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# SOD Mimetic Improves the Function, Growth, and Survival of Small-Size Liver Grafts After Transplantation in Rats

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**Background.** Small-for-size syndrome (SFSS) may occur when graft volume is less than 45% of the standard liver volume, and it manifests as retarded growth and failure of the grafts and more mortality. However, its pathogenesis is poorly understood, and few effective interventions have been attempted.

**Aims.** The present study aimed to delineate the critical role of oxidant stress in SFSS and protective effects of a superoxide dismutase mimetic, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), on graft function, growth, and survival in the recipient rats.

**Methods.** Small size graft liver transplantation (SSGLT) was performed to determine the survival, graft injury, and growth. MnTBAP was administered in SSGLT recipients (SSGLT+MnTBAP).

**Results.** Serum alanine aminotransferase levels were sustained higher in SSGLT recipients, which were correlated with an increased apoptotic cell count and hepatocellular necrosis in liver sections. Malondialdehyde content, gene expression of tumor necrosis factor  $\alpha$  and interleukin  $1\beta$ , and DNA binding activity of nuclear factor- $\kappa$ B in the grafts were increased significantly in SSGLT recipients compared with sham-operated controls. Both phosphorylated p38 mitogen-activated protein kinase and nuclear c-Jun were increased in SSGLT. All these changes were strikingly reversed by the administration of MnTBAP, with an increase in serum superoxide dismutase activity. Moreover, in situ bromodeoxyuridine incorporation demonstrated that graft regeneration was much more profound in the SSGLT+MnTBAP group than in the SSGLT group. Finally, the survival of recipients with MnTBAP treatments was significantly improved.

**Conclusions.** Enhanced oxidant stress with activation of the p38/c-Jun/nuclear factor- $\kappa$ B signaling pathway contributes to SFSS-associated graft failure, retarded graft growth, and poor survival. MnTBAP effectively reversed the pathologic changes in SFSS-associated graft failure.

**Keywords:** Living-donor liver transplantation, Small-for-size syndrome, Oxidant stress, SOD mimetics.

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Liver transplantation is the only established treatment of liver failure as a result of acute toxicity, hepatitis, or end-stage liver disease. More than 25,000 patients are on the waiting list in the United States, and only 6341 received

transplants in 2011, with a waiting duration up to 6 years ([www.unos.org](http://www.unos.org)). To cope with the severe shortage of donor livers, living-donor liver transplantation (LDLT) emerges as an alternative method to shorten the waiting duration and

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The authors declare no conflicts of interest.

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Y.-Y.C. participated in designing and performing most of the experiments, collecting data, and preparing the article. J.-M.Q. participated in providing supervision and technical guidance for liver transplantation. A.-H.Y., X.-M.Z., and Q.D. participated in liver transplantations and animal care. X.-F.Q. participated in performing histologic examination. Z.-Y.M. participated in partially analyzing data. Y.Z. participated in liver transplantations and measuring liver malondialdehyde concentration. J.Z. participated in animal care and performing electrophoretic mobility shift assays. S.W. participated in overall supervision and providing funding support. J.W. participated in making the conceptual vision, analyzing data, preparing the article, and providing partial funding support.

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improve the donor liver quality (1). However, the associated ethnical concerns, surgical complexity, and complications in both donors and recipients still limit its wide application. In fact, the number of LDLT performed in the United States has declined in the recent 8 years (www.unos.org). One of the complications associated with LDLT is small-for-size syndrome (SFSS), which manifests as malfunction, loss, or retarded growth of the graft and is thought to be partially caused by excessive portal blood flow and obstructed hepatic venous outflow (2). Both changes may lead to disordered hemodynamics of the portal circulation (2). However, the transient increase in portal circulation could not explain the quick loss and delayed growth of the small-size grafts (SSGs), and the molecular mechanisms underlying the injury and delayed growth in SFSS, which happens when the ratios of graft volume over standard liver volume is less than 45%, are poorly understood.

Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) is a synthetic nonpeptidyl mimetic of superoxide dismutase (SOD) with a low molecular weight. It overcomes the extreme short half-life of scavenging activity of the enzyme and may have a wider clinical application than natural SOD (3, 4). MnTBAP is also nonimmunogenic and may cross the plasma membrane to neutralize reactive oxygen species intracellularly. However, there is no study that investigates whether MnTBAP improves the graft function, survival, and growth in SSG liver transplantation (SSGLT), which is a valuable animal model of LDLT. Our previous studies have documented that significant oxidant stress is one of pathogenic mechanisms in ischemia-reperfusion (I/R)-induced graft injury in SSGLT recipient rats (5). We have used the SSGLT model to investigate the protective effects of a pre-ischemic maneuver against subsequent I/R-induced injury (5) and microRNA regulation of graft growth in rats (6). In this study, MnTBAP was administered in the SSG recipients to determine its protective effects on graft function, growth, and survival of small-for-size (SFS) graft recipient rats to explore the critical role of oxidant stress in the SFSS development.

## RESULTS

### SFS-Associated Graft Injury and Its Attenuation by MnTBAP

Graft injury and failure with increased mortality is often seen in SFS liver transplantations with less than 45% graft volume in rats (5). Recipient rats with small-size liver transplantation at 30% of graft volume experienced severe graft damage with elevated serum levels of alanine aminotransferase (ALT) activity (Fig. 1B), increased apoptotic cell counts (Fig. 2E,F) compared with the sham-operated animals, and focal to massive cell death in graft histologic condition for 1 week after transplantation (Fig. 2A–C). The graft failure led to a poor survival rate (40%, 6/15) during the first 2 weeks after transplantation (Fig. 1A). However, with MnTBAP pretreatment in the donor and the subsequent treatment in recipients after transplantation, serum ALT levels were much lower than in SSGLT recipients without MnTBAP treatment, as were the apoptotic cell counts (65.5±12.7 vs. 41.5±8.6,  $P<0.01$ ) 3 days after transplantation (Fig. 2G,H). The improvement in the graft damage was evidenced by hematoxylin-eosin staining (Fig. 2D). All these

contributed to an improved survival rate (66.7%, 10/15) in recipient rats with MnTBAP treatments ( $P<0.05$ ) (Fig. 1A). Thus, it is evident that the graft failure and poor survival in 30% SSGLT recipient rats were strikingly improved by a series of MnTBAP treatments.

### Oxidant Stress Is Responsible for SFS-Associated Graft Damage

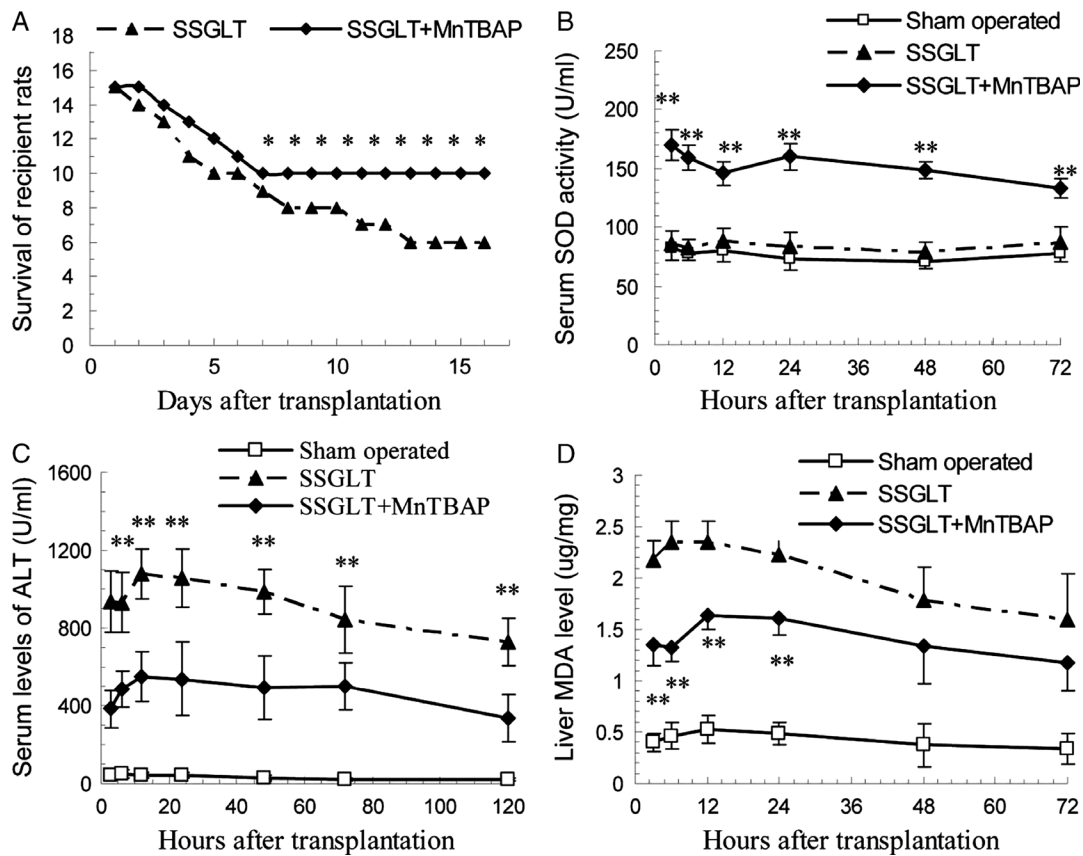
To determine whether oxidant stress is a crucial factor causing severe damage in SSGLT, malondialdehyde (MDA) content in SSGs was assayed over time after transplantation. As shown in Figure 1D, MDA content was markedly increased for 3 hr and sustained during the first 3 days after transplantation. In contrast, MnTBAP treatment significantly lowered MDA levels in the graft tissues over time. As a proof of the effectiveness of MnTBAP treatment, elevated serum SOD activity was observed in recipient rats during the first 3 days compared with the sham-operated or SSGLT recipient rats (Fig. 1C). These data indicate that elevated oxidant stress may be one of critical factors responsible for the marked damage in the SSGs after transplantation and that the dismutation of superoxide anions or other antioxidant effects by MnTBAP seems to be the prime mechanism for its protection as indicated in a long-term animal study with a fatty liver model (7).

### TNF- $\alpha$ and IL-1 $\beta$ are the Inflammatory Mediators for SFS-Associated Graft Injury

Enhanced oxidant stress in the SFSS grafts results in up-regulation of inflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ , which in turn attract inflammatory infiltration and activate intracellular signaling pathways. As shown in Figure 3, the gene expression of both TNF- $\alpha$  and IL-1 $\beta$  cytokines was markedly up-regulated as early as 3 hr after the transplantation and was sustained for 3 days. The downstream signaling molecule, such as p38 mitogen-activated protein kinase (MAPK), was phosphorylated as evidenced by the fact that phosphorylated p38 MAPK content in the cytoplasm was increased significantly (Fig. 4A,B). c-Jun is a transcription factor that binds to the enhancer heptamer motif, and rallies the activating signaling to further activate genes involved in stress and inflammatory responses. It is also evident that c-Jun in the nuclear fraction of SSGs was strikingly increased, indicating that the nuclear translocation of this transcription factor was enhanced over time after transplantation (Fig. 4A,C). Moreover, nuclear factor (NF)- $\kappa$ B is a transcription factor responsible for a strong inflammatory response to oxidant stress and was activated as documented by enhanced DNA binding activity of its p65 subunit in the nuclear extract as shown in Figure 4D. In contrast, enhanced expression of TNF- $\alpha$  and IL-1 $\beta$  genes and the activation of p38 MAPK, c-Jun, and NF- $\kappa$ B in the SSGs were all abrogated markedly by the treatment with SOD mimetic, MnTBAP (Figs. 3 and 4). These data indicate that antioxidant treatment not only minimized oxidant stress but also reduced the inflammatory cytokine release and abolished intracellular signaling molecules that are key inflammatory regulators, such as NF- $\kappa$ B (8).

### Improved Graft Growth Contributing to a Better Survival in SSGLT Recipients

One critical issue in SSG transplantation is whether the graft will grow after transplantation. The growth rate and



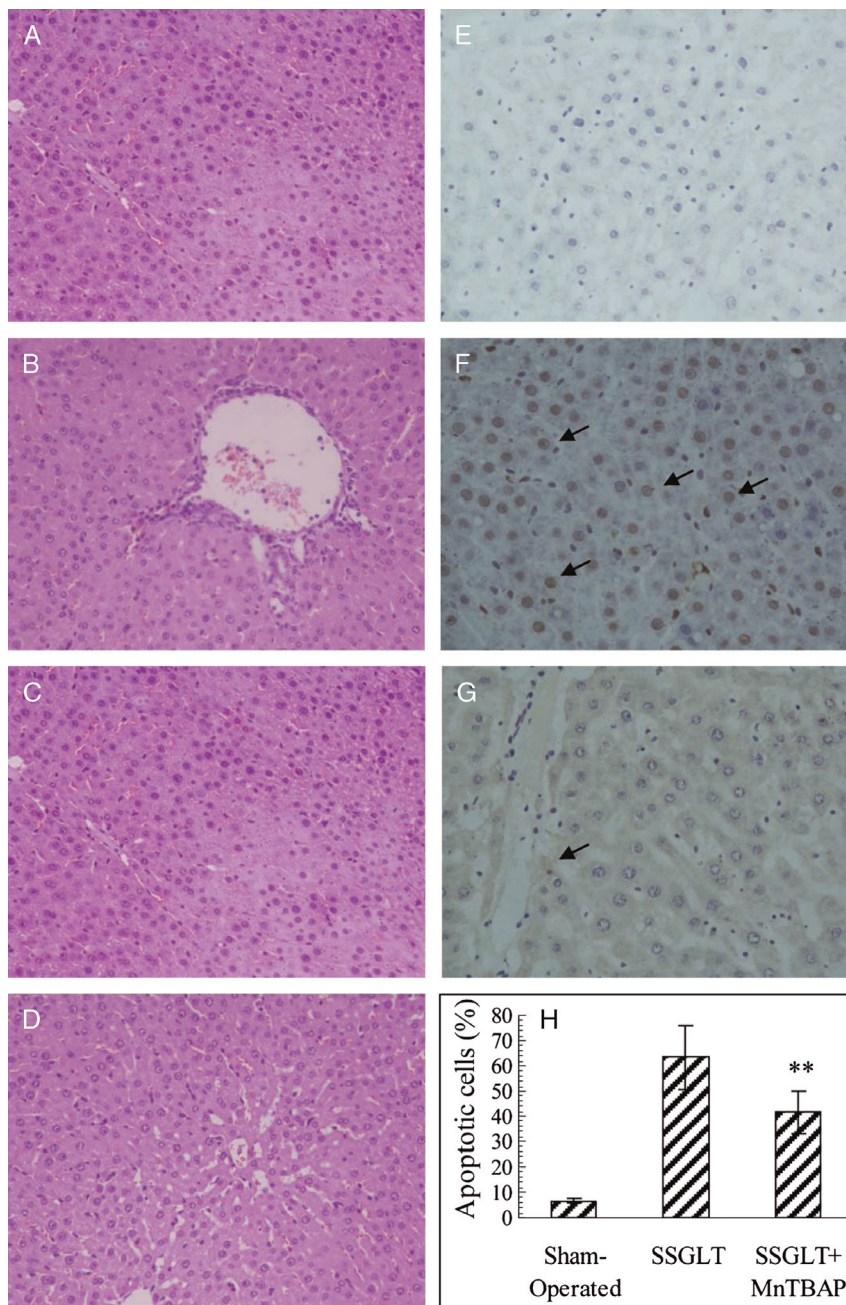
**FIGURE 1.** Animal survival, serum superoxide dismutase (SOD) activity, injury, and malondialdehyde (MDA) content of small-for-size hepatic graft. **A**, Survival of small-size graft liver transplantation (SSGLT) recipient rats with Mn(II)tetrakis (4-benzoic acid)porphyrin chloride (MnTBAP) treatment. MnTBAP was given once daily after the transplantation until sacrifice. Improved survival was seen in those with MnTBAP treatment 8 days after the treatment. \*  $P < 0.05$  compared with the SSGLT group ( $n = 15$ ). **B**, Serum alanine aminotransferase (ALT) levels at different time points after SSGLT. Data were presented as mean (SD),  $n = 6$  in each group. \*  $P < 0.05$  and \*\*  $P < 0.01$  compared with the SSGLT group. **C**, Increased serum SOD activity in SSGLT recipient rats with MnTBAP treatment was shown. Serum SOD activity was determined at indicated time points after the transplantation.  $n = 6$ . \*\*  $P < 0.01$  compared with SSGLT. **D**, Decreased liver MDA levels in SSGLT recipient rats with MnTBAP treatment. Liver MDA was determined at indicated time points spectrophotometrically to represent oxidant stress,  $n = 6$  in each group. \*\*  $P < 0.01$  compared with SSGLT alone.

capacity of the SSGs affect the overall graft function and animal survival (6). We used an in situ 5-bromo-20-deoxyuridine (BrdU) incorporation assay to determine the graft regeneration after transplantation. As shown in Figure 5, BrdU-positive cells in SSGs were increased approximately 3-fold at day 7 in SSGLT without MnTBAP treatment compared with that at 12 hr after transplantation, and then the BrdU-positive cell count started to decline. In contrast, BrdU-positive cell count was significantly higher than in those without MnTBAP treatment from day 2 to day 7, which clearly demonstrates that graft growth was more profound in the recipient rats with MnTBAP treatments than in those without the treatment and that the improved graft growth positively affected the animal survival as shown in Figure 1.

## DISCUSSION

The scarcity of donor livers drives the use of small, split, or margin grafts for transplantation in orthotopic or LDLT, which often leads to the onset of SFSS (9), presenting as malfunction and retarded growth of the grafts with signifi-

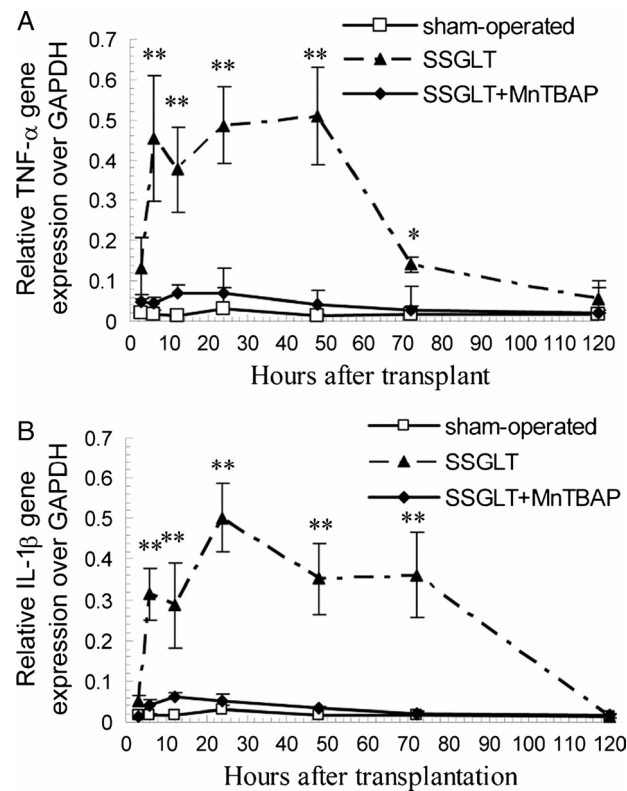
cant morbidity and mortality (10). Although the causes of SFSS may be multifactorial in a clinical setting, including lower graft volume/standard liver volume (<30%–40%), more than 30% steatosis, and prolonged I/R time in graft-related factors, insufficient graft volume is the key factor leading to pathophysiological changes, such as portal hyperperfusion with impaired venous outflow and hepatic artery flow, in turn causing sinusoidal congestion and endothelial dysfunction, as well as parenchymal cell death (9, 10). Surgical procedures, for example, portosystemic shunt and splenectomy, were implemented for reducing hyperperfusion in preventing the occurrence of SFSS (10, 11). Therefore, these studies support the hypothesis that an increase in portal flow with increased portal pressure (see SDC, <http://links.lww.com/TP/A693>) may be one of crucial factors contributing to the development of SFSS. On the other hand, portal hyperperfusion in SSGs at 29% graft volume is the major cause and stimulus for the graft growth in pig recipients (2). Thus, it seems that disordered hemodynamics in the portal circulation plays a significant role in SFSS development, although there is no close correlation between



**FIGURE 2.** Representative micrographs of liver histologic condition after small-size graft liver transplantation (SSGLT) with or without Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) treatment. A–D, Hematoxylin-eosin staining for small-size liver graft sections at different time points after transplantation. A, 24 hrs after transplantation. B, 3 days after transplantation. C, 5 days after transplantation. D, 3 days after transplantation with MnTBAP treatment. E–G, Representative micrographs of in situ terminal deoxynucleotidyl transferase dUTP nick end labeling staining of apoptotic cells in small-size grafts. Each section was examined for 10 high-power fields, both apoptotic and all cells were counted. E, Sham-operated control. F, Small size graft at 30% volume 72 hr after transplantation. G, Small size graft at 30% volume from recipient rats with MnTBAP treatment ( $\times 400$ ). H, Apoptotic cell count was expressed as follows: apoptotic cell count (%)=(apoptotic cells/total cell count) $\times 100$ . Average apoptotic cell count (%) in each group 3 days after transplantation. \*\*  $P < 0.01$  compared with SSGLT.  $n = 6$  in each group.

portal pressure and flow in the liver transplantation setting (12). Moreover, the portal hyperperfusion hypothesis could not explain all changes in SFSS, such as significant apoptosis or necrosis and retarded growth. There is no study available

so far to reveal the underlying molecular mechanisms of retarded graft growth and to delineate the critical role of oxidant stress in the mediation of graft dysfunction and delayed growth. Studies aiming at exploring the molecular

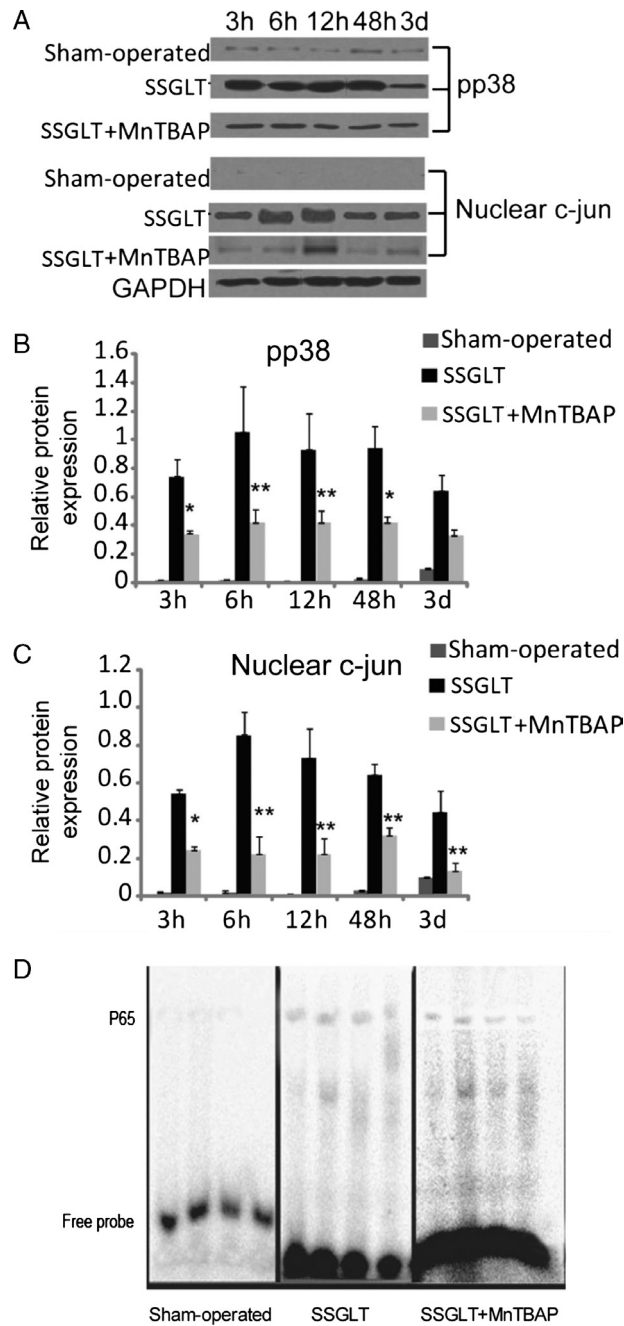


**FIGURE 3.** Up-regulated expression of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  genes in the mediation of small-size graft injury. Increased TNF- $\alpha$  (A) and IL-1 $\beta$  (B) gene expression was demonstrated at an early phase (2–3 days) after small-size graft liver transplantation (SSGLT) and Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) significantly attenuated the enhanced TNF- $\alpha$  and IL-1 $\beta$  gene expression as demonstrated by real-time reverse transcriptase polymerase chain reaction analysis in the graft tissue. The *GAPDH* gene was used as a housekeeping control.  $n=6$ , \*  $P<0.05$ , \*\*  $P<0.01$  compared with the SSGLT group. The sequences of the forward and reverse primers are: 5'-CGA TTT GCC ATT TCA TAC CAG-3' and 5'-AGT ACT TGG GCA GGT TGAC-3' for TNF- $\alpha$  (amplicon size, 180 base pair [bp]); 5'-TGT GGA TCC CAA ACA ATA CCC-3' and 5'-TAT GTC CCG ACC ATT GC-3' for IL-1 $\beta$  (amplicon size, 175 bp); and 5'-TGT GCA GTG CCA GCC TCG TCT-3' and 5'-TTG CCG TGG GTA GAG TCA TAC-3' for *GAPDH* (amplicon size, 190 bp).

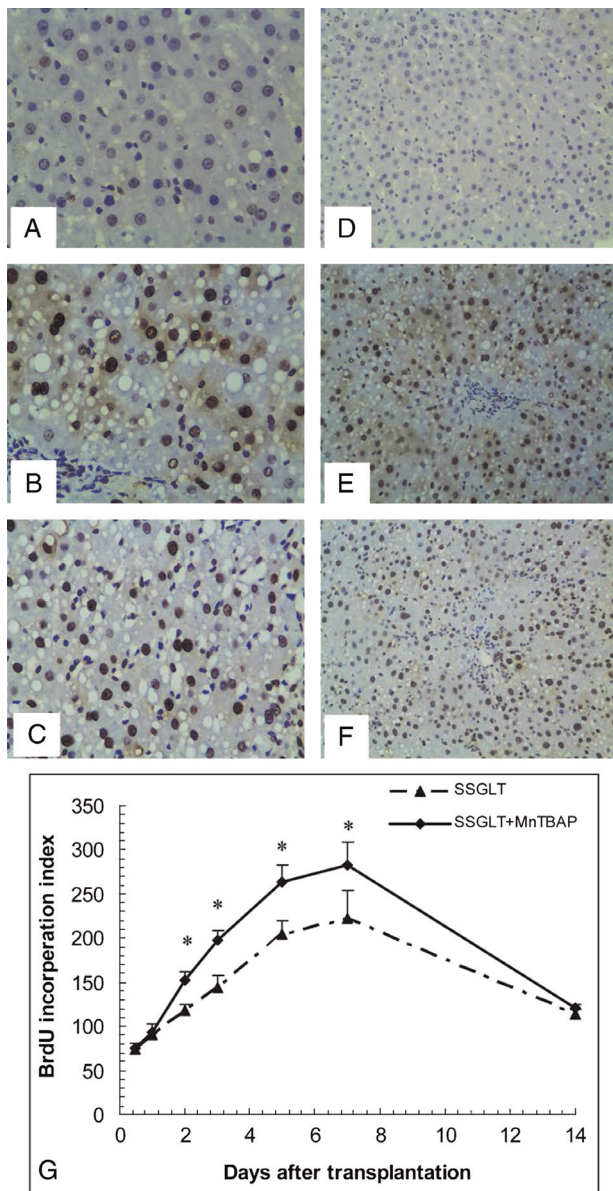
mechanisms are hampered by the surgical difficulties in establishing rodent models of SSGLT.

In the present study, we used a rat model of SSGLT at 30% graft volume to investigate the critical role of oxidant stress and downstream signaling mechanisms in the SFSS development. Our data have demonstrated that marked graft injury occurred with highly elevated serum ALT levels, increased apoptotic cell counts and focal to massive cell death in histologic condition during the first 5 days after the transplantation. The survival rate of the recipients was approximately 40% for 2 weeks. These changes were in parallel with an increase in liver MDA content, which is the classic indicator of enhanced lipid peroxidation, increased expression of inflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , and activation of p38 MAPK, c-Jun, and NF- $\kappa$ B. The enhanced TNF- $\alpha$  and IL-1 $\beta$  expression in the SSG caused the activation of downstream signaling molecules, such as p38 MAPK and c-Jun activation. c-Jun is the substrate of c-Jun N-terminal kinase (JNK), which binds to and phosphorylates c-Jun at serines 63 and 73 located within its transactivation domain under stressful and inflammatory stimuli and triggers the translocation of c-Jun into the nuclei to activate the genes involved in stress and inflammation (13). JNK plays an

important role in the stress response and is activated by reactive oxygen species and cytokines, such as TNF- $\alpha$  (14), both of which were obviously increased in the SSGs (5, 15), contributing to pronounced apoptosis in the graft histologic condition as shown by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling staining in the present study and our previous studies (16). At the same time, the activation of p38 MAPK and JNK elicits the translocation of NF- $\kappa$ B 65 subunit into the nuclei to turn on stress or inflammatory genes and leads to the initiation and perpetuation of the inflammatory responses (8). In contrast, the treatment with MnTBAP partially reversed all these changes and resulted in much lower serum ALT levels, reduced apoptotic cell counts and improved histologic condition, increased serum SOD activity and lower liver MDA contents, and a much improved survival rate of the recipients. All of these data demonstrate that oxidant stress plays a pivotal role in the SFSS development and that TNF- $\alpha$ /IL-1 $\beta$ -initiated activation of the p38 MAPK/JNK/c-Jun-NF- $\kappa$ B pathway is attributable for the oxidant stress-elicited signaling mechanisms during the onset of SFSS. The treatment with MnTBAP or other SOD mimetics proves to be effective in ameliorating oxidant stress in vitro (17) and radiation-induced oxidant



**FIGURE 4.** Western blot analysis of activation of p38 mitogen-activated protein kinase (MAPK) and c-Jun signaling pathway in small-size graft tissue. A, Phosphorylated p38 MAPK and nuclear c-Jun levels in small-size graft tissue at various time points were determined by Western blot analysis using glyceraldehyde-3-phosphate dehydrogenase as a loading control (shown are the representative images). Please note that the activation of p38 MAPK was observed during the first 48 hr, whereas nuclear c-Jun was enhanced during the first 12 hr after transplantation. The activation of both p38 MAPK and c-Jun was attenuated by the treatment with Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP). B and C, Densitometrical analysis of the Western blot images of p38 MAPK and c-Jun was performed with Image-Pro Plus software (n=6). \*  $P < 0.05$  and \*\*  $P < 0.01$  compared with the small-size graft liver transplantation (SSGLT) group. D, Electrophoretic mobility shift assays of NF- $\kappa$ B in small-size graft tissue after transplantation. The biotin 3'-end-labeled double-stranded specific DNA probe is 5'-TTGTTACAAGGGACTTTCGGCTGGGACTTTCAGGGAGGCGTGG-3' (the underlined indicates NF- $\kappa$ B-binding sites). Electrophoretic mobility shift assay image of NF- $\kappa$ B p65 subunit in Sham-operated, SSGLT, and SSGLT+MnTBAP groups (n=4). Early activation of NF- $\kappa$ B was demonstrated in the graft tissue of SSGLT recipient rats compared with Sham-operated group, and decreased DNA binding activity was shown in SSGLT+MnTBAP group in comparison with SSGLT.



**FIGURE 5.** Graft regeneration assessed by in situ bromodeoxyuridine (BrdU) incorporation. The BrdU-positive cell counts were significantly increased in Mn(III)tetrakis (4-benzoic acid)porphyrin chloride (MnTBAP)-treated rats compared with those without MnTBAP treatments after transplantation. Immunohistochemical staining for BrdU-positive cells in liver tissue from the control (A and D), small-size graft liver transplantation (SSGLT) (B and E), and SSGLT with MnTBAP treatment (C and F) groups 48 hr after transplantation (original magnification  $\times 400$  [A–C] and  $\times 200$  [D–F]). The BrdU-positive cells were counted in each section for 10 high-power fields. \*  $P < 0.05$  compared with the SSGLT group at the same time point,  $n = 5$  at each time point of the two groups.

stress in rats (18). To our best knowledge, to date, we are the first group in using this potent antioxidant in improving graft function and survival in SSGLT recipients.

The growth of SSG is critical for the overall function and well-being of the recipients in LDLT (6). One of the factors that give rise to SFSS is the delayed regeneration re-

sponse after the implantation (19). The I/R-induced graft damage, hyperperfusion-associated volume stress, and poor quality of donor organs (with steatosis or extended cold ischemia duration) may all contribute to the further damage and impaired growth of the SSGs in recipients (10). In the present study, we found that the treatment with MnTBAP markedly improved the growth of the SSGs as evidenced by significantly increased BrdU in situ incorporation. The improved growth of the SSGs in these recipients may be the results of reduced oxidant stress, minimized stress and inflammatory responses, as well as injurious process in the grafts. However, it remains to be investigated whether MnTBAP acts on hepatocytes directly or through other molecular pathways, such as inhibiting oxidant stress or ameliorating the injury process, in promoting graft growth (6).

In conclusion, the results presented in this study demonstrate that enhanced oxidant stress with activation of the p38 MAPK/c-Jun/NF- $\kappa$ B signaling pathway contributes to SFS-associated graft failure, retarded graft growth, and poor survival. MnTBAP is an effective antioxidant, which strikingly reversed the pathological changes of SFS-associated graft failure and may have potential clinical application in a transplant setting.

## MATERIALS AND METHODS

### Animals and SSGLT

Male Sprague-Dawley rats (weighing 220–250 g) were purchased from Slack Shanghai Laboratory Animal Co., Ltd. (Shanghai, China), and fed standard pellet diet on a 12-hr light/dark cycle with free access to water and food. The animal experiment protocol was approved by the Institutional Ethical Committee of Animal Experimentation, and the experiments were performed strictly according to governmental and international guidelines. Animals were fasted overnight before surgical procedures (allowing free access to water). All surgical procedures were performed under sterile conditions. Animals were divided into sham-operated ( $n = 6$ ), SSGLT ( $n = 36$ ), and SSGLT+MnTBAP ( $n = 36$ ) groups. Additional 30 animals were used for in situ BrdU incorporation experiment. SSGLT was performed with “two-cuff” method as we previously described (5). The median lobe of the liver was retained to ensure that graft volume/standard liver volume was no higher than 30% (20). Rats were restrained in the supine position after administration of anesthetics (ketamine and xylazine) as we previously reported (15). MnTBAP with 99% purity, purchased from Alexis Biochemicals (San Diego, CA) was dissolved in normal saline and injected intraperitoneally at 10 mg/kg, 4 hr before transplant in donor rats and once daily until sacrifice in recipient rats. The SSGLT group received the same volume of normal saline injections each time when MnTBAP was given to rats in the SSGLT+MnTBAP group. After transplant surgery, rats were kept warm for 1 hr, and provided with 10% glucose in drinking water. In the sham-operated group, the ligaments around the liver were separated. The abdomen was closed after the liver lobes were turned upside down and returned back. Normally, recipient rats did not receive any antibiotics unless confirmed infection occurred. Otherwise, gentamycin was injected intraperitoneally at 10 mg/kg, once daily for 3 to 5 days. Fifteen recipient rats from each group were used to observe the survival rate, and the remaining were sacrificed at specific time points to collect liver graft tissue for histologic examinations. Blood samples were collected for serum levels of ALT and SOD activity assays at time points as indicated in the result section.

### Serum ALT

Serum ALT was assayed with a routine method using an autoanalyzer (Hitachi 7600-10; Hitachi High-Technologies Corporation, Tokyo, Japan).

### Liver Histologic Condition

Liver tissues were fixed in 10% neutralized formalin at 4°C, paraffin-embedded, sectioned at 4  $\mu$ m in thickness, and stained with hematoxylin-eosin using a standard method.



### Terminal Deoxynucleotide Transferase dUTP Nick-End Labeling Assay

Frozen sections of SSGs were fixed with 4% paraformaldehyde in phosphate-buffered saline and stained to determine apoptotic cells with an in situ cell death detection kit from Roche-Boehringer Mannheim (Indianapolis, IN) as we previously described (21).

### Serum SOD Activity and MDA Content in the Liver Graft Tissue

Serum SOD activity was determined with the SOD Assay Kit (Jiancheng Biotechnology, Nanjing, China). The SOD activity was expressed as units per milligrams of protein as we previously described (15). MDA content was determined spectrophotometrically by a commercially available kit from Jiancheng Biotechnology.

### Western Blot Analysis for Phosphorylation of p38 MAPK and c-Jun

Total and nuclear protein was extracted from liver graft tissue, and protein concentration was determined as we described previously (22). Western blot analysis was performed according to the method we described previously (6). The primary antibodies against phosphorylated p38 MAPK, c-Jun (Abcam, Cambridge, MA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA) were used.

### Quantitative Reverse-Transcriptase Polymerase Chain Reaction of TNF- $\alpha$ and IL-1 $\beta$ Gene Expression

Total RNA was extracted from snap-frozen liver graft tissue by TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription reactions and real-time polymerase chain reaction were run as we previously reported (23). The rat GAPDH was used as the housekeeping gene control. TNF- $\alpha$ , IL-1 $\beta$ , and  $\beta$ -actin primers were designed using Primer 3 software, synthesized by Sangon Biotechnology Co. (Shanghai, China).

### Electrophoretic Mobility Shift Assays

To determine NF- $\kappa$ B activation, we performed electrophoretic mobility shift assays as we described previously (5, 24). Competition reaction mixtures contained a 100-fold molar excess of nonlabeled double-stranded oligo deoxynucleotide.

### In situ BrdU Incorporation for Determination of Graft Growth

To examine mitogenic response after liver transplantation, recipient rats in SSGLT or SSGLT+MnTBAP groups were intravenously injected with BrdU (50 mg/kg) at 12, 24, and 48 hr and 3, 5, 7, and 14 days after transplantation. They were sacrificed 2 hr after the injection, and the liver tissue was collected and snap frozen. Frozen sections at 10  $\mu$ m were prepared in a Cryostat and stained with antibodies against BrdU as previously described (25). Labeling index was determined by counting the number of BrdU-positive nuclei per 1000 nuclei in 10 randomly selected high-power fields under a light microscope.

### Statistical Analysis

All data are presented as mean (SD) and analyzed with the SPSS statistical package. Comparisons of same parameters at corresponding time points between two groups were analyzed by unpaired Student *t* test. Differences among more than two groups were analyzed with one-way analysis of variance with subsequent Newman-Keuls tests. The survival curves of recipient animals were estimated with the Kaplan-Meier method and analyzed by the log-rank test. *P* values less than 0.05 were considered statistically significant.

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