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ORIGINAL ARTICLE

Hepatic steatosis is associated with intrahepatic cholestasis and transient hyperbilirubinemia during regeneration after living donor liver transplantation

Jai Young Cho,[1] Kyung-Suk Suh,[1] Hae Won Lee,[1] Eung-Ho Cho,[1] Sung Hoon Yang,[1] Yong Beom Cho,[1] Nam-Joon Yi,[1] Min A Kim,[2] Ja-June Jang[2] and Kuhn UK Lee[1]

1 Department of Surgery, Seoul National University College of Medicine, Seoul, Korea
2 Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

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Correspondence
Kyung-Suk Suh MD, PhD, Department of Surgery, Seoul National University, College of Medicine, Seoul National University Hospital, 28 Yongon-dong, Chongno-gu, Seoul, 110–744, Korea. Tel.: 82 2 2072 3789; fax: 82 2 766 3975; e-mail: kssuh@plaza.snu.ac.kr

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Summary

A clear understanding of the mechanisms in steatotic livers that trigger cholestasis or hyperbilirubinemia after living donor liver transplantation (LDLT) remains elusive. We hypothesized that microarchitectural disturbance might occur within regenerating steatotic livers without impairment of hepatic proliferative activity. Liver biopsy specimens from 67 LDLT recipients taken at the 10th postoperative day were scored for the numbers of portal tracts per area (nPT/A) of liver tissue and for intrahepatic cholestasis, and immunostained by proliferating cell nuclear antigen (PCNA) and Ki-67. The preoperative degree of macrovesicular steatosis (MaS) was independently associated with cholestasis after LDLT ($P < 0.001$). Serum total bilirubin results on the 1st, 3rd, and 7th days post-LDLT in MaS+ (5–30% of MaS; $n = 37$) patients were significantly higher than those in MaS− (<5% of MaS; $n = 30$) patients ($P = 0.030$, 0.042, and 0.019, respectively). Mean numbers of positively stained hepatocytes were 53.1 ± 12.0 in patients with MaS and 48.0 ± 17.1 in those without MaS by PCNA ($P = 0.390$), and 24.4 ± 10.5 and 24.0 ± 14.0 by Ki-67 ($P = 0.940$). However, a significant negative correlation was found between the degree of MaS and nPT/A ($P = 0.013$), and nPT/A was correlated with the grade of histological cholestasis ($r = 0.350$, $P = 0.039$). Intrahepatic cholestasis and hyperbilirubinemia after LDLT could be caused by scanty morphologic change of portal tract during steatotic liver regeneration.

Introduction

Liver transplantation (LT) has become an accepted alternative therapy for patients with end-stage liver disease. However, the scarcity of deceased donors has resulted in a high mortality rate among patients awaiting LT. In an effort to cope with the shortage of deceased donor organs, especially in countries where deceased donors are limited, living donor liver transplantation (LDLT) has become the predominant modality, and suboptimal donor livers such as steatotic livers are sometimes used for LT. Hepatic steatosis has two subtypes, macrovesicular steatosis (MaS) and microvesicular steatosis (MiS) [1]. Of these, MaS is the more prevalent and the more important subtype from the clinical standpoint [2,3].

Grafts with severe steatosis are frequently associated with primary graft nonfunction, delayed graft function, and postoperative morbidity in deceased donor liver transplantation [4–6]. Although hepatocellular proliferation was unaffected by mild hepatic steatosis [7], hepatic steatosis was associated with a longer operation time, increased postoperative morbidity, and hyperbilirubinemia after partial hepatectomy (PH) for a hepatic neoplasm [8–10].

In addition to its key functions of metabolic control and the synthesis of important polypeptides including albumin and clotting factors, one of the most important functions of the liver is the continuous formation and excretion of bile. Liver regeneration is accompanied by a complex remodeling of hepatic tissue and a concomitant transient breakdown of the lobular architecture [11,12]. This complex process is impaired after PH [13] and results in transient cholestasis with diminished overall bile flow and increased serum bile acid levels after PH in rats [14]. Unfortunately, studies on the mechanisms that trigger hepatic dysfunction during regeneration of steatotic livers in human subjects are scarce.

Given these facts, we speculated that architectural disturbance might occur within regenerating steatotic livers after PH. To examine this hypothesis, we quantified hepatocellular proliferation activities and the number of portal tracts per unit area (nPT/A) of liver biopsy specimens during liver regeneration in a cohort of patients with chronic hepatitis B (HBV), and then correlated these factors with the severities of intrahepatic cholestasis and steatosis and with other variables.

Materials and methods

Patients, clinical, and laboratory data

Of 94 cases of consecutive LDLT performed at our institution between September 2002 to August 2004, 67 adult LDLT recipients who were diagnosed as having HBV-related end-stage liver disease, with an available liver biopsy which was performed on the 10th postoperative day, were enrolled in this study. Eighteen pediatric recipients and nine adult recipients who were diagnosed as having end-stage liver disease not related with HBV were excluded in this study. Currently, for LDLT, our institution uses grafts with up to 30% of MaS, regardless of the degree of MiS, as long as the graft to recipient weight ratio (GRWR) exceeds 0.8.

Preoperative liver biopsies were performed on the donors who were suspected of having steatosis as a result of a preoperative imaging study in order to exclude potential donors with severe hepatic steatosis. After informed consent was obtained from each patient by the surgical team, liver biopsy was performed in recipients on the 10th postoperative day. All biopsy specimens from recipients had been obtained using a uniform procedure at two different sites using an 18-gauge percutaneous biopsy needle.

Clinical outcomes known to be associated with intrahepatic cholestasis after LT [15], namely ischemia/reperfusion injury, bacterial infection, acute cellular rejection, cytomegalovirus infection, small-for-size graft, biliary strictures, hepatic artery thrombosis or stenosis, ABO blood group incompatibility, and recurrent HBV were retrospectively reviewed in our prospective morbidity data base. Ischemia/reperfusion injury was assessed by comparing cold and warm ischemic times, and graft size relative to recipient weight was evaluated by GRWR. Hepatic function was assessed by evaluating serial liver function tests, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT), which were performed on the 1st, 3rd, 7th, 30th, 180th, and 365th days after LDLT.

Histopathological analysis

Fresh liver sections were embedded in paraffin, sectioned, and stained with hematoxylin and eosin to delineate the hepatic histology. Sections were analyzed by an experienced hepatopathologist who was unaware of laboratory parameters and clinical data. All donors underwent an intraoperative wedge liver biopsy ($n = 67$) at the time of the hepatic resection. The intraoperative degree of steatosis in graft livers was quantified in terms of the percentage of hepatocytes affected according to the type (MaS or MiS), and scored as absent (<5% of hepatocytes affected) or present (≥5% of hepatocytes affected) [16].

Postoperative intrahepatic cholestasis was defined as bile pigment in the biliary canaliculi, and was graded in liver biopsy specimens obtained from recipients 10 days after LDLT as follows: grade 1 (<1/3 affected in fields at 400× magnification); grade 2 (1/3–2/3 affected); grade 3 (>2/3 affected).

Immunosuppression

Immunosuppression was based on a flexible double-drug protocol. The maintenance immunosuppressive agents used during the study period consisted primarily of a calcineurin inhibitor and a corticosteroid. The primary immunosuppressant was tacrolimus in 44 patients and cyclosporine in 23. Methylprednisolone was given before the portal and arterial reperfusion, twice as a bolus of 0.5 g, which was then tapered over 6 days. Oral prednisone (20 mg/day) was initiated at the 7th postoperative day and was tapered out over 6 months.

Immunohistochemical staining and quantification

Immunohistochemical staining for nuclear antigens, such as Ki-67 and proliferating cell nuclear antigen (PCNA), is used to monitor hepatic regenerative activity. All hepatic sections (5 μm) obtained on the 10th postoperative day from recipients ($n = 67$) were immunostained for Ki-67 and PCNA, using a monoclonal anti-Ki-67 antibody (clone MIB-1, DAKO, Glostrup, Denmark) and a

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monoclonal anti-PCNA antibody (clone PC10, Amersham, Westbury, NY, USA). Hepatocellular proliferative activity was assessed by counting Ki-67 or PCNA-positive hepatocyte nuclei in 10 random lobular fields at a magnification of 400×.

Quantification of the nPT/A (cm^2)

All tissue sections obtained from recipients 10 days after LDLT were photographed using a PixeLink Colour Digital Camera (Total Turnkey Solutions, Mona Vale, NSW, Australia). To quantify the total area of liver specimen per biopsy, nonoverlapping fields of the entire biopsy were photographed at 100×. IMAGE ANALYSIS software (Image-Pro Plus 5.1, Media Cybernetics, Inc., Silver Spring, MD, USA) was used to assess the total area (cm^2) per biopsy. In each case, the total number of portal tracts was counted at two histologic levels, and nPT/A in liver tissue was obtained. Both portal triads and portal dyads were included in the total count of portal tracts [17]. Incomplete portal tracts at the edges of biopsies were not counted as portal tracts. Total 536 portal tracts (mean: 8.0 ± 4.3 per biopsy; range: 2–26) were observed in liver tissues taken 10 days after LDLT from recipients.

Statistical methods

Continuous normally distributed variables are represented as means ± SD. Grade of cholestasis is represented by median values. A chi-squared goodness of fit test was used to determine whether there was a difference in distribution of steatosis among the gender and type of graft. To compare group means, analysis of variance (ANOVA) or Student's *t*-test was used. Pearson's correlation coefficient was used to determine correlations between continuous normally distributed variables. Degrees of association between nonparametric or ordinal variables were assessed using Spearman's correlation.

Multivariate analysis was performed, after correcting for age, gender, and the model for end-stage liver disease (MELD) score in recipients, and for age, gender, and body mass index (BMI) in donors, and for degree of MaS, degree of MiS, GRWR, type of graft, cold ischemic time, and warm ischemic time in grafts. All variables except for gender and the type of graft were used for the continuous number. Independent effects of normally distributed variables were assessed by multiple linear regression. A backward elimination approach was used to remove nonsignificant variables and determine the most parsimonious model including both fixed factors and covariates. Ordinal linear regression was used to assess the relative influences of variables on categorical data. All analyses were carried out using SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL, USA) and differences were considered significant at a *P*-value of <0.05.

Results

Mean age, MELD score, donor age, and donor BMI, and the distribution of donor gender are shown in Table 1. Of the 67 patients, 43 (64.2%) were male, and mean patient age was 49.5 ± 9.4 years (range: 24–77). Patients

Table 1. Demographic, donor, and graft characteristics of patients with chronic HBV who underwent living donor liver transplantation (LDLT).

	Total	Without MaS	With MaS	*P*-value*
No. of patients, *n*	67	30	37	
Recipient factors				
Age (years)	49.5 ± 9.4	48.6 ± 7.1	51.5 ± 10.6	0.249
Gender (male/female)	43/24	18/12	25/12	0.521
MELD score	23.7 ± 8.7	21.2 ± 7.2	24.5 ± 8.9	0.141
Immunosuppression (tacrolimus/cyclosporine)	44/23	20/10	24/13	0.831
Donor factors				
Age (years)	30.1 ± 9.1	28.5 ± 9.3	29.3 ± 8.1	0.740
Gender (male/female)	51/16	20/10	31/6	0.102
BMI (kg/m^2)	23.6 ± 3.1	22.5 ± 2.5	24.4 ± 3.4	0.032
Graft factors				
GRWR (%)	1.08 ± 0.26	1.10 ± 0.25	1.09 ± 0.24	0.790
Type of graft (right/left liver)	55/12	25:5	30:7	0.066
Cold ischemic time (min)	79.2 ± 29.4	82.5 ± 25.9	77.3 ± 34.3	0.543
Warm ischemic time (min)	40.1 ± 12.7	42.2 ± 11.2	38.9 ± 12.7	0.308

BMI, body mass index; MELD, the model for end-stage liver disease; GRWR, graft-to-recipient weight ratio; MaS, macrovesicular steatosis.

*Statistical tests were *t*-test for age, MELD score, body mass index, degree of steatosis, graft to recipient weight ratio, and ischemic time, and the chi-squared test for gender and graft type.

were followed for a median 24 months (range: 0–36). Thirty-seven (55.2%) of the 67 grafts had ≥5% of MaS (range: 5–30%). No significant difference was found between recipient demographic factors in the MaS-negative (<5% of MaS) and MaS-positive (≥5% of MaS) groups in terms of age, gender, or MELD score. No significant difference was also found between donor demographic factors in these two groups in terms of age or gender, but mean BMI was significantly higher in donors with MaS ($P = 0.032$). Graft parameters, including GRWR, graft type, and cold and warm ischemic times were similar in the two groups. Preoperative laboratory test results including AST, ALT, TB, ALP, and GGT were similar in the two groups ($P > 0.05$). In addition, the nPT/A of the liver specimens taken intraoperatively from the donors with (56.8 ± 24.0) or without MaS (64.1 ± 26.0; $P = 0.393$) was similar. Moreover, there was no association between the presence or absence of MaS and the other donor factors, such as the degree of MiS, age, gender, or BMI.

The grade of intrahepatic cholestasis in the liver biopsy specimens obtained from the recipients 10 days after LDLT in the patients with MaS (median: 2; range: 1–3) was significantly higher than those without MaS (median: 1; range: 1–2; $P < 0.001$). However, the clinical outcomes, including bacterial infections, acute cellular rejection, cytomegalovirus infection, biliary strictures, hepatic artery thrombosis or stenosis, ABO blood group incompatibility, and recurrent HBV, which are known to be associated with post-LT intrahepatic cholestasis, were similar in the patients regardless of the presence or absence of MaS (Table 2). After univariate analysis, the degree of MaS ($P < 0.001$), MiS ($P = 0.016$), and MELD score ($P = 0.020$) were found to be significantly associated with the grade of histological cholestasis. After multivariate analysis, degree of MaS and MELD score remained independently associated with the grade of histological cholestasis ($P < 0.001$ and $P = 0.024$; Table 3). Transient hyperbilirubinemia was observed in patients with MaS during the first week after LDLT, but this did not persist thereafter (Fig. 1). Serum TB results on the 1st, 3rd, and 7th days post-LT in patients with MaS were significantly higher than those of patients without MaS ($P = 0.030$, 0.042, and 0.019, respectively), but serum TB results obtained 30, 180, and 365 days post-LT were not different in the two groups. Changes in serum AST, ALT, ALP, and GGT levels on the 1st, 3rd, 7th, 30th, 180th, and 365th days after LDLT were similar in the two groups.

Table 2. Clinical outcomes of patients with chronic HBV that underwent LDLT.

	Total ($n = 67$)	Without MaS* ($n = 30$)	With MaS ($n = 37$)	*P*-value†
Bacterial infection	7	2	5	0.247
Acute cellular rejection	7	5	2	0.215
Cytomegalovirus infection	0	0	0	1.00
Biliary strictures	12	5	7	0.796
ABO incompatibility	0	0	0	1.00
Recurrent viral hepatitis B	2	1	1	0.983
Hepatic artery stenosis	3	1	2	0.573

*MaS, macrovesicular steatosis.
†Determined using the chi-squared test.

Table 3. Association between the degree of cholestasis and demographic and histological variables.

	P-value	Adjusted *P*-value*
BMI (kg/m^2)	0.045	0.075
MaS (%)	<0.001	<0.001
MiS (%)	0.016	0.981
Donor gender	0.811	0.680
Donor age	0.676	0.600
Recipient gender	0.388	0.815
Recipient age	0.942	0.882
MELD score	0.020	0.024
GRWR	0.288	0.644
Cold ischemic time (min)	0.878	0.566
Warm ischemic time (min)	0.475	0.410
Type of graft	0.796	0.640

BMI, body mass index; MaS, macrovesicular steatosis; MiS, microvesicular steatosis; MELD, the model for end-stage liver disease; GRWR, graft-to-recipient weight ratio.
*Adjusted for age, gender, and MELD score in recipients, age, gender, and BMI in donors, and degree of MaS, degree of MiS, GRWR, type of graft, cold ischemic time, and warm ischemic time in grafts.

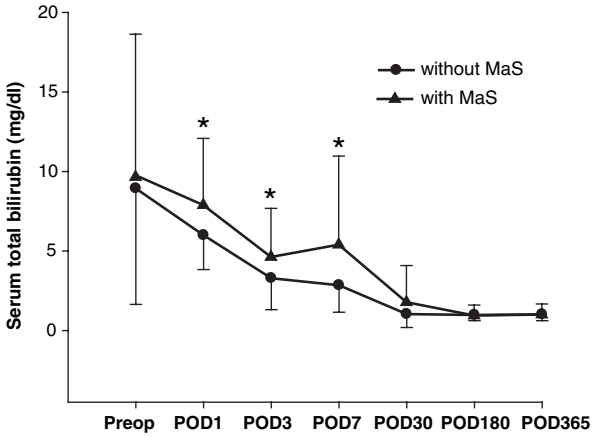

Figure 1 Changes in serum total bilirubin levels after transplantation in patients with or without macrovesicular steatosis (MaS). Serum TB results on the 1st ($P = 0.030$), 3rd ($P = 0.042$), and 7th days ($P = 0.019$) postoperatively in patients with MaS were significantly higher than that in patients without MaS. Data are expressed as means ± SD. *$P < 0.05$ between the two patient groups.

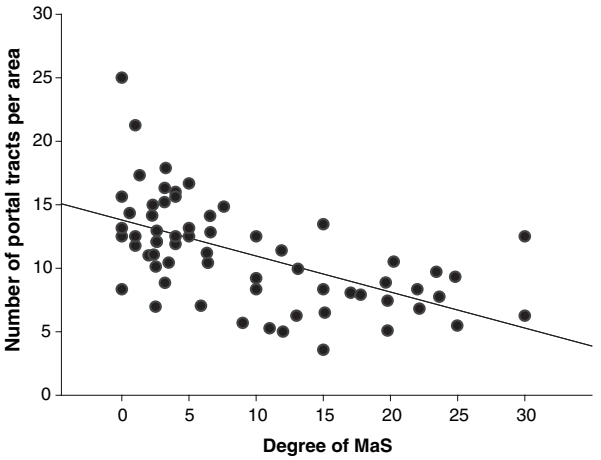

Figure 2 Correlation between the degree of MaS and the mean number of portal tracts per area taken from recipients 10 days after transplantation. MaS degree was found to be inversely correlated with the number of portal tracts ($r = 0.476$, $P = 0.004$).

To determine whether or not hepatocyte proliferative activity in steatotic livers was impaired during liver regeneration, hepatocyte proliferation was evaluated by PCNA and Ki67 immunohistochemistry, and the numbers of reactive nuclei were determined. No difference in hepatocellular proliferative activity was observed between patients with or without MaS. The mean numbers of positively stained hepatocytes in 10 high power fields were 48.0 ± 17.1 in patients without MaS and 53.1 ± 12.0 in patients with MaS according to PCNA antibody staining ($P = 0.390$), and 24.0 ± 14.0 in patients without MaS and 24.4 ± 10.5 in patients with MaS according to Ki-67 antibody staining ($P = 0.940$).

However, the degree of MaS, assessed as both a score and as a percentage ($r = 0.476$, $P = 0.004$; Fig. 2), and donor gender ($P = 0.021$) showed a significant correlation with nPT/A of liver specimen taken at 10 days postoperatively. After multivariate analysis, the preoperative degree of MaS in grafts alone remained independently associated with nPT/A in liver specimens taken at 10 days postoperatively ($P = 0.013$; Table 4). Moreover, nPT/A was found to be significantly associated with grades of histological cholestasis ($r = 0.350$, $P = 0.039$).

Table 4. Associations between the number of portal tracts per area (cm^2) of liver tissue taken 10 days after transplantation and demographic and histological variables.

	r	*P*-value	Adjusted *P*-value*
BMI (kg/m^2)	0.033	0.852	0.987
MaS (%)	0.476	0.004	0.013
MiS (%)	0.249	0.149	0.423
Donor age	0.030	0.864	0.852
Recipient age	0.113	0.518	0.933
MELD score	0.001	0.996	0.802
GRWR	0.057	0.753	0.302
Cold ischemic time (min)	0.073	0.693	0.457
Warm ischemic time (min)	0.279	0.116	0.243
	nPT/A		
Type of graft			
Right liver	11.8 ± 4.9	0.669	0.652
Left liver	11.0 ± 3.4		
Donor gender			
Male	12.2 ± 4.6	0.021	0.312
Female	7.9 ± 2.6		
Recipient gender			
Male	11.4 ± 4.1	0.657	0.649
Female	12.1 ± 5.6		

BMI, body mass index; MaS, macrovesicular steatosis; MiS, microvesicular steatosis; MELD, the model for end-stage liver disease; GRWR, graft-to-recipient weight ratio; nPT/Area, the number of portal tracts per area.

*Adjusted for age, gender, and MELD score in recipients, age, gender, and BMI in donors, and degree of MaS, degree of MiS, GRWR, type of graft, cold ischemic time, and warm ischemic time in grafts.

Discussion

Recently, increasing evidence indicates that hepatic steatosis is more vulnerable to factors that lead to inflammation and fibrosis [18]. When another liver disease is present, co-existent steatosis may exacerbate the liver injury [19]. Because healthy livers typically regenerate and recover completely from acute inflammation [20], normal regenerative response to injury might be impaired in steatotic livers. It was recently suggested that impaired hepatocyte replication in steatotic livers promotes the activation of hepatic progenitor cells as a compensatory response that helps limit progressive liver disease [21,22]. This study compared the hepatocellular proliferation activity, as measured by the PCNA and Ki-67 levels, in patients with or without MaS. The results showed no difference between the two groups. This confirms our previous reports on the hepatic regeneration power of mild steatotic livers [7,23].

In patients after LT, interference in the uptake, transfer, or secretion of bile caused by hepatocyte and/or cholangiocyte cell injury can result in cholestasis [24]. And, although the majority of such events remain subclinical, severe cholestasis may be associated with irreversible liver damage requiring retransplantation. Known causes of intrahepatic cholestasis after LT include ischemia/reperfusion injury, bacterial infection, acute cellular rejection, cytomegalovirus infection, small-for-size graft, drugs administered, intrahepatic biliary strictures, hepatic artery thrombosis, ABO blood group incompatibility, and recurrent disease [15]. In the present study, after collecting all

possible modifiers, MaS degree and MELD score were found to be independently associated with the development of intrahepatic cholestasis after LT. Our findings suggest that although grafts with <30% MaS are widely accepted for LDLT in current practice, grafts with mild hepatic steatosis transplanted into critically ill patients could add another risk factor of prolonged cholestasis and hyperbilirubinemia after LDLT.

One would expect a correlation between a higher MELD score and cholestasis (hyperbilirubinemia) because serum bilirubin is a factor in the MELD calculation. The survival rate in adults is influenced not only by the graft size, but also by the disease severity in the recipient; those with high MELD scores or Child-Pugh class B and C recipients have poorer outcomes [25]. Patients with cirrhosis have insufficient hepatocyte function to meet the increased metabolic demands after a PH, and have significantly lower levels of hepatic regeneration after a liver resection, making them extremely vulnerable to posthepatectomy liver failure [26]. Regeneration is often defective and may not occur at all in cases of severe disease. On the other hand, there may simply be a delay in full regeneration. Hyperbilirubinemia tends to be prolonged, with extreme increases in the serum bilirubin level in patients who ultimately do not survive the resection. Cirrhotic patients also become severely protein deficient after surgery, which is a feature not usually observed in patients with a normal liver function [27].

During liver regeneration, the re-establishment of the normal vascular architecture is accomplished by the subsequent invasion of surrounding endothelial cells into hepatocyte islands. The network of bile canaliculi, which interconnects the plurality of hepatocytes in a lobule, is also affected by lobular remodeling [12]. Experimental studies on steatotic animal models have shown an inverse correlation between degree of steatosis and sinusoidal blood flow, which is believed to be caused by ballooned hepatocytes containing fat droplets compressing and distorting the sinusoidal lumen and increasing intrahepatic portal resistance [28,29]. Moreover, it is known that the formation of bile depends on the generation of osmotic gradients within the bile canaliculus and on the active secretion of ATP-binding cassette protein superfamily [30,31], and that the expressions of these molecules are altered during hepatic regeneration [32,33]. This study demonstrates a statistically significant negative correlation between the preoperative degree of MaS in grafts and the numbers of portal tracts per area in liver tissues after partial LT. The normal liver graft, therefore, appears to regain standard liver volume in the adult recipient through proliferation of hepatocytes, thus increasing the distance between portal tracts so that the fully restored allograft contains the reduced portal tracts [13]. Scanty morphologic change of portal tract during liver regeneration may be aggravated by the presence of steatosis in the grafts. A sustained spatial disturbance of the bile canalicular networks during the regeneration of a steatotic liver has been observed in rats, and intrahepatic cholestasis during regeneration in patients with steatosis may be, at least in part, a consequence of disturbance of the microarchitecture, including bile canalicular networks, causing mechanical obstruction of the biliary drainage route [34]. The role of the biliary epithelium in bile production is such that, although damage to the biliary epithelium reduces bile flow, impaired fluid secretion by cholangiocytes of the biliary epithelium will induce qualitative changes in the biliary fluid that may predispose the biliary epithelium itself to damage by other concurrent events [35].

In summary, hepatic steatosis was found to be associated with microscopically intrahepatic cholestasis and functionally transient hyperbilirubinemia during regeneration, although hepatocellular proliferation was found to be unaffected by the presence of mild steatosis. Scanty morphologic change of portal tract during liver regeneration may be aggravated by the presence of steatosis in the grafts, providing clues as to why a number of grafts with steatosis develop graft dysfunction after LT, and why a fraction of patients with steatosis eventually experience increased morbidity and hyperbilirubinemia after PH.

Potential conflict of interest

Nothing to report.

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