

## Graft Calcifications and Dysfunction Following Liver Transplantation

a report by

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### Background

The molecular events following ischaemia and reperfusion (I/R) of the liver during transplantation are largely unknown. There is evidence that apoptotic and necrotic events may take place and occasionally result in primary graft dysfunction. Two cases are reported herein, where significant I/R injury correlated with the development of liver calcification and primary liver dysfunction.

### Case Presentation

Both patients with clinical and biochemical evidence of primary graft dysfunction demonstrated calcification at light microscopy (LM) and electron microscopy (EM) levels. In addition, one patient showed macroscopic evidence of calcification on cross-sectional imaging. Both patients died secondary to the sequelae of the graft dysfunction.

### Conclusions

Severe I/R-induced injury to the liver clinically leads to graft dysfunction. This is due to advanced apoptotic and/or necrotic events at the hepatocyte level that may, in the most severe form, lead to calcification. The study of microcalcification at the early post-transplant period could provide insight in the events taking place following significant ischaemia/reperfusion-induced injury to the graft.

### Background

Liver transplantation (LT) remains the only treatment for end-stage liver disease. However the transplant procedure mandates cold perfusion, hypothermic storage, warm ischaemia and warm reperfusion of the graft, resulting in I/R-induced injury to the transplanted graft. Although the introduction of the University of Wisconsin solution (UW) has improved clinical outcomes, I/R injury remains one of the major clinical problems following LT leading (in cases of marginal donor quality or prolonged cold or warm ischaemic time) to the development of graft dysfunction or non-function.

Recently, the cellular events following liver I/R during LT have been brought into sharp focus. Nevertheless, it is still controversial if the major mode of cell death during I/R is apoptosis and/or necrosis.<sup>1</sup> It has been shown in an animal model of viral-induced haemorrhagic liver necrosis that the liver undergoes several morphologic changes including mineralisation.<sup>2</sup> Furthermore, previous clinical reports have documented the development of calcifications in liver upon extensive ischaemia.<sup>3,4</sup> These observations may reflect a high degree of cell damage leading to an enhanced apoptotic cell engulfment by non-professional phagocytes of the liver<sup>5</sup>, with subsequent necrosis and biomineralisation.

In this report, using a combination of cross-sectional imaging, histochemistry and LM as well as transmission electron microscopy (TEM), liver calcification was identified in two grafts with severe I/R injury following LT.

### Case Presentations

#### Case One

A 65-year-old man of Asian origin underwent LT in June 2002 for decompensated hepatitis B-related cirrhosis. The patient did not have a history of abnormal calcium metabolism or hyperparathyroidism. Upon listing, his serum-ionised calcium was 1.07mmol/L. The donor was a previously healthy 42-year-old male, who suffered intracranial bleeding following a motor vehicle crash. He did not suffer any period of hypoxia but was hypotensive (systolic blood pressure (SBP) = 90mm Hg) prior to procurement. His serum liver function tests were normal prior to harvesting (acute systemic toxicity (AST) = 32 units per litre (U/L), alkaline aminotransferase (ALT) = 40 U/L). During procurement the liver was found to be well perfused, with no focal injuries and no macroscopic evidence of steatosis.

The recipient underwent an uncomplicated, conventional LT without the use of a venovenous bypass. There were no periods of hypoxia or severe hypotension during transplantation. The cold

ischaemic time of the graft was eight hours while the warm ischaemic time was 45 minutes. The graft reperused well and no biopsies were taken. Intravenous methylprednisolone (500mg) was administered intra-operatively and the patient received induction with antithymocyte globulin post-operatively. During transplantation, the patient received a total of four units of packed red blood cells (PRBCs) and six units of fresh frozen plasma (FFP).

At the time of LT, the international normalisation ratio (INR) was 1.53, while serum total bilirubin was 103  $\mu\text{mol/L}$ . On the second post-operative day, the patient had a serum peak AST (3,469 U/L) and at this point he had further biochemical evidence of primary graft dysfunction, with the inability to normalise his INR and with progressive elevation of his total serum bilirubin. Repeated ultrasonographic examination revealed a patent hepatic artery and portal vein, as well as patent hepatic veins. At this point, a liver biopsy demonstrated severe reperfusion injury with several apoptotic bodies, several dystrophic calcifications and no evidence of acute cellular rejection. His clinical status deteriorated, he developed multi-organ system failure and died 12 days after his transplantation. No septic focus was identified. Both kidneys harvested from the same donor did not present any signs of delayed graft function after transplantation.

### Case Two

A 55-year-old man of Greek origin underwent LT for ethanol and hepatitis B-related cirrhosis, as well as a large hepatocellular carcinoma. The patient did not have a history suggestive of hyperparathyroidism or abnormal calcium metabolism and, upon listing, his serum ionised calcium was 0.94mmol/L. The donor was a previously healthy 52-year-old male who suffered a closed head injury during a motor vehicle crash. Prior to procurement the donor did not suffer any periods of hypoxia or hypotension. His liver function tests were normal prior to harvesting (AST = 36 U/L, ALT = 45 U/L). During procurement the liver was found to be well perfused, with no evidence of aberrant vascular anatomy and no evidence of trauma. Macroscopic examination of the liver did not show any evidence of steatosis. The recipient underwent a conventional LT without venovenous bypass and without intra-operative hypotension. The cold ischaemic time was 10 hours while the warm ischaemic time was 40 minutes. Following reperfusion, the graft appeared well perfused and again no biopsies were taken. Intra-operatively the patient received 500mg of methylprednisolone and post-operatively he was induced with antithymocyte globulin. During transplantation the recipient received three units of PRBCs and six units of FFP. In the early post-operative period the patient had to

be explored once for retro-peritoneal bleeding associated with hypotension (SBP < 90mm Hg).

At the time of LT, the patient had an INR of 2.7 and a serum total bilirubin of 104  $\mu\text{mol/L}$ . Following LT, the patient developed severe primary graft dysfunction with rising serum bilirubin and INR, while his serum ALT and AST peaked during the second and third post-operative day (4,700 U/L and 6,598 U/L respectively). At that time, ultrasonography demonstrated uniformly patent vessels (hepatic artery, portal and hepatic veins), while computed tomography (CT) showed areas in the right lobe of the liver isodense with the spinal column. The patient was listed for re-transplantation. During the re-transplant procedure, the explant liver graft had a 'bony' consistency in the involved right lobe. Cross-sections of the right lobe showed a 'clay-like' parenchyma with clear evidence of calcification. LM and TEM investigation of ultrathin sections obtained from specimens selected from biopsies at the interface of calcified and non-calcified tissues showed extensive intracellular calcification within the hepatic cells. High-resolution TEM (HRTEM) images and selected-area electron diffraction (SAED) combined with energy dispersive spectroscopy (EDS) analysis demonstrated the presence of hydroxyapatite (HA) as a solid phase in the calcified region. The adjacent non- or partially calcified hepatic cells displayed extensive nuclear condensation, suggestive of significant apoptosis, as well as severe vacuolisation, suggestive of an extensive apoptotic and necrotic process.

The patient had a complicated post-operative course and finally died from ventricular fibrillation, unresponsive to electrical cardioversion. Both kidneys harvested from the initial donor were transplanted without any evidence of delayed or primary graft dysfunction/non-function.

### Conclusions

Currently, more than 16,000 candidates are listed with the United Network for Organ Sharing, awaiting liver transplantation. Nevertheless, only 4,800 cadaveric liver transplants are performed annually in the US. Due to this discordance between organ demand and supply, it is estimated that approximately 10% of patients on the waiting list will die before obtaining an organ. As a result, novel strategies to expand the donor pool have been explored. With the exception of live donor liver transplantation, the remaining strategies involve the use of older cadaveric grafts, allografts with mild steatosis or even donors with evidence of past hepatitis B or C infection.<sup>6</sup> This is why one of the major obstacles to be tackled is the development of clinically significant ischaemia and reperfusion injury, which is even more important for 'marginal' grafts. Every progress towards understanding the molecular

events following not only cold storage but also cold and warm reperfusion of the graft could have a significant impact on the current transplantation practices. Indeed, recent data suggest that following I/R there is a balanced apoptosis and occasionally necrosis of hepatocytes translating into cell swelling, distension of various cellular organelles, clumping and random degradation of nuclear DNA, extensive plasma membrane endocytosis and autophagy.<sup>7</sup> Furthermore, when these events become predominant, they can lead (at least in animal models) to the development of calcifications as observed in livers of rabbits infected with rabbit haemorrhagic virus.<sup>2</sup>

This case report has shown the events that can take place in human subjects following liver transplantation. According to the authors' knowledge, these are the first reported cases in the literature of liver calcification following liver transplantation, presumably secondary to I/R injury. Not only did both patients have biochemical evidence of severe graft I/R injury, they also had biopsy proven I/R induced injury associated with the development of calcifications. Furthermore, both succumbed to the sequelae of the injury. Both recipients received grafts from donors with normal serum biochemistries and no evidence of hepatic trauma or steatosis. Both donors had no evidence of crystal deposition or storage disease and, although donor liver biopsies were not performed, the grafts appeared macroscopically normal and perfused well with UW solution. Corroborating this is the fact that all four kidneys (from both donors) were transplanted without any problems. Neither recipient had any evidence of calcium metabolism problems, since both had normal serum calcium upon listing. Both recipients had an anticipated intra-operative course without periods of hypotension and without massive transfusion requirements. Finally, both grafts did not demonstrate any vascular problems in the post-operative period by ultrasonography or CT scan examination or any evidence of intrahepatic thrombosis in post-mortem or explant examination.

Ischaemic stress has been previously reported to induce calcium accumulation at the cell level, either by impaired energy metabolism and/or plasmalemmal alterations. This elevated intracellular calcium concentration is responsible for cytoskeletal modifications, which alter cell shape for the activation of phospholipases, which in turn results in perpetuation of membrane damage and finally, mitochondrial calcification.<sup>8</sup>

Although the crystal shape, composition and organisation of HA in the samples are similar to those observed in bone and cartilage<sup>9</sup>, as well as synthetic HA formed in serum<sup>10</sup>, the intracellular precipitation of HA within hepatic cells is unique

and has not been reported from other physiological and pathological tissues.

The observation of calcified, vacuole-like structures in hepatic cells from these two livers could be suggestive of mitochondrial calcification. In addition, the extensive presence of phagocytic structures in the pre-calcified regions of these livers suggests an intense apoptotic/necrotic process undergone after I/R injury in these regions. However, further investigation is required to understand the mechanism(s) and the mode of calcification in the liver.

In conclusion, the authors believe that the described phenomenon is under-reported, at least in liver transplant literature. Furthermore, it appears that there is a correlation between the development of severe I/R injury leading to apoptosis and/or necrosis and calcifications detectable even by light microscopy. The development of microcalcifications should be studied more extensively in the context of I/R injury following liver transplantation. Although such a phenomenon appears to correlate with significant I/R injury evident through biochemical data, it has the potential to provide further information on the pathways of severe I/R injury post-transplant. ■

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