels at 24 h Post-LT

In transplant screening experiments (SCS protocol), transaminase levels were statistically lower in recipients receiving livers treated with FASsiRNA-1 (5 transplants) than in those in the FASsiRNA-2 group (3 transplants) and in the PBS treated group (5 transplants). In preliminary experiments, all recipients transplanted with livers treated with FASsiRNA-1 associated with InvivoFectamine (3 transplants) presented the highest levels of transaminases, which suggests a probable dose-associated toxicity of the InvivoFectamine used in our experiments (Figure 2; Table S1, SDC, <http://links.lww.com/TP/C435>) as shown in other studies.^{18,19} Therefore, all subsequent experiments were performed using naked FASsiRNA-1 sequence (without InvivoFectamine), hereinafter referred to in the text below as FASsiRNA.

Surgical Parameters and Survival Rates Were Similar in Both Experimental Protocols

The anhepatic time was similar in all groups, and the increased operating time observed in the HOPE groups can be explained by the additional time required to disconnect the graft from the MP (Figure S2; Table S1, SDC, <http://links.lww.com/TP/C435>). At 24 h post-LT, all recipients in the SCS groups and in the HOPE groups were in good clinical condition with no signs of distress or suffering. Two animals in the SCS groups died from surgical complications, one from uncontrolled bleeding and the other from the pneumothorax; both were excluded from the analysis.

FASsiRNA Treatment Reduces the Intensity of IRI Post-LT

In the SCS protocol, prolonged CIT was used to increase the deleterious effects of IRI, as measured by the post-LT levels of transaminases, considered as surrogate markers of liver injury in the current study. The SCS-FASsiRNA group presented significantly lower levels of aspartate aminotransferase and alanine aminotransferase at all time points post-LT than the SCS-PBS groups in the SCS experiments (Figure 3A; Table 2). These findings were associated with reduced levels of the following proinflammatory cytokines/chemokines: interleukin (IL)-1a, IL-2, tumor necrosis factor alpha, monocyte chemoattractant protein 1, and C-X-C motif chemokine 10 at 24 h post-LT in the SCS-FASsiRNA group (*P* = 0.009, 0.002, 0.037, 0.024, and 0.017, respectively). Conversely, in the HOPE protocol, recipients of FASsiRNA-treated livers did not present significantly different levels of transaminases at 24 h post-LT compared with the untreated livers (Figure 3B; Table 2). In addition, reduction in inflammatory cytokines levels was less intense in the HOPE protocol than in the SCS protocol and did not reach statistical significance between FASsiRNA-treated and FASsiRNA-untreated groups; however, levels of anti-inflammatory cytokines IL-4 and IL-10 were increased in the HOPE-FASsiRNA-treated group compared with the untreated group (fold change 2.84 and 0.37, respectively). On the other hand, in the SCS protocol, these anti-inflammatory cytokines levels were decreased in the SCS-FASsiRNA group (fold change -1.58 and -2.82, respectively), with a significantly reduced level of IL-10 in the FASsiRNA-treated group (*P* = 0.004; Table 3).

Effect of FASsiRNA Treatment on the Hepatic Cell Apoptosis

The intensity of apoptosis in the liver parenchyma was assessed using TUNEL assay. Cells showing nuclear fluorescence with an intact cell membrane were considered

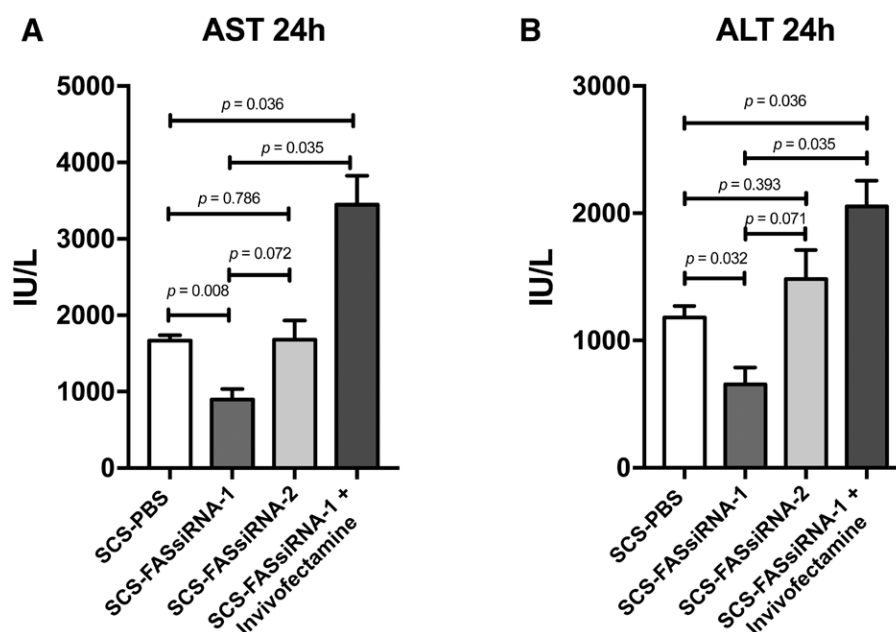


FIGURE 2. FASsiRNA screening experiments. A and B, Chart displaying the AST and ALT levels at 24 h after liver transplantation for the different groups performed for the siRNA sequence screening of transplants performed for the siRNA screening/validation. ALT, alanine aminotransferase; AST, aspartate aminotransferase; SCS, static cold storage; siRNA, small interfering RNA.

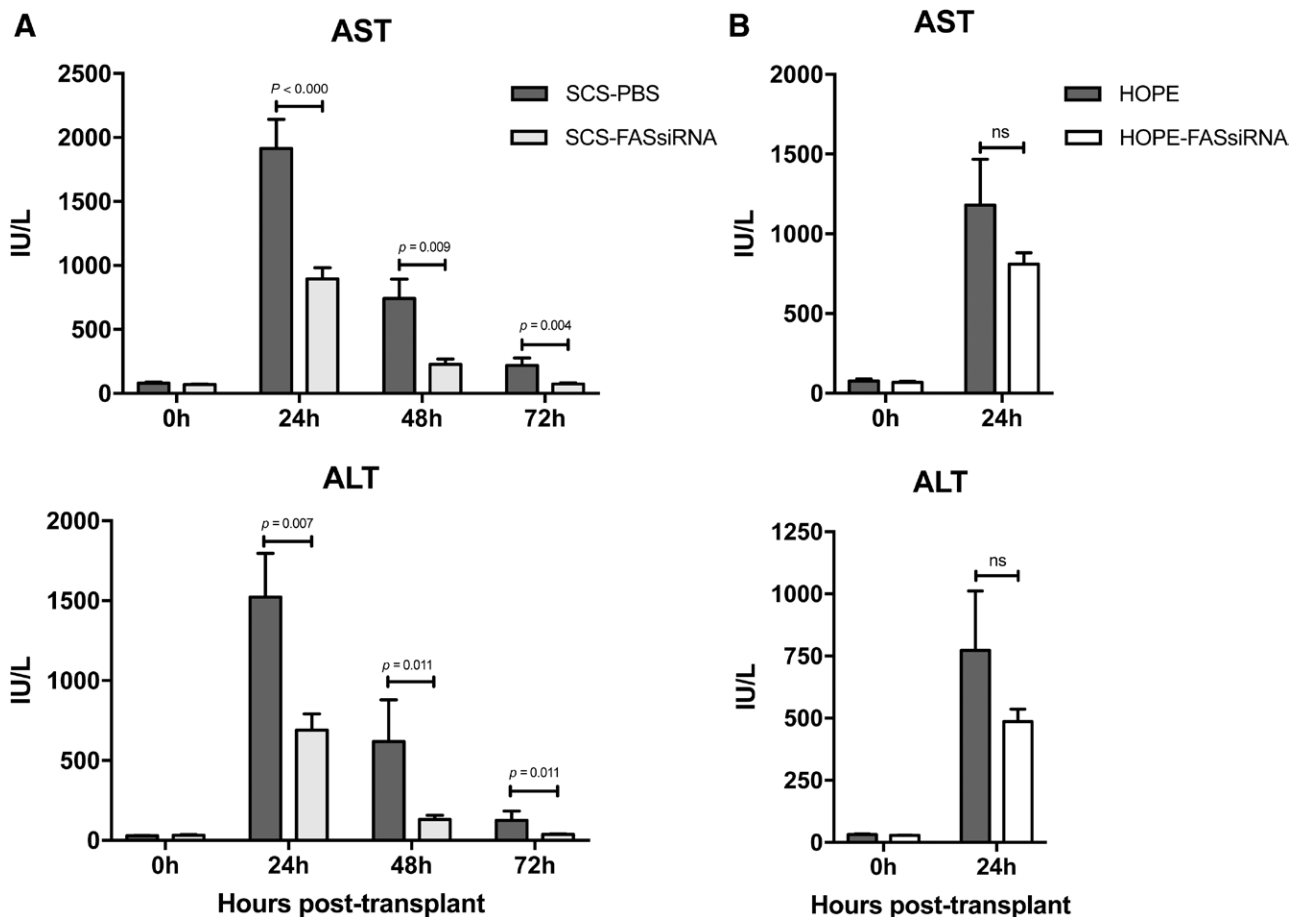


FIGURE 3. Post-LT transaminases kinetics. A, Chart showing the AST and ALT evolution during the LT per-operative period in the SCS protocol. B, Charts showing the AST and ALT levels at time and 24 h after the LT procedure in the hypothermic oxygenated machine perfusion (HOPE) protocol. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LT, liver transplantation; SCS, static cold storage.

as TUNEL⁺ cells. There were no statistical differences on the TUNEL⁺ cells in recipients of FASsiRNA-treated livers compared with untreated livers in each protocol (SCS $P=0.720$ and HOPE $P=0.580$). Furthermore, in the SCS protocol, the apoptotic index returned to baseline levels in both groups on the third day post-LT (Figure 4), in accordance with the lower cytolyses observed at that time.

FASsiRNA Treatment Effect on Necrosis and Inflammatory Infiltration Post-LT

Initial histological analyses were performed using the Suzuki score,¹⁷ which showed that congestion was already present at 24 h in the HOPE-FASsiRNA group and only at 72 h in the SCS-FASsiRNA group. Conversely,

vacuolization was only demonstrated at 72 h in the SCS protocol, as no groups in both protocols showed vacuolization at 24 h. Regarding the extent of necrosis, no differences were detected between the groups (Table 4). Next, we measured the percentage of necrotic areas in 10 microscopic fields at $\times 20$ magnification in 2 liver sections per animal to obtain a more accurate reading of the extent of necrosis. We did not observe differences on the necrosis levels in the SCS-FASsiRNA group compared with the control group at 24 h ($P=0.054$). After 72 h, necrotic areas continued to be similar in both groups ($P=0.330$). There were no differences in necrosis between the HOPE-FASsiRNA and control groups ($P=0.230$) (Figure 5A). In addition, there were no differences on the rate of neutrophil infiltration

TABLE 2.

Surgical parameters and post-LT transaminases levels for all groups in SCS and HOPE protocols

	SCS	SCS + FASsiRNA	P	HOPE	HOPE + FASsiRNA	P
Rec. weight (g)	246.5 ± 18.2	255.8 ± 12.4	0.530	250.8 ± 21.5	248 ± 13.8	0.990
Anhepatic time (min)	17.6 ± 1.2	17.3 ± 2	0.970	17.8 ± 2.2	16.7 ± 1.4	0.670
Operative time (min) protocols	88.1 ± 1.2	—	—	99.6 ± 3.4	—	0.000
Operative time (min) groups	89.6 ± 6.1	86.7 ± 24.5	0.990	102 ± 8.27	97.2 ± 14.8	0.990
AST (IU/L) (at 24 h post-LT)	1914 ± 229	894.6 ± 88.7	<0.000	1178.8 ± 287.8	810 ± 71.7	ns
ALT (IU/L) (at 24 h post-LT)	1522 ± 274	795 ± 139.6	0.007	772.8 ± 49.8	486.2 ± 49.8	ns

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOPE, hypothermic oxygenated perfusion; LT, liver transplantation; ns, not significant; SCS, static cold storage; siRNA, small interfering RNA.

TABLE 3.
Serum levels of cytokines measured by Luminex assay from siRNA-1 treated and nontreated rat liver recipients

Symbol	Name	FC SCS protocol	P	FC HOPE protocol	P
IL-1α	Interleukin 1 alpha	−5.16	0.009	ND	ND
IL-2	Interleukin 2	−17.12	0.002	0.09	0.965
IL-4	Interleukin 4	−1.58	0.372	2.84	0.414
IL-6	Interleukin 6	−3.23	0.056	−1.62	0.372
IL-10	Interleukin 10	−2.82	0.004	0.37	0.589
IL-17	Interleukin 17	−0.41	0.175	−0.21	0.965
CXCL10	C-X-C motif chemokine 10	−1.48	0.017	−0.01	0.818
MCP-1	Monocyte chemoattractant protein 1	−0.88	0.024	ND	ND
IFNγ	Interferon gamma	−2.85	0.827	−0.89	0.738
TNFα	Tumor necrosis factor alpha	−3.04	0.037	−0.13	0.372

FC is exhibited as log₂ of the siRNA-treated/nontreated values.
FC, fold change; HOPE, hypothermic oxygenated perfusion; ND, not dosed; SCS, static cold storage; siRNA, small interfering RNA.

in livers treated with FASsiRNA compared with untreated livers in all protocols (Figure 5B).

Hepatocyte Uptake of siRNA Compounds Despite Hypothermic Conditions

Liver samples collected at 24h after transplantation showed that double-stranded FASsiRNA conjugated to the fluorescent marker CY3 on the sense strand underwent

discrete uptake in liver parenchyma after 1 h HOPE compared with perfused livers without FASsiRNA. Uptake was persistently demonstrated in sinusoidal endothelial cells and surrounding central veins. The siRNA appeared to be confined to the cytosol, which would maximize the efficiency of FAS silencing via the formation of a highly specific RNA-induced silencing complex. The distribution of FASsiRNA appeared to be equally diffuse between the

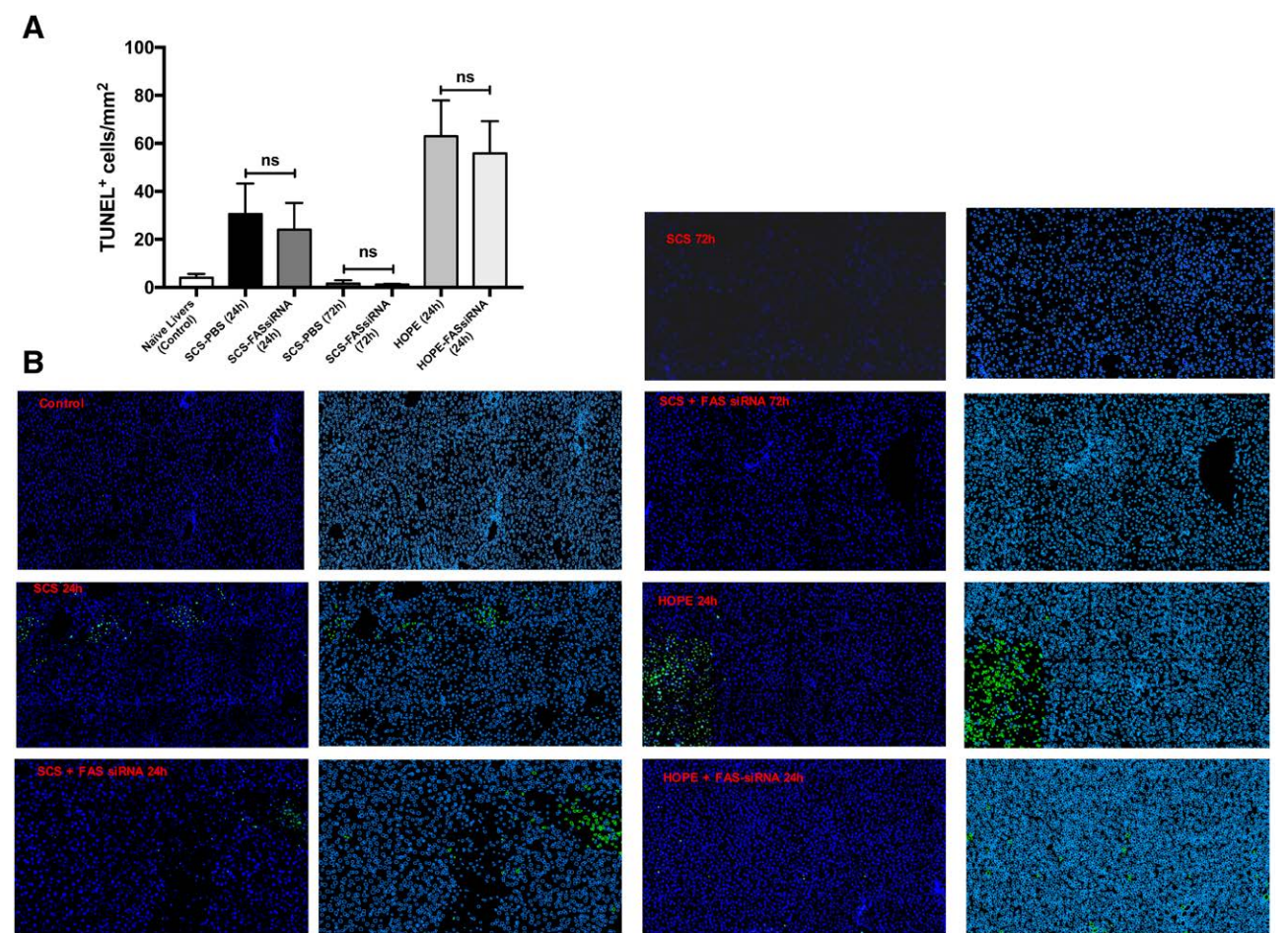


FIGURE 4. TUNEL assay. A, Chart showing the number of hepatic positive TUNEL cells per mm² in all in all experimental groups. B, TUNEL+ nuclei staining and the quantification mask illustration of each experimental group described in (A). HOPE, hypothermic oxygenated machine perfusion; SCS, static cold storage; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase DUTP nick end labeling.

TABLE 4.**Histological readout of the ischemia-reperfusion injury in liver parenchyma by the Suzuki score**

Injury score	SCS (24 h) (n = 6)	SCS + FASsiRNA (24 h) (n = 6)	SCS (72 h) (n = 5)	SCS + FASsiRNA (72 h) (n = 6)	HOPE (24 h) (n = 6)	HOPE + FASsiRNA (24 h) (n = 6)
Congestion						
No	3	6	1	3	0	0
Minimal	2	0	3	2	4	4
Mild	1	0	1	1	2	2
Moderate	0	0	0	0	0	0
<i>P</i> value	0.135		0.561			1.0
Vacuolization						
No	6	6	0	0	6	6
Minimal	0	0	3	1	0	0
Mild	0	0	2	3	0	0
Moderate	0	0	0	2	0	0
<i>P</i> value	1.0		0.210		1.0	
Necrosis						
No	0	2	0	1	0	1
Minimal (<5%)	2	2	3	3	3	3
Mild (6%–33%)	4	2	2	2	2	2
Moderate (34%–66%)	0	0	0	0	1	0
<i>P</i> value	0.260		0.630		0.560	

HOPE, hypothermic oxygenated perfusion; SCS, static cold storage; siRNA, small interfering RNA.

left and right lobes and equal along the length of the liver sinusoids (Figure 6).

Effect of the FASsiRNA Treatment on Transcription of FAS (CD95) Protein

We attempted to verify whether the previously observed changes in all the parameters analyzed were associated with a reduction in the transcription rate of the FAS protein (CD95), a hepatocyte membrane receptor associated with apoptosis, due to treatment with FASsiRNA. The concentration of FAS protein, measured in samples collected at 24 and 72 h after LT, was not different in the SCS-FASsiRNA and in the HOPE-FASsiRNA groups compared with their respective control groups at 24 h ($P = \text{NS}$; Figure 7).

DISCUSSION

RNAi is a process in which genes are cleaved by small double-stranded RNA sequences to prevent subsequent protein expression.²⁰ Synthetic siRNA sequences have been used in experimental models of acute liver failure, hepatic segmental ischemia, and reperfusion and even after LT to silence specific genes associated with liver damage, such as the apoptosis pathway.^{11,15,21,22} Furthermore, mounting experimental and clinical evidence has confirmed that MP restores the cellular energy load, facilitates organ viability assessment, and can be used to deliver specific organ therapies to improve organ function.^{4,5,13,23,24} More specifically, our group has actively worked on variations in organ perfusion modalities and pioneered feasibility studies on siRNA treatment during ex situ MP of rat liver grafts.^{25,26}

In the current study, we readdressed apoptosis inhibition using RNAi therapy to alleviate IRI in a rat liver transplant experimental protocol that could eventually be replicated in clinical practice. Unlike Li et al,¹⁵ who administered FASsiRNA 48 h earlier and preserved livers for only 3 h, our donors received a single dose of siRNA only 2 h before

hepatectomy, and the CIT was extended from 3 to 22 h to intensify the harmful effects of IRI. The 22 h CIT was also chosen because shorter periods of CIT did not induce a strong IRI in our arterialized transplant model, which could make comparisons between treated and untreated groups difficult. Transaminase levels have been considered a surrogate functional biological marker of liver damage.^{11,27-31} Therefore, from our screening experiments, we chose the siRNA sequence that exhibited the lowest transaminase levels at 24 h post-LT. Using this sequence, transaminase levels were significantly lower in the SCS-FASsiRNA group at each time point in the 3-d follow-up experiments than in the control group. On day 3, the transaminase levels tended to be within the normal ranges in both groups, which correlated with the good clinical conditions observed in all animals. Therefore, we did not proceed with a long-term survival experiment to avoid futile use of animals. Only a few deaths occurred during liver implantation due to technical complications. Although arterial anastomosis is non-essential in the rat liver transplant model, rearterialization of liver grafts contributes to excellent graft function and survival rate.³²⁻³⁴ Although whether or not to arterialize rat liver grafts remains a debate in the literature, and this discussion may be beyond the scope of this study, we did so for the following reasons: (1) we wanted to stay as close as possible to the clinical scenario, (2) we believe that graft oxygenation can influence some of the parameters measured, so what we would be showing in the case of nonarterialization would not reflect the real mechanisms of injury that we can find in the clinical setting. However, we were unable to repeat the same experiments shown here in nonarterialized groups to test our hypotheses regarding arterialization of rat liver grafts.

IRI is a phenomenon that involves different interactions between cells and molecules through pathways that are not yet fully understood. In the early stages, damaged hepatocytes release necrotic intracellular debris that induces the

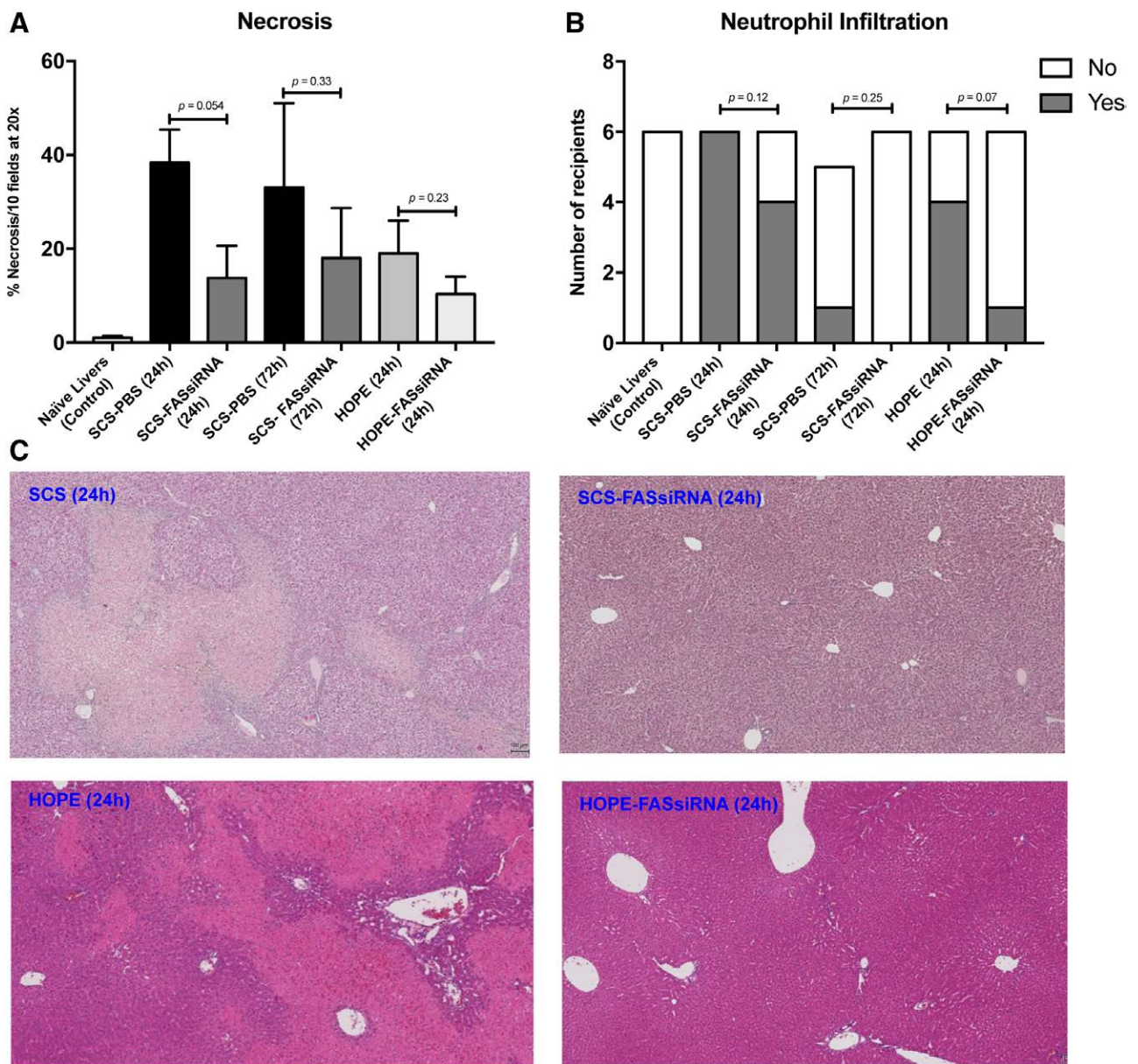


FIGURE 5. Histological analysis. A, Chart displaying the average percentage of necrosis for all experimental groups. B, Chart displaying the distribution of inflammatory infiltration in all transplanted animals in all experimental groups. C, Liver section pictures of a liver section at 24 h representing the SCS (upper left), SCS-FASsiRNA (upper right), HOPE (lower left), and HOPE-FASsiRNA groups (lower right). HOPE, hypothermic oxygenated machine perfusion; SCS, static cold storage; siRNA, small interfering RNA.

expression of many cytokines and chemokines in non-parenchymal liver cells. This proinflammatory environment facilitates cytotoxic cell activation and recruitment, amplifying IRI.^{35,36} Ultimately, activated apoptotic factors contribute to sinusoidal endothelial cell death and subsequent hepatocyte death associated with apoptosis.³⁷ In the SCS protocol, FASsiRNA treatment induced a significant reduction in the serum levels of various proinflammatory cytokines compared with controls. Previous studies have also shown that inhibition of a single gene using RNAi can prevent the expression of several proinflammatory cytokines. In a murine model of hepatic segmental IRI, lower levels of proinflammatory cytokines were induced by downregulation of caspase-1 and nuclear factor kappa B activities after inhibition of NOD-, LRR- and pyrin domain-containing protein 3 gene.³⁸ The same effect was

observed for high-mobility group box 1.²¹ Although the FAS receptor is defined as a classical factor for the apoptosis-programmed cell death pathway, NOD-, LRR- and pyrin domain-containing protein 3 and high-mobility group box 1 have also been associated with other cell death pathways, such as pyroptosis and necroptosis.^{39,40}

Given that many of these inhibited proinflammatory cytokines act as chemoattractants for hepatic polymorphonuclear leukocyte infiltration, we hypothesized that less tissue infiltration can occur in the treated groups. Unfortunately, when we looked at the neutrophil infiltration, we did not find a statistical difference between the FASsiRNA groups and their respective control groups. Liver histological lesions are expected to be a corollary of all molecular and cellular damage occurring during IRI. We then analyzed 2 samples per animal collected in the

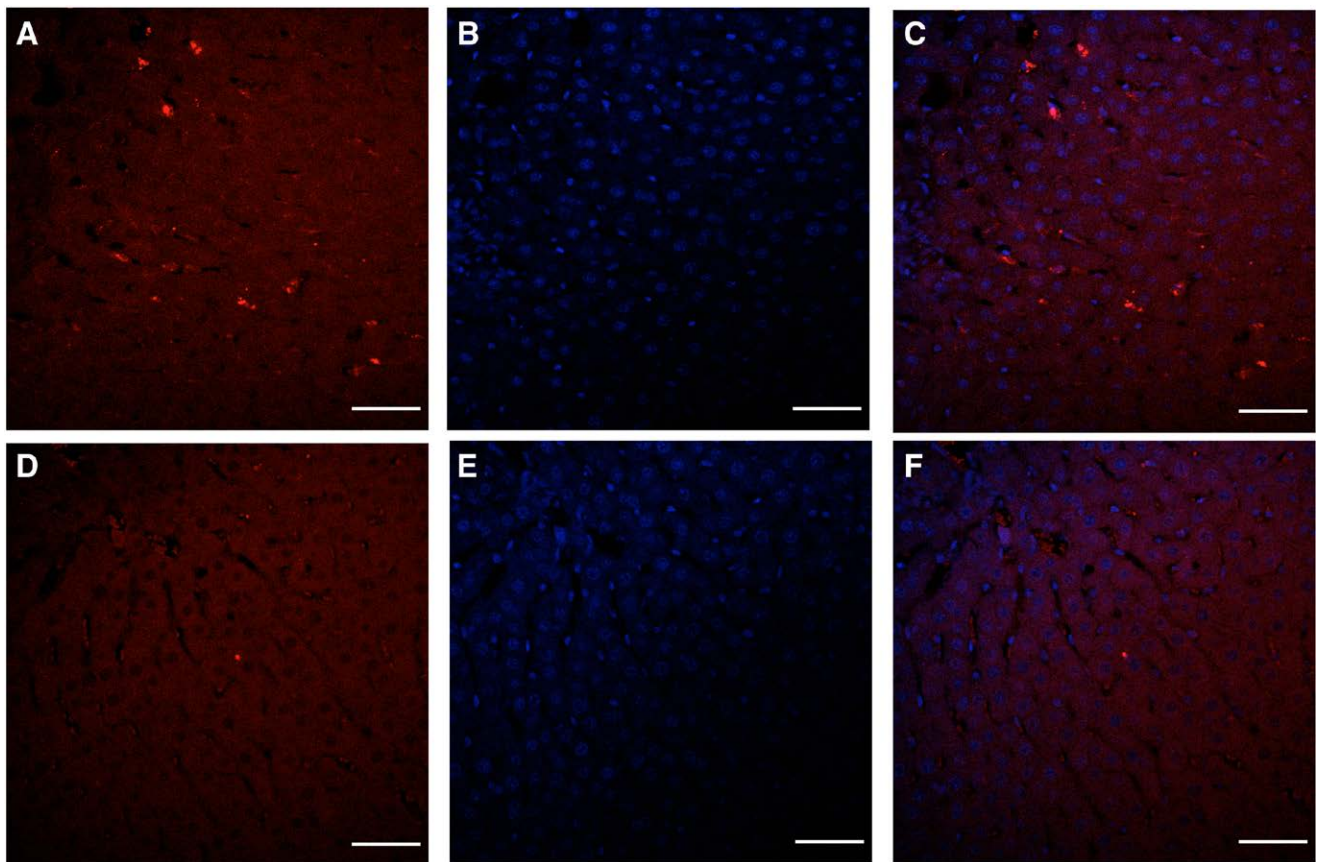


FIGURE 6. Confocal microscopy analysis of liver samples from the HOPE protocol: HOPE-FASsiRNA-treated livers at 24h posttransplant (A) show uptake of Cy3-conjugated FASsiRNA compared with livers perfused with Belzer MPS Machine Perfusion Solution, Bridge to Life (MPS) alone (D). Nuclei are visualized in blue for treated (B) vs control (E). Merged images of treated (C) vs control (F) transplanted animals. HOPE, hypothermic oxygenated machine perfusion; siRNA, small interfering RNA.

right and in the left side of the liver, with the aim of eliminating any potential variation in reperfusion to different rat liver lobes, and in this way had a more representative histological reading. Initial histological evaluation using the 4-level graded Suzuki score did not detect significant differences in any of the experimental groups with respect to parenchymal necrosis, sinusoidal congestion, and cytoplasmic vacuolization. Therefore, we verified the histological damage by calculating the percentage of necrosis in 10 microscopic fields per sample. This methodology did not allow us to identify statistical differences in the necrotic damage in the FASsiRNA groups while the P was borderline ($P=0.054$) in the SCS protocol. Although this topic is beyond the scope of the current study, future studies are required to define whether graded scores are appropriate for assessing histological damage in similar conditions. In the same way, there were no differences on the levels of apoptotic positive cells in the FASsiRNA groups comparing with the untreated groups. Taken together, these results may suggest that insufficient FAS silencing was related to the use of an inappropriate siRNA dose. Another less likely explanation for some anti-inflammatory effects in the absence of antiapoptotic effects in our model is that there was deactivation of FAS apoptosis-independent inflammatory pathways, as shown in other studies.⁴¹⁻⁴³

Although our results confirmed some positive effect of FASsiRNA therapy using a potentially replicable SCS experimental protocol in clinical practice, it may still pose

some logistical and ethical issues. It could be an ethical challenge to administer siRNA treatment to a multiorgan donor, as the agreement of all the different transplant teams is required, including the consent of all future recipients of each organ from such donors. Furthermore, it would substantially increase costs because high doses would be necessary to treat the donor with the aim of achieving an effective benefit in the target organ. Therefore, ex situ MP could be a valuable option to overcome the aforementioned problems because a single organ will be treated with siRNA compounds. In addition, it can improve siRNA uptake efficiency by the perfused organ, which can decrease the required therapeutic doses and, consequently, reduce costs. Consent is required from only 1 patient, decreasing the significant ethical and logistical burden for treating the multiorgan donor.

In the case of RNAi therapy, the physiological temperature during normothermic MP could theoretically be better for cellular siRNA uptake and function than during hypothermic MP; however, the presence of RNases in blood-derived perfusates can eventually inactivate siRNA compounds. Furthermore, experimental normothermic perfusion is technically complex and costly.⁴⁴ Based on our previous study showing siRNA hepatic uptake during hypothermic perfusion,²⁶ we maintained the same perfusion modality. Next, in a proof-of-concept experiment, we used a well-established experimental protocol of a 4 h SCS period followed by 1 h HOPE.^{12,13,45} Unfortunately, while

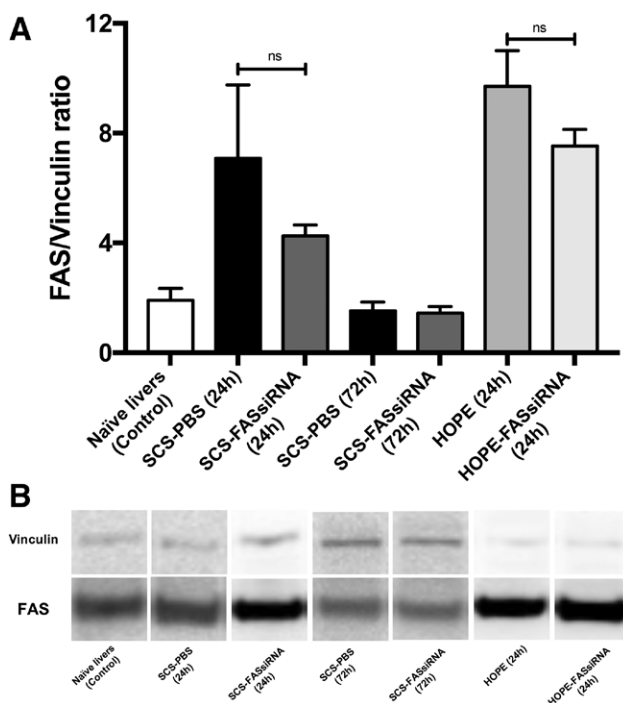


FIGURE 7. FAS protein expression after liver transplantation (LT). A, Chart displaying the average ratio of FAS/vinculin for all experiment groups. B, Gel representation of the Western blot analysis of the FAS protein expression and the vinculin. HOPE, hypothermic oxygenated machine perfusion; ns, not significant; SCS, static cold storage; siRNA, small interfering RNA.

using this protocol, we did not observe statistical differences on the post-LT transaminase levels between the HOPE-FASsiRNA group compared with controls. Based on that, we can hypothesize several possible explanations for the discrepancy between the results of the SCP and HOPE groups: (1) inability to perform power analysis to determine adequate sample size calculations due to a lack of similar studies using siRNA during MP, (2) insufficient FAS silencing during hypothermic MP, (3) the low IRI associated to the very short period of SCS (4h) maybe being one of the reasons for the low transaminase levels observed in the HOPE protocol, (4) transaminases washout proportionated by the HOPE perfusion session. In this model, the transaminases accumulated in the graft are flushed out the graft to the perfusate, which is discarded before transplant. Indeed, in a liver porcine normothermic machine perfusion experimental model, an infusion of a high concentration transaminases solution in the perfusate system resulted in a progressive reduction of the transaminases levels over time. Although they observed a slight increase of transaminases levels in the control group, the authors hypothesized about a potential binding of transaminases to the components of the perfusions system.⁴⁶ These same factors could be evoked to explain the lack of differences on the proinflammatory cytokine levels observed between the 2 groups in the HOPE protocol, the FASsiRNA group and the untreated group; however, unlike the SCS groups, IL-10 levels unexpectedly increased significantly in the HOPE FASsiRNA group, suggesting activation of anti-inflammatory mechanisms by adding FAS-siRNA during HOPE. Indeed, IL-4 and IL-10 have been associated with the inhibition of apoptosis associated with FAS and less liver damage in *in vitro* and *in vivo* studies.^{47,48} In the context of liver MP, Jassem et al⁴⁹ found

a negative correlation between CIT and the frequency of IL-10-producing CD4⁺ T cells in livers undergoing SCS preservation; conversely, an expanded pool of regulatory T cells was observed in livers conserved with normothermic machine perfusion with the potential to enhance IL-10 production. Histological damage was minimal in the HOPE-FASsiRNA group, with only 1 animal showing neutrophil infiltration compared with 4 animals in the control group. In a model of concanavalin-A-induced acute liver injury, Song et al¹¹ also reported less hepatic neutrophil infiltration in FASsiRNA-treated mice. These findings highlight the association between FAS and inflammation, not only with apoptosis.^{42,43} In the same way, we did not observe differences on the necrosis levels and in the apoptotic index between the FASsiRNA treated group and the untreated group. Taken together, the HOPE protocol results were not as strong as the SCS results. This can be explained by a shorter CIT of 4h (instead of 22) and by the intrinsic beneficial effect of HOPE on post-LT IRI as mentioned above.^{12,13}

Although hypothermia could affect cellular uptake of siRNA compounds, our qualitative confocal microscopic evaluation showed the presence of siRNA compounds in liver samples collected 24h post-LT in the HOPE-FASsiRNA group. Furthermore, reduced, albeit nonsignificant, levels of FAS protein in the FASsiRNA groups were correlated with beneficial changes in transaminase and proinflammatory cytokine serum levels, apoptotic index, hepatic inflammatory infiltration, and necrosis compared with the control groups.

The design of siRNA compounds is constantly evolving with the goal of achieving better tissue distribution and gene specificity with fewer off-target effects.^{50,51} Here, we preliminarily tested 2 siRNA sequences already proven to be effective in alleviating liver damage in 2 different experimental models (a murine acute liver failure and a rodent liver transplant models).^{11,15} Although these 2 sequences were already tested against nonsense siRNA controls in their original studies, we must acknowledge that we did not conduct experiments with nonsense siRNA compounds because of logistical and costs limitations. We used the most effective one, which was delivered in a “naked way” by the rapid hydrodynamic injection method.^{10,11} The use of the naked siRNA compound, without any associated molecule such as *N*-acetylgalactosamine used to improve tissue absorption and gene specificity,⁵² may partly explain the limitations of the extent of the protective effects of the FASsiRNA with the inconclusive results showed by our model. We also acknowledge that just 1h of HOPE, though it is one of the most common HOPE experimental protocols used, might not be sufficient for optimal uptake of FASsiRNA from hepatocytes under low metabolic conditions promoted by hypothermia.^{12,13,53} Despite this, we were able to verify some beneficial effect of FASsiRNA therapy to alleviate post-LT IRI in the SCS protocol (lower transaminases and less proinflammatory cytokines) and liver tissue absorption of FASsiRNA in the HOPE protocol; however, hepatic uptake of siRNA did not translate into a significant decrease in FAS mRNA or protein or decreased hepatocyte apoptosis, which would have confirmed the feasibility of this therapeutic protocol associating siRNA during HOPE organ preservation to alleviate post-LT IRI. Further studies using newly

developed strategies to deliver more efficiently specific siRNA compounds (eg, nanoparticles, siRNA associated with *N*-acetylgalactosamine for better cellular absorption) could optimize RNAi organ therapy during MP. Because there were no similar studies of siRNA therapy during MP before to guide us with the effective siRNA dose and determination of the sample size, it might be that our study was underpowered.

CONCLUSION

Although siRNA therapy and MP technology have already proven their beneficial effect in alleviating post-LT IRI in a rodent liver transplant model when used individually, their association as a potential strategy to further improve function of liver graft proved to be very challenging in our experiment model. This was demonstrated by the inconclusive results in favor of our hypothesis that gene modulation during MP can be effective and feasible with the aim of improving graft function; however, the fact that we were able to demonstrate that liver siRNA absorption happens during hypothermic oxygen perfusion in a transplant model may suggest that the combination of MP and RNAi therapy can be a useful therapeutic strategy to decrease the severity of IRI associated with poor quality organs such as steatotic livers and deceased after circulatory death livers. Further studies are required to prove the effectiveness of this strategy, which should include a better understanding of the pharmacokinetics of siRNA compounds specifically designed for a better cellular uptake during hypothermic and normothermic perfusions.

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