

The Extent of Vacuolation in Non-Heart-Beating Porcine Donor Liver Grafts Prior to Transplantation Predicts Their Viability

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Livers exposed to prolonged warm ischemia (WI), such as those from non-heart-beating donors (NHBDs), are at higher risk of primary graft nonfunction (PNF). In a pig model of liver transplantation (LTx) from NHBDs, hepatocellular vacuolation, focal hepatocyte dropout, congestion, and sinusoidal dilatation appeared on biopsies taken after exposure to WI. In functioning grafts, vacuolation and sinusoidal dilatation were reversible after LTx, in contrast to PNF grafts. We studied whether the extent of these morphological signs and particularly vacuolation, present on pre-LTx biopsies, was associated with WI length and able to predict PNF, hepatocellular damage, and survival. Pre-LTx biopsies from pig livers exposed to incremental periods of WI were reviewed retrospectively. The extent of vacuolation was quantified blindly by a pathologist's semiquantitative score, validated by stereological point counting and digital image analysis, and then used to predict PNF and hepatocellular damage. On biopsies taken after WI, stereological point counting and digital analysis scoring contributed significantly in predicting PNF ($P = 0.027$ and $P = 0.043$, respectively) versus the pathologist's semiquantitative score ($P = 0.058$). Stereological point counting and digital image analysis predicted the extent of hepatocellular damage ($P < 0.0001$ and $P = 0.001$) versus the pathologist's semiquantitative score ($P = 0.085$). In conclusion, the extent of parenchymal vacuolation present on WI liver grafts reflects the severity of hepatocellular damage and predicts pig liver graft viability before LTx. Further studies are now warranted to evaluate whether these anoxic changes that are associated with liver graft viability in pigs also apply to human NHBD liver biopsies. *Liver Transpl* 14:1256-1265, 2008. © 2008 AASLD.

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See Editorial on Page 1240

Liver transplantation (LTx) is an effective treatment for end-stage liver disease and acute liver failure. Primary graft nonfunction (PNF) is an early complication after LTx causing death in the absence of rapid retransplantation, thereby increasing the pressure on the scarce donor pool.¹ During the last decade, the incidence of

PNF has generally decreased, except for liver grafts from non-heart-beating donors (NHBDs). Such livers were recently advocated to expand the organ pool but were also found to be associated with an increased incidence of PNF experimentally² and clinically.³ Viability prediction of such livers prior to transplantation, preventing the transplantation of grafts that are likely to fail, therefore remains a holy grail.⁴

At present, donor liver biopsies are sometimes used

Abbreviations: AST, aspartate aminotransferase; ATP, adenosine triphosphate; CI, cold ischemia; H&E, hematoxylin-eosin; LTx, liver transplantation; NHBD, non-heart-beating donor; PNF, primary graft nonfunction; WI, warm ischemia.

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clinically to assess the suitability of grafts prior to LTx. However, except for obvious macrovesicular steatosis, no reliable histological criteria have been defined to predict viability prior to LTx. Samples of preservation solution or perfusion dynamics during, for example, machine perfusion of grafts are other potential candidates for the pre-LTx prediction of viability but are not yet used clinically. Histological findings on donor liver biopsies are thought to merely reflect preexisting damage that might compromise the outcome after LTx and not illustrate possible deleterious peroperative or ischemia/reperfusion injury-related problems. Nevertheless, donor liver biopsies have been shown to be beneficial by ruling out preexisting damage such as severe macrovesicular steatosis and centrilobular necrosis.⁵

Because NHBD-LTx entails an increased risk of PNF due to the exposure to warm ischemia (WI), of which the duration is not always exactly known in human NHBD-LTx, it is crucial to determine whether histological parameters easily obtainable prior to LTx are associated with the risk of PNF. Assessing these histological parameters on donor liver biopsies could enable us to predict the viability of NHBD livers.

We therefore evaluated donor biopsies obtained in our previously validated pig model of NHBD-LTx and compared them with the outcome after LTx.² In this preclinical model, exposure of the liver to increasing lengths of WI before a short fixed period of cold ischemia (CI) was associated with more hepatocellular injury, an increasing risk of PNF, and subsequently poorer recipient survival. Direct hepatocellular damage, present immediately before and after reperfusion, was reflected by aspartate aminotransferase (AST) levels in serum measured early after reperfusion independently of the ensuing ischemia/reperfusion injury. On biopsies taken immediately after the exposure of livers to WI (but before CI and LTx), we previously reported several qualitative morphological changes (hepatocellular vacuolation and dropout, congestion, and sinusoidal dilatation) in livers exposed to prolonged WI. Moreover, vacuolation and sinusoidal dilatation were reversible in recipients with a functioning graft, in contrast to PNF grafts.² A similar qualitative observation was made later in a rat model of NHBD-LTx.⁶ The appearance of vacuoles in the cytoplasm of hepatocytes has already been reported to be induced by anoxia, as described in the morphogenesis of postmortem hepatocyte vacuolation.⁷ To our knowledge, this morphological feature of ischemia has never been used to predict the viability of NHBD liver grafts.

The aim of this study was to determine whether histopathological alterations (hepatocellular vacuolation and dropout, congestion, and sinusoidal dilatation) observed in NHBD liver grafts prior to LTx could predict the viability and function of these grafts after LTx. In particular, this study was designed to validate whether the extent of WI-induced vacuolation on liver biopsies taken prior to LTx could predict PNF, hepatocellular damage, and recipient survival. Therefore, a pathologist's semiquantitative score, which is of potential use in clinical practice, was used and validated by stereo-

logical point counting and digital image analysis, 2 more objective and quantitative approaches.

MATERIALS AND METHODS

Animal Model

As described previously, 60 Landrace pigs (25-30 kg) were used as donors and recipients.² After the induction of cardiac arrest, livers were exposed to different lengths of WI (0, 15, 30, 45, and 60 minutes; 5 experimental groups with 6 animals in each group) and flushed with a histidine-tryptophan-ketoglutarate solution. Donor livers were then procured and transplanted after 4 hours of CI. As clinically relevant endpoints, we used PNF of the liver, hepatocellular damage, and recipient survival.

PNF was defined in the recipient as persisting encephalopathy, irreversible metabolic acidosis, profound hypoglycemia, severe coagulopathy, reduced/absent bile production, and high serum AST levels. Persisting encephalopathy in the pig was defined as the inability of the recipient to wake up after LTx and cessation of all anesthetics. All PNF recipients displayed, within 180 minutes after reperfusion, irreversible metabolic acidosis [for example, excessive lactate accumulation (>7 mmol/L) and low pH (<7.1)]. This acidosis could not be corrected by standard medical treatment. In PNF recipients, profound hypoglycemia (<30-50 mg/dL) persisted despite attempts to correct blood sugar levels. Severe coagulopathy was clinically observed in PNF recipients by rapidly deteriorating coagulation after reperfusion, which contributed to hemorrhagic ascites. Biochemically, the prothrombin time progressively declined (<20%-30%) within 180 minutes after reperfusion. In all PNF recipients, bile production was lower or absent in comparison with non-PNF recipients. AST serum levels were measured early (15 minutes) after reperfusion and were also used as a second clinical endpoint for this study. In our hands, this early increase of AST levels reflected the liver injury inflicted (hepatocellular damage) and the length of WI, as described previously.² In all PNF recipients, AST levels measured in serum at 15 minutes after reperfusion were found to be higher than 500 U/L, directly reflecting the WI (≥ 30 minutes) damage already present, and thus independent of the subsequent ischemia reperfusion injury following LTx.

PNF was absent after 0 and 15 minutes of WI and was observed in 50% after 30 and 45 minutes of WI. PNF was observed in all pigs after 60 minutes of WI. This resulted in a posttransplant day 4 survival of 100% after 0 minutes of WI (control animals), 83% after 15 minutes of WI, 33% after 30 and 45 minutes of WI, and 0% after 60 minutes of WI. On posttransplant day 1, 3 recipients died with a functioning graft: one died because of pulmonary edema (15-minute WI group); one in the 45-minute WI group could be awakened but not weaned from the ventilator (possibly because of a brainstem injury) and had to be sacrificed; and one died without a clear cause being identified (30-minute WI

TABLE 1. Screening Scoring System

Parameter		Location Within Lobulus	Score
1. Vacuolation	No vacuoles		0
	Microvesicular	Acinar zone 3 or zones 3 and 2	1
	Microvesicular to mediovesicular		
	Mediovesicular	Acinar zones 3, 2, and 1 (panlobular)	2
	Mediovesicular to macrovesicular		3
2. Congestion	Macrovesicular		
	No congestion		0
	Focal sinusoidal congestion		1
	Patchy sinusoidal congestion	<50% of the slide	2
		>50% of the slide	3
3. Cell dropout	No cell dropout		0
	Group of cells		1
	Centrolobular necrosis		2

NOTE: After a first retrospective screening analysis, a preliminary pathological score was developed, taking into account the degree of vacuolation, degree of congestion, and degree of cell dropout in accordance with the location within the lobulus. *Vacuolation* was defined as macrovesicular when the vacuoles occupied the whole cytoplasm of a hepatocyte or blew up the hepatocyte, mediovesicular when about half the cytoplasm was taken by the vacuoles, or microvesicular when the vacuoles were smaller than half of the cytoplasm. *Congestion* was defined as stasis of red blood cells. *Cell dropout* was defined as destruction of the normal architecture of the parenchyma.

group). The observation period was limited to 4 days (76 hours) to minimize confounding effects due to, for example, rejection and infection. All surviving recipients were sacrificed under general anesthesia, but all not surviving animals were autopsied to identify the possible cause of death. No technical reasons for graft loss and/or recipient death were found. All experiments were ethically approved by the local animal care committee.

Morphology

Large wedge liver biopsies were taken before and after exposure to WI and after CI and immediately fixed accordingly for light and electron microscopy. In the control group (0 minutes of WI), livers were not exposed to WI, and no biopsies were thus available to assess the extent of vacuolation after exposure to WI.

Light Microscopy and Pathological Score

Biopsies were fixed in 6% formalin, embedded in paraffin, and stained in hematoxylin-eosin (H&E). In a first screening analysis, (1) the appearance of vacuoles in the hepatocytes' cytoplasm (number and size per hepatocyte and distribution of hepatocytes displaying vacuoles within the hepatic lobule), (2) the degree of congestion and sinusoidal dilatation, and (3) the amount of cell dropout were found to depend on the WI length. These morphological features related to WI were previously described.² Thereafter, a preliminary pathological score was developed by a clinical pathologist who then finally scored all biopsies blindly (Table 1). However, it was anticipated that such a score would be prone to large interobserver variability. In order to simplify the

score, it was decided to focus on all 3 histopathological features of the score individually. As shown later, the extent of vacuolation was not only found to highly correlate with the length of WI and hepatocellular damage but also allowed a more objective quantification by semiquantitative scoring as well as some more detailed morphometric analyses such as stereological point counting and digital image analysis.

Electron Microscopy

The nature of the vacuoles was studied with electron microscopy. For electron microscopy, small fragments of liver biopsies were trimmed into slices of 1-mm thickness or less and immediately fixed in 2.5% glutaraldehyde (0.1 mol %/L phosphate buffer) at 4°C. After 1 hour of postfixation in 1% osmium tetroxide (0.1 mol %/L phosphate buffer) at 4°C, the samples were dehydrated in a graded series of alcohols and embedded in an epoxy resin. Ultrathin sections (50-60 nm), cut and stained with uranyl acetate and lead citrate, were examined at 50 kV with a Zeiss EM 900 electron microscope. Images were recorded digitally with a Jenoptik Progress C14 camera system operated with Image-Pro express software.

Quantification of the Vacuolation and Predictive Value

To further quantify the extent of vacuolation on H&E-stained biopsies and to validate the potential value of hepatocellular vacuolation as a tool for predicting viability, 3 different methods were used: (1) pathologist's semiquantitative scoring, (2) stereological point counting, and (3) digital image analysis. These scores were

performed on biopsies taken after WI alone and after WI and CI. We then studied whether these scores were associated with the post-LTx outcome (PNF, recipient survival, and hepatocellular damage as reflected by AST release in serum early after reperfusion). Baseline biopsies taken in the donor liver, before exposure to WI, served as controls.

Pathologist's Semiquantitative Scoring. Similar to a frequently used fat deposition score,⁸ the amount of vacuoles was expressed as a percentage of the total surface of the parenchyma taken in by vacuoles. This score, expressed as a percentage, also allows comparison with quantification scores by stereological point counting and digital image analysis. The amount of vacuoles was blindly and independently estimated by 2 independent pathologists.

Stereological Point Counting. All light microscopy images were viewed with an Olympus BX61 microscope (Olympus, Germany) and captured with an integrated digital camera. The images were then analyzed with morphometrical imaging software (AnalySIS D, Olympus). For the stereological point counting, 5 images of nonoverlapping lobules were randomly selected from each biopsy (final magnification, $\times 400$). Within each image, 1 periportal zone and 1 centrilobular zone were selected and captured distinctively. A point grid consisting of 204 crossings $25\ \mu\text{m}$ apart was superimposed on each of these zones with the AnalySIS D morphometrical imaging software. The stereological point counting method followed was similar to the one described for steatosis by Franzen et al.⁹ Because anoxic vacuoles generally appear to be smaller than macrovesicular steatosis, for example, the described semiquantitative method was adapted for this particular study by a reduction of the distances between the lines of the grid ($25\ \mu\text{m}$ instead of $100\ \mu\text{m}$), which allowed higher sensitivity. Vacuoles were identified on the basis of their shape, location within the hepatocytes, and content. The number of vacuoles bonding to the grid crossings was counted by an independent observer and expressed as a percentage of the total number of crossings. Crossings outside the hepatocellular parenchyma (such as the portal tract, central vein, sinusoid, or artifact) were excluded (Fig. 1). For the analysis, an average across the images used for the periportal and centrilobular zones was calculated, resulting in a total stereological point score.

Digital Image Analysis. All H&E-stained biopsies were completely and automatically scanned with a Zeiss Axiovert 200M microscope and saved as high-resolution images (Fig. 2A). Hepatocellular vacuoles were then automatically extracted from the image on the basis of their color, density, shape (degree of circularity), and size. Only vacuoles greater than $1\ \mu\text{m}^2$ and less than $30\ \mu\text{m}^2$ (similar to the surface of the nucleus) were taken into account, the sinusoids thereby being excluded (Fig. 2B). Therefore, an image analysis macro was written in Zeiss KS400 software. The software al-

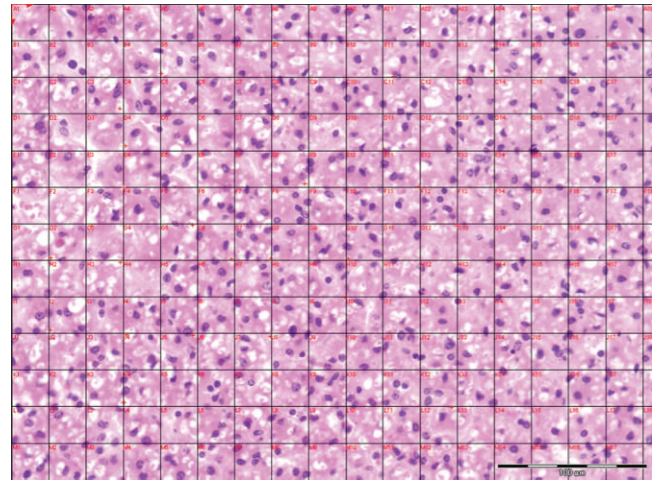


Figure 1. Stereological point counting (magnification, $\times 400$). Three periportal and 3 centrilobular images were taken from 5 nonoverlapping, randomly selected lobules and superimposed by a point grid (204 crossings $25\ \mu\text{m}$ apart). The number of vacuoles bonding to the grid crossings was counted (red crosses) and expressed as a percentage of the total number of crossings after the exclusion of crossings outside the hepatocellular parenchyma.

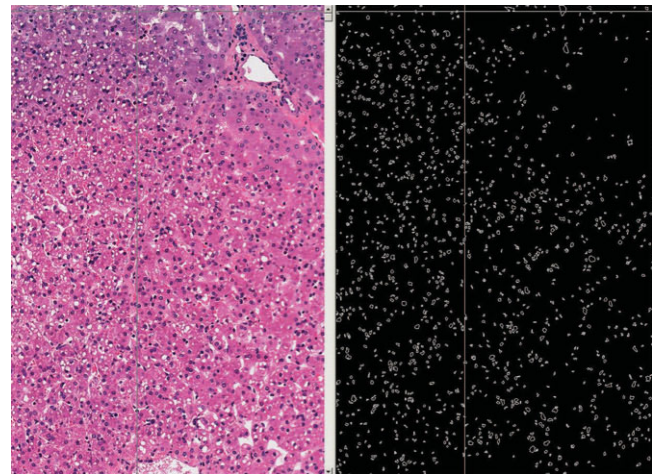


Figure 2. Digital image analysis. (A) All biopsies were first scanned completely and saved as high-resolution images. **(B)** Hepatocellular vacuoles were then automatically selected on the basis of their shape, the absence of cytoplasm, and their intrahepatocellular location. The total surface taken in by vacuoles was expressed as a percentage of the total biopsy surface. The digital image analysis score for vacuoles was designed and performed by Innogenetics (Gent, Belgium).

lowed the following parameters to be evaluated: the number of vacuoles per biopsy surface unit, the vacuole surface/biopsy surface ratio as a percentage, and the morphometric parameters of each vacuole (surface/density). As a result, the number of vacuoles per square millimeter of parenchyma was calculated. The digital image analysis scoring was an adapted version of a steatosis image analysis macro. Adaptations for measuring hepatocyte vacuolation included changes in the size and circularity parameters from the original

method developed by GENimmune, a subsidiary of Innogenetics (Gent, Belgium).

Data Analysis

Chi-square analyses were conducted for comparisons between the preliminary pathological scores and the incidence of PNF and length of WI.

To determine predictors of PNF, logistic regressions were conducted with PNF (yes or no) as the criterion and the different vacuolation scores consecutively as predictors (pathologist's semiquantitative score, stereological point counting, and digital image analysis). The Wald statistical test is reported with a *P* value to indicate whether the predictor has a significant relationship with the criterion (PNF). The estimated odds ratio represents the effect size (>1 indicates that PNF is more likely to occur when the predictor increases with a 1-unit change).

Similar to the analyses for PNF, linear regression analyses to evaluate which of the predictors significantly contributed in predicting AST (continuous variable) were conducted. Values of the *t* statistic and *P* are reported to indicate whether the predictor has a statistically significant relationship with the criterion. The standardized coefficient (beta) is a measure that represents the amount which the predictor actually contributes in the AST measure. Of note, the scores for the control group (0 minutes of WI) were not included in the analysis (they were not included in the logistic or linear regression because the livers in this group were not exposed to WI).

Survival rates were calculated by Kaplan-Meier modeling, and the influence of different categorical variables on survival was evaluated by the log-rank test. Because all surviving animals were sacrificed at 76 hours, these were censored at that time point in the statistical analyses. Variables that were significant in the log-rank test were used in a multivariate Cox proportional hazard regression model.

To compare the 5 experimental groups with different WIs with respect to the vacuolation scores (semiquantitative, stereological point counting, and digital image analysis), analyses of variance were conducted on the different scores with the 5 groups as between-subjects factor (0, 15, 30, 45, and 60 minutes of WI) and with the ischemic times (WI alone or WI and CI) as within-subjects factors. A *t* test was used to compare the extent of vacuolation after WI alone and after WI and CI within the same experimental group.

Statistical software [JMP6 (SAS) and SPSS 12] was used for the analyses.

Data are expressed as means $\pm$ standard deviation. A *P* value < 0.05 was considered significant.

RESULTS

Light Microscopy and Pathological Score

A higher pathological score (sum of vacuolation, congestion, and dropout scores; see Table 1) was found to be associated with a longer exposure to WI (1.57 ± 1.39 ,

4.16 ± 1.6 , 2.66 ± 2.1 , and 5.66 ± 1.36 for groups with 15, 30, 45, and 60 minutes of WI, respectively; *P* = 0.03). Longer WI times were associated with higher scores for vacuolation (1.2 ± 0.44 , 1.8 ± 0.4 , 1.6 ± 0.5 , and 2.66 ± 0.5 for groups with 15, 30, 45, and 60 minutes of WI, respectively; *P* < 0.0001) and higher scores for congestion (1.0 ± 1.0 , 1.33 ± 1.03 , 1.2 ± 0.83 , and 2.5 ± 0.83 for groups with 15, 30, 45, and 60 minutes of WI, respectively; *P* = 0.0216). Longer WI times were also associated with higher scores for hepatocellular dropout (0 ± 0 , 1 ± 0.63 , 0.4 ± 0.89 , and 0.5 ± 0.54 for groups with 15, 30, 45, and 60 minutes of WI, respectively; *P* = 0.05). As the extent of vacuolation, in contrast to congestion and dropout scores, displayed more variation, allowing discrimination between all groups (for example, 0 and 15 minutes of WI), vacuolation was further evaluated as a potential parameter to predict viability.

Electron Microscopy

In livers exposed to WI, many hepatocytes contained small, medium, and/or large vacuoles in their cytoplasm. Their distribution within the cell was variable: at the sinusoidal or lateral pole of the hepatocytes, next to the nucleus, and next to bile canaliculi. Some vacuoles seemed to fuse with each other, forming very large vacuoles displacing the nucleus and organelles present in the cytoplasm (Fig. 3A). The vacuoles were surrounded by a smooth membrane and contained amorphous material of a low electron density (Fig. 3B).

Quantification of the Vacuolation and Predictive Value

Quantification of Vacuoles

According to the semiquantitative score system, the extent of vacuolation after 15, 30, 45, and 60 minutes of WI was $34.5\% \pm 16.05\%$, $41.33\% \pm 17.68\%$, $34\% \pm 8.9\%$, and $36.83\% \pm 22.42\%$, respectively (*P* = 0.895; Fig. 4A). Following an additional 4 hours of CI, the extent of vacuolation was 0%, $42.5\% \pm 15\%$, $42.5\% \pm 15.73\%$, $55\% \pm 11.18\%$, and $39.16\% \pm 19.6\%$ for the 0-, 15-, 30-, 45-, and 60-minute WI groups, respectively (*P* = 0.418).

When the stereological score data were used, a linear trend was observed with increasing WI: $4.1\% \pm 1.9\%$ for 15 minutes of WI, $8.53\% \pm 4.2\%$ for 30 minutes of WI, $18.62\% \pm 6.95\%$ for 45 minutes of WI, and $26.28\% \pm 8.85\%$ for 60 minutes of WI (*P* = 0.002; Fig. 4B). A pairwise multiple comparison method according to Dunn revealed that the extent of vacuolation was significantly higher (*P* < 0.05) in the 60-minute WI group versus the 15- and 30-minute WI groups. After WI and CI, the extent of vacuolation was $4.86\% \pm 2.6\%$ for 0 minutes of WI, $14.87\% \pm 9.39\%$ for 15 minutes of WI, $21.66\% \pm 4.56\%$ for 30 minutes of WI, $30.13\% \pm 5.05\%$ for 45 minutes of WI, and $29.53\% \pm 4.2\%$ for 60 minutes of WI (*P* < 0.001). A pairwise multiple comparison method according to Tukey revealed that the extent of

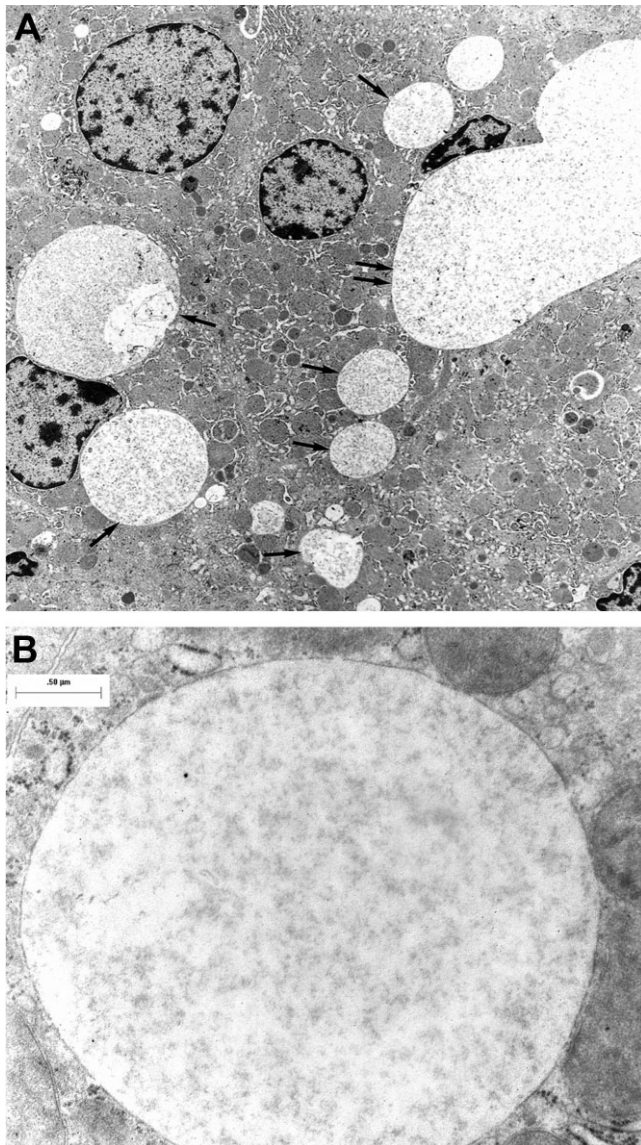


Figure 3. (A) Representative electron micrograph of the parenchyma of a liver exposed to 30 minutes of warm ischemia. Large, medium, and small vacuoles (arrows) are obvious in the cytoplasm of the hepatocytes (original magnification, $\times 6250$). (B) Detail of a cytoplasmic vacuole with a clear surrounding membrane and an amorphous, low-electron-density material in the lumen.

vacuolation after WI and CI was significantly higher ($P < 0.05$) in the 60-minute WI group versus the 0- and 15-minute WI groups, in the 45-minute WI group versus the 0- and 15-minute WI groups, and in the 30-minute WI group versus the 0-minute WI group. After additional CI, the extent of vacuolation in the 30- and 45-minute WI groups increased significantly ($P < 0.05$) in comparison with the extent of vacuolation after WI alone.

When the digital image analysis was used, the extent of vacuolation was 518 ± 284 , 941 ± 321.47 , 963.83 ± 574.68 , and 1613 ± 543.37 after 15, 30, 45, and 60 minutes of WI, respectively ($P = 0.471$; Fig. 4C). After an

additional 4 hours of CI, the extent of vacuolation was 22.33 ± 7.048 , 816 ± 200.35 , 1447 ± 568.14 , 1590 ± 480.89 , and 1598.3 ± 610.86 for the 0-, 15-, 30-, 45-, and 60-minute WI groups, respectively ($P = 0.004$). A pairwise multiple comparison method according to Tukey revealed that the extent of vacuolation after WI and CI was significantly higher ($P < 0.05$) in the 60-minute WI group versus the 0- and 15-minute WI groups, in the 45-minute WI group versus the 0- and 15-minute WI groups, in the 30-minute WI group versus the 0-minute WI group, and in the 15-minute WI group versus the 0-minute WI group.

Risk of Developing PNF

In animals with livers exposed to WI, PNF could be predicted with all vacuolation scores on biopsies taken after WI. The stereological point counting and digital analysis scores were found to be significant predictors (Wald = 4.905 and 4.090, $P = 0.027$ and 0.043, and odds ratio = 1.528 and 1.003, respectively) versus the semiquantitative score (Wald = 3.602, $P = 0.058$, and odds ratio = 1.065; Table 2). PNF could no longer be predicted on biopsies taken after WI and CI. As expected from the logistic regression statistics, we observed that all livers exposed to WI with a vacuolation score higher than 50%, 12.5%, and 1529 for the semiquantitative, stereological point counting, and digital image analysis scores, respectively, developed PNF. These cutoff values were consequently used for the survival analysis.

Survival

As shown in Fig. 5, recipients with vacuolation scores above the aforementioned cutoff values died very quickly, whereas the other recipients survived longer, and most of them reached the time of sacrifice ($P = 0.0163$, $P < 0.0001$, and $P = 0.0021$ for the semiquantitative, stereological point counting, and digital image analysis scores, respectively). In a multivariate analysis, only the stereologically determined vacuolation remained significant ($P = 0.0046$ and hazard ratio = 11 with a confidence interval of 2-59).

Prediction of Hepatocellular Damage

Stereological point counting and digital image analysis scores for biopsies taken after WI were highly significant predictors of hepatocellular damage (as reflected by serum levels of AST 15 minutes after reperfusion; Table 3). The digital image analysis scores for biopsies taken after WI and CI were also significant predictors for hepatocellular damage (Table 3).

DISCUSSION

At present there are no reliable pre-LTx predictors to assess the viability of liver grafts from NHBDs. In our validated pig model of NHBD-LTx, we evaluated whether particular histopathological features could be used to predict viability. After an initial assessment by light microscopy, a multifactorial score system based

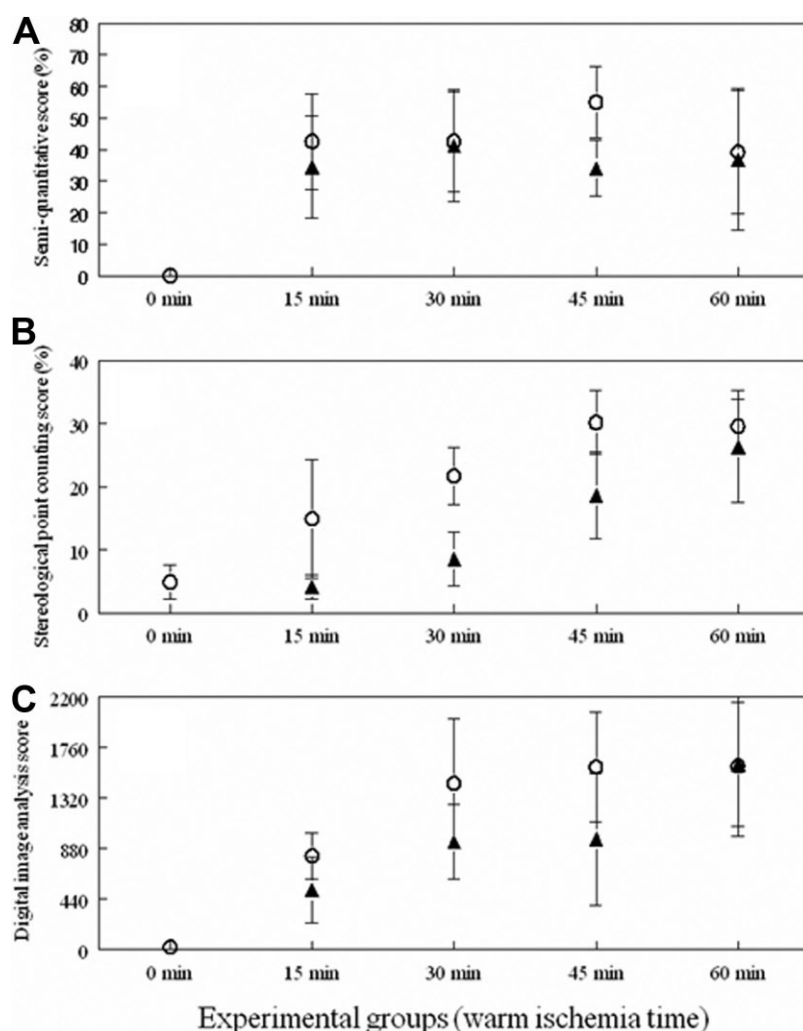


Figure 4. (▲) The extent of vacuolation between the different experimental groups after warm ischemia is compared with (○) the extent of vacuolation after warm ischemia and cold ischemia. The values are based on (A) the semiquantitative scoring method, (B) the stereological point counting scoring method, and (C) the digital image analysis scoring method.

TABLE 2. Comparison of the Predictive Values for Primary Graft Nonfunction of Different Scoring Methods Used To Quantify the Extent of Vacuolation on Liver Biopsies Taken After Exposure to WI or After Exposure to WI and CI

	Timing of Biopsies	Wald	P	Odds Ratio
Semiquantitative score	After WI	3.602	0.058	1.065
	After CI and WI	1.028	0.324	0.971
Stereological point counting	After WI	4.905	0.027	1.528
	After CI and WI	0.259	0.611	1.042
Digital image analysis	After WI	4.090	0.043	1.003
	After CI and WI	1.374	0.241	1.001

Abbreviations: CI, cold ischemia; WI, warm ischemia.

on 3 different parameters [hepatocellular vacuolation (size and distribution within the lobulus), sinusoidal congestion, and focal hepatocyte cell dropout] was developed. All these features appeared to be associated with increasing lengths of WI. Of these 3 features, the extent of vacuolation after WI was found to highly correlate with PNF and the degree of hepatocellular damage. Although such a histopathological score allows clinical application within the time frame of a trans-

plantation procedure, it was anticipated to be not easily reproducible and subject to large intraobserver and interobserver variability. Therefore, different methods to assess the extent of vacuolation were evaluated, although in a limited number of experimental groups and animals. This was done first to confirm the value of assessing the extent of vacuolation in predicting viability and second to evaluate whether a pathologist's semiquantitative score indeed is reproducible by more

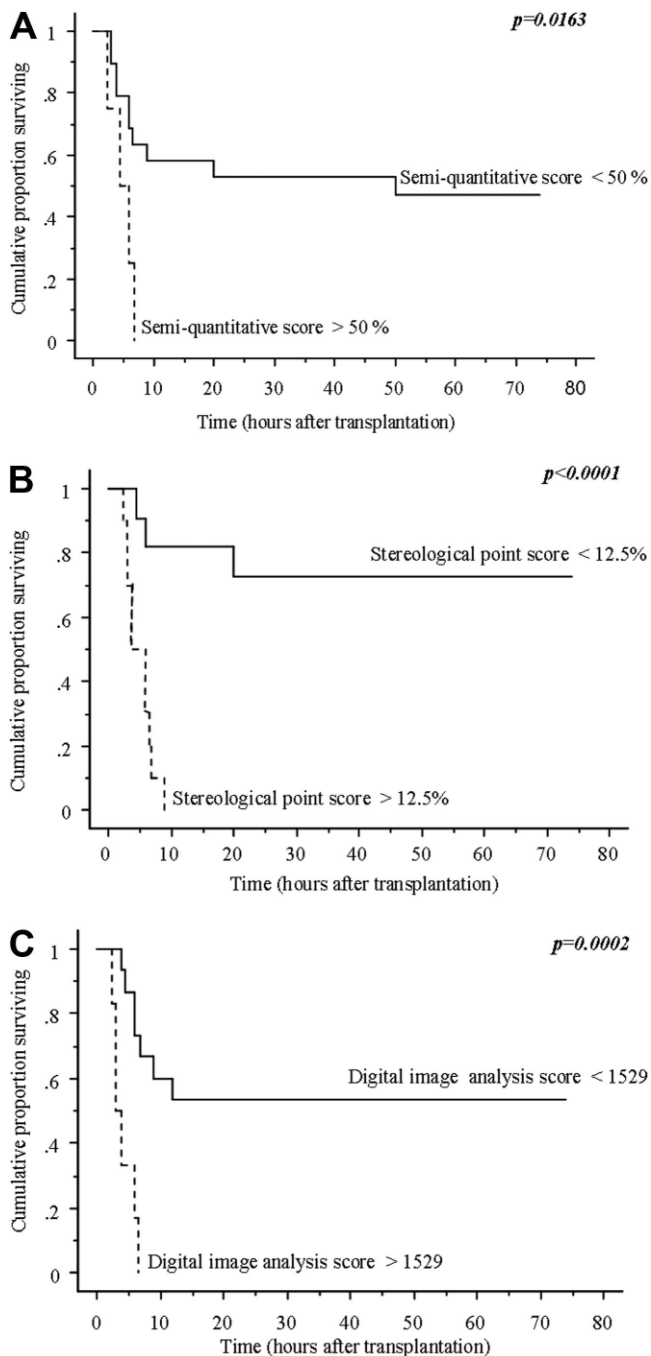


Figure 5. Correlation between recipient survival and the extent of vacuolation according to (A) the semiquantitative scoring method using a cutoff value of 50%, (B) the stereological point counting scoring method using a cutoff value of 12.5%, and (C) the morphometrical digital image analysis scoring method using a cutoff value of 1529.

objective quantification methods. First, a semiquantitative method, a well-known method for scoring liver steatosis, was applied. Clinically, this assessment is relatively simple and logistically easy in comparison with stereological point counting and digital image analysis. In addition, the continuous data obtained are more suitable for statistical analysis. However, the po-

tential but inherent disadvantages and drawbacks of this scoring system include not only the observer dependency but also the risk of overestimating the degree of macrovesicular steatosis, as pointed out by Franzen et al.⁹ In our hands, the semiquantitative scoring allowed us to predict viability and recipient survival (cutoff > 50%), but this score could discriminate the extent of vacuolation between the different experimental groups exposed to >15 minutes of WI. Furthermore, we also applied 2 morphometric analyses (stereological point counting and digital image analysis) to validate whether vacuolation allows us to predict the risk of developing PNF and to verify the eventual accuracy of the pathologist's semiquantitative score. Similar assessments have already been done on donor liver biopsies to assess the degree of steatosis on biopsies obtained immediately before and after graft reperfusion to provide additional information on the risk of developing PNF in LTx.¹⁰ The drawback of such morphometric analysis is that it is very unlikely to be available in a logistically acceptable time frame under clinical circumstances. Because in most transplant centers a clinical pathologist can rapidly review biopsies within the time frame of a transplantation, it was also interesting to verify the eventual accuracy of this pathologist's score.

Histological alterations and the extent of parenchymal vacuolation in particular reflected the severity of hepatocellular damage induced by WI and allowed us to predict pig liver graft viability before LTx. We first observed that vacuolation of the hepatocyte cytoplasm was associated with incremental WI, an increased risk of developing PNF leading to the recipient's death, and concomitant hepatocellular damage. At first glance, it appears surprising that biopsies taken before LTx and thus prior to the ensuing ischemia/reperfusion injury reflect viability post-LTx. This is in accordance with previous findings in our experimental model of NHBD-LTx.¹¹ In this model, we observed that the degree of hepatocellular damage and Kupffer cell activation determined whether the grafts were viable or not. Of note, this hepatocellular damage and the activation of Kupffer cells were present prior to reperfusion, as reflected by the release in serum immediately after reperfusion of AST and β -galactosidase, respectively. Second, we observed that the stereological point counting and digital image analysis scores on the whole biopsy were significantly correlated with PNF and hepatocellular damage (AST) in contrast to the semiquantitative score.

Third, we observed that an additional period of CI seems to dilute the predictive effect of the vacuolation score after WI alone. As discussed later, the etiology of vacuolation after WI is unknown, although both anoxia and a high intrahepatic venous blood pressure play important roles. In our model, livers are flushed, taken out of the donor, and cold-stored. During this *ex vivo* cold storage, there is no longer a venous pressure that contributes to the extent of vacuolation, which then may be relatively underestimated. Therefore, transplant surgeons should rely on the extent of vacuolation

TABLE 3. Comparison of the Predictive Values for Hepatocellular Damage of Different Scoring Methods Used To Quantify the Extent of Vacuolation on Liver Biopsies Taken After Exposure to WI or After Exposure to WI and CI

	Timing of Biopsies	AST at 15 Minutes		
		<i>t</i>	<i>P</i>	Standardized Coefficient
Semiquantitative score	After WI	1.805	0.085	0.367
	After CI and WI	0.421	0.679	0.099
Stereological point counting	After WI	6.665	<0.0001	0.837
	After CI and WI	2.026	0.062	0.476
Digital image analysis	After WI	3.931	0.001	0.670
	After CI and WI	2.641	0.017	0.528

Abbreviations: AST, aspartate aminotransferase; CI, cold ischemia; WI, warm ischemia.

as assessed immediately after WI to discriminate between viable and nonviable grafts.

Finally, we observed that after the additional CI, the extent of hepatocellular vacuolation increased in the groups exposed to 15, 30, and 45 minutes of WI when the stereological point counting method was used. This could perhaps in part clarify why we (publisher abstract,¹² 2008) and others have also observed that the outcome of livers exposed to longer (>4 hours) periods of CI following relatively short periods of WI (<30 minutes) is not favorable experimentally^{12,13} or clinically.³ This observation should definitely encourage limiting the CI time of NHBD liver grafts in clinical LTx. Of note, vacuolation was never observed after exposure to CI alone as in our control group or in another study with a similar setup but a longer period (8 hours) of CI (data not shown). In any case, the exposure to WI is regarded as a prerequisite before CI has an additional effect on the extent of vacuolation.

The etiology of vacuolation of hepatocytes has never been completely clarified, and neither has this finding ever gained any potential clinical application as suggested for the first time in this work. Several pathways have been postulated to clarify its origin. Mechanisms such as lysosomal degradation (also called autophagic vacuoles),¹⁴ the internalization of apical cell membranes due to adenosine triphosphate (ATP) depletion,¹⁵ and anoxia in general with influx of plasma into the cell have been postulated. On electron microscopy assessment, no cytosol and/or cellular organelles were observed in the vacuoles of NHBD livers. Therefore, the autophagic process is not likely to account for the development of the vacuoles observed in our NHBD-LTx model. Nevertheless, autophagic vacuoles are regarded as an important survival mechanism during short-term starvation by degrading some nonessential components to provide the cell with vital nutrients, although this probably requires a minimum of cellular energy. The internalization of the membrane, observed in ischemic (ATP-depleted) rat cholangiocytes, could be another plausible explanation for the vacuole formation in the hepatocyte cytoplasm that we observed. In our study, smooth membranes surrounded the vacuoles on electron microscopy assessment. Contrary to Doctor et

al.,¹⁵ we were not able to stain these membranes immunohistochemically for normal membrane-bound proteins because anti-porcine antibodies that react with membrane-bound proteins are not readily available. Another plausible origin of the vacuoles observed in our experimental setting can be attributed to the anoxic conditions arising after cessation of circulation and heartbeat. Similar vacuoles have been described in the liver after coal-gas poisoning,¹⁶ in anoxic-resuscitated liver grafts in a porcine LTx model,¹⁷ in cells in tissue cultures injured by ischemia or ATP depletion, in human proximal nephron cells in acute renal failure,¹⁸ and as a postmortem feature in rats.⁷ The conditions during which we observed the appearance of vacuoles and its association with incremental WI are similar to the postmortem vacuolation of hepatocytes observed in rats during anoxic euthanasia conditions (anoxic chamber).⁷ Similarly to us, Li et al.⁷ described a time-dependent progression of vacuolation together with other factors (type of euthanasia used as well as fasting or nonfasting) influencing the variation of the vacuolation. Besides the anoxia, a high intrahepatic blood pressure was regarded as a second critical factor for developing these anoxic postmortem hepatocyte vacuoles.¹⁶ A high pressure in the intrahepatic sinusoids is the result of a rising venous blood pressure after death. This can be observed as sinusoidal congestion/dilatation, and plasma is forced out of the sinusoids into the hepatocytes' cytoplasm. This may account for the granular aspect of the vacuole, which is similar to plasma. In addition, it explains why vacuolation is associated with hepatocellular damage. As we observed that vacuolation is reversible after reperfusion in functioning grafts, it implies to some extent the reversibility of the membrane's damage. However, the same can be assumed when vacuolation is not reversible in PNF grafts; this allows the release of AST out of the cells with irreversibly damaged membranes. Finally, the anoxic vacuoles described did not contain fat, as observed on electron microscopy assessment and confirmed by oil red O staining on light microscopy (data not shown).

Until now, histological data on biopsies from human NHBD liver grafts taken before LTx have been scarce. However, in a landmark article, Casavilla et al.¹⁹ com-

mented on the histological findings of back-table biopsies of 3 failing liver grafts from uncontrolled NHBDS. Although these back-table biopsies were reported to be normal, mild and scattered spotty acidophilic necrosis and minimal microvesicular steatosis (10%-15%) were described. In retrospect, these signs could be similar to the focal cell dropout and vacuolation that we observed in our model and that in our hands appear to be reliable predictors of graft viability.

The current findings, suggesting that anoxic changes are associated with liver graft viability after exposure to WI, need to be confirmed and further explored in clinical samples of human NHBDS liver biopsies.

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