

Hepatectomy and liver regeneration: from experimental research to clinical application

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Introduction

Liver regeneration is defined as an orchestrated response induced by specific external stimuli, leading to sequential changes in gene expression, growth factors production, as well as morphological structure (anatomy).¹⁻³ Liver regeneration occurs through the proliferation of all existing mature cellular populations composing the intact organ. These include hepatocytes, biliary epithelial cells, fenestrated endothelial cells with large cytoplasmic gaps that allow maximal contact between circulating blood and hepatocytes, Kupffer cells, oval cells, liver precursor cells, and Ito cells. These cells synthesize connective tissue proteins and secrete several growth factors, such as hepatocyte growth factor (HGF), epidermal

Abstract

Background: The mechanisms and kinetics of hepatic growth have continuously been investigated. This study concerns liver regeneration in animal and patients who underwent partial hepatectomy evaluated by the hepatic extraction fraction (HEF) calculated through radioisotopic methods.

Methods: Thirty normal Wistar rats were submitted to an 85% hepatectomy, and 95 patients with primary and secondary liver tumours were included. In animal study, the liver regeneration kinetics was assessed by HEF using ^{99m}Tc-mebrofenin, the ratio liver/bodyweight and by using bromodeoxyuridine deoxyribonucleic acid incorporation. In patient study, the liver regeneration was evaluated by calculation of HEF before surgery, 5 and 30 days after hepatectomy.

Results: In animal, we verified a positive correlation between HEF kinetics and liver/bodyweight ratio or hepatocyte proliferation evaluated by bromodeoxyuridine deoxyribonucleic acid staining after 85% hepatectomy. In the clinical arm, no statistical differences of the HEF before hepatectomy, 5 and 30 days after hepatectomy, were observed.

Conclusions: Our results support the view that human liver regeneration commences early, is fast, non-anatomical and functionally complete 5 days after hepatectomy. The fast functional liver regeneration may have a high clinical impact particularly concerning the post-operative oncological therapeutic approaches.

growth factor receptor ligands, epidermal growth factor, transforming growth factor, alpha tumour necrosis factor and acidic fibroblast growth factor. Besides this cellular intervention, humoral involving interleukin 6, norepinephrine, insulin and bile acids are also important.⁴⁻⁸

The mechanisms and kinetics of hepatic growth have continuously been investigated by using either primary hepatocyte cell cultures or partially hepatectomized animals, or by evaluating liver volumes in recipient or donor patients after living donor transplantation, as well as in patients following liver resections.⁹⁻¹³

As we know, liver resection remains the treatment of choice in a number of primary and secondary liver tumours, and hepatectomy is currently considered the standard of care and remains the only

potential curative therapy for liver metastases of colorectal cancer. Complete surgical resection of isolated or multiple hepatic metastases has been associated with a 26–51% 5-year survival, which confers a clear survival advantage over 0–5% survival in patients who do not undergo liver resection.^{1–3} Similarly, liver resection can be considered the principal therapy for a specific group of patients with hepatocellular carcinoma. Although liver transplantation is accepted as the best curative option for carefully selected patients with hepatocellular carcinoma, this treatment cannot always be offered due to either patients' advanced age or scarcity of liver donors, and as a result, even in this group, hepatic resection continues to be the treatment of choice for the majority of patients (yielding 5-year survival rates as high as 60% in some series).^{4–6}

Currently, nuclear medicine methodologies are able to give dynamic functional information, with the possibility of lesion characterization and also cell function. In this context, the ^{99m}Tc-mebrofenin, a derivative of iminodiacetic acid labelled with ^{99m}Tc, is transported into the hepatocyte by a high-capacity carrier-mediated anionic clearance mechanism, organic anion transporter polypeptide. After the uptake, acid iminodiacetic derivative is transported into the bile canaliculi by active membrane transport. These compounds will have the same hepatocyte uptake, transport and excretion pathways as bilirubin. Using ^{99m}Tc-mebrofenin, a derivative of iminodiacetic acid labelled with ^{99m}Tc is possible to evaluate the hepatic extraction fraction (HEF) after intravenous injection. The presence of radiotracer in the excreted biliary fluid represents good hepatocyte function, that is tracer extraction and excretion.^{14–16} Changes in hepatocyte function modify the tracer cellular management, reflecting the dynamic process referred to. Therefore, nuclear imaging, which also provides information on biliary kinetic, is a good investigating alternative for the hepatocellular function.

Based on this, we conducted a combined experimental and clinical study to evaluate the liver regeneration using quantification of HEF after partial hepatectomy.

Methods

Animal study

Thirty male Wistar rats of 2 months of age were used for the experimental research. The animals were fed a laboratory diet with water and food *ad libitum* until surgery. They were kept under constant environment conditions with 12-h light–dark cycle. All the procedures were performed in accordance with the national and international animal care laws, and were approved by the local animal ethics committee.

The induction of liver regeneration by partial hepatectomy depends on the reduction of functional liver mass. In the rats, the liver mass makes up circa 4% of the bodyweight, the individual liver lobes constant proportions and each having a long vascular hilum with segmental portal vein, hepatic artery and bile duct.¹³ The animal model adopted was a major hepatectomy (partial hepatectomy of 85%), which was already described by Cantré *et al.*, referred this hepatectomy as an excellent model of liver regeneration, like those who reported 70% hepatectomy in rat models.^{4,9}

All the animals were anaesthetized with methoxyflurane and submitted to the major hepatectomy. Briefly, the abdomen was entered

through a midline incision, and the liver was freed from its ligaments. The left lateral lobe, median lobe and right superior lobe were then resected by placing 3/0 suture ties most proximally to their origin (left lateral and median lobes), or at short distance from their origin (right superior lobe), guaranteeing adequate blood flow to neighbouring residual lobe.⁹ Care was taken to avoid disruption of the hilar structures, including portal vein, hepatic artery and biliary tract. No animal was excluded due to mortality or morbidity. After surgery, the animals were divided into five subgroups according to the time they were sacrificed post hepatectomy: 12 h (six animals), 1 day (six animals), 2 days (six animals), 3 days (seven animals) and 5 days (five animals).

Liver regeneration study in animal

After sacrificing them, the remnant livers were procured and used to evaluate liver regeneration kinetics through DNA incorporation of 5-bromo-2'-deoxyuridine (BrdU), and the ratio of liver and bodyweight. In order to evaluate HEF, the animals were studied 5 days before surgery and 1 h before the sacrifice through an injection of ^{99m}Tc-mebrofenin in a tail vein.

Overall liver regeneration was evaluated only on the animal models. This information was obtained by two different approaches: first, by weighting the animals and the remaining livers after the hepatectomy and the calculation of liver to bodyweight ratio (LW/AW) after euthanasia; and second, by analysing the DNA synthesis using BrdU incorporation as described.¹² Briefly, BrdU (Roche) was dissolved in phosphate-buffered saline and injected (2 h before sacrificing the animals) in a concentration of 0.1 mg/g of the animal bodyweight. Samples were collected and fixed in 10% tamponated formaldehyde in order to be evaluated by fluorescence microscopy to count the number of visualized nucleoli. For this analysis, five independent optical fields were studied.

Patient study

Ninety-five patients were enrolled in this study from January 2007 to May 2009. The mean age was 63.2 ± 11.3 years (range: 32–83); 63 were men and 32 women. Sixty-nine patients had colorectal liver metastases, sixteen had hepatocellular carcinoma, two had cholangiocarcinoma, two had liver metastases from gastric cancer, two had hepatic hemangioma and four patients had other tumours. Ten patients were cirrhotic, diagnosed by radiological and histological investigations.

The selection of patients for surgery was based on liver functional status, radiological evidence of tumour resectability using computer tomography (CT) or magnetic resonance, study of hepatic volumetry by CT, absence of major contraindications precluding surgery, and in the cases of hepatocellular carcinoma patients had to be outside the Milan criteria¹⁷ and classified as Child–Pugh score grade A.¹⁸

Thirty-five patients (37%) underwent major hepatic resection (resection of three or more liver segments) (MAHR): right extended hepatectomy (*n* = 9), right hepatectomy (*n* = 11), central hepatectomy (*n* = 2), extended left hepatectomy (*n* = 1), left hepatectomy (*n* = 7) and trisegmentectomy (*n* = 5).

The remaining 60 patients (63%) underwent minor hepatic resection (MHR): bisegmentectomy (*n* = 22), left lobectomy (*n* = 8), segmentectomy (*n* = 25) and subsegmentectomy (*n* = 5).

There was no immediate or 3-month post-operative mortality. Post-operative morbidity was 5.2% ($n = 5$), which included wound infection in two patients, respiratory complications in two patients and bleeding peptic ulcer in one patient (treated conservatively).

The preoperative evaluation was done by standard liver biochemistry (plasma levels of prothrombin, albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, total and direct/indirect bilirubin, and lactic acid dehydrogenase) and Child–Pugh score.

All patients had HEF evaluated by radioisotopic methods with intravenous injection at antecubital vein of ^{99m}Tc -mebrofenin, after fasting for at least 6 h, before surgery (T0), 5 days after surgery (T5) and 1 month after surgery (T30). The HEF was evaluated 5 days after surgery because of convenience to patient.

Data analysis was performed considering major and minor hepatectomy patients globally and separately.

The study protocol was approved by the ethics committee of Coimbra University Hospital and Coimbra Faculty of Medicine. All patients were informed about the study and signed an informed consent.

Calculation of HEF

For HEF calculation, an iminodiacetic acid derivative, the 3-bromo-2,4,6-trimethylphenylcarbamoylmethyl 1-iminodiacetic acid labelled with technetium-99 m (^{99m}Tc -mebrofenin) was used. The labelling procedure was performed by adding 1110 MBq of ^{99m}Tc freshly eluted to a mebrofenin kit (GE-Amersham Health, Eindhoven, The Netherlands). For the patients studied, 185 MBq was administered, and for the animals studies the administered dose was 37 MBq. Related to dosimetry, the target organ that has the highest estimated absorbed dose is the large bowel (9.2 E-02 mGy/MBq), according to the Medical Internal Radiation Dose Committee guidelines.^{15,19}

Data acquisition for experimental and clinical groups was performed by a single detector gamma camera (GE Maxicamera 400 AC, Milwaukee, WI, USA) coupled with a low-energy, high-resolution, parallel-hole collimator, controlled by a genieACQ computer (GE, Milwaukee, WI, USA). Data were dynamically collected after the bolus injection of ^{99m}Tc -mebrofenin for $128 \times 128 \times 16$ matrix, every second during the first minute, followed by 59 frames of 60 s each without zoom. After the dynamic acquisition, two 256×256 static images, anterior and posterior, were performed with an individual duration of 67 s for decay correction.

All patients were positioned supine with the upper abdomen and cardiac area included in the gamma camera field of view. For the animals, the position was ventral decubitus directly over the collimator, with the whole body in the gamma camera field of view.

Energy discrimination was centred on 140 keV with a 20% energy window. After acquisition, the data were transferred to a work station, eNTEGRA, for processing.

On the dynamic sequence of images, regions of interest (ROIs) were flagged over the cardiac region and right hepatic lobe, avoiding the gall bladder and main intrahepatic biliary ducts of patients. For the animals studied, the entire liver remnant is included as they do not have gall bladder. Time-activity curves were generated from these ROIs, and the HEF was calculated through deconvolution analysis of the first pass curve. This measurement also gives values

of the T1/2 (time needed to reduced liver activity to half, assuming that the activity was instantaneously injected in the hepatic artery or portal vein as a Dirac delta function) and T_{max} (time of maximum liver activity).

In the experimental arm involving animal subjects, normal HEF, the T1/2 and T_{max} values were of $98 \pm 5\%$, 20.9 ± 14.6 min and 18 ± 4.6 min, respectively. Normal HEF values were of $99 \pm 0.4\%$, the T1/2 values were 39 ± 19 min and T_{max} values were 15 ± 6 min for the patients.¹⁵

This technical approach was used in both, experimental and clinical studies, to evaluate liver regeneration after hepatectomy, as described before in animal studies.^{19–21}

Statistical analysis

Continuous data were presented as mean and standard deviation. Repeated measures analyses of variance were obtained by generalized linear models within effects (time) and one factor (hepatectomy) on variables HEF (%), T_{max} (min) and T1/2 (min) in all patients. *P* values of ≤ 0.05 were considered statistically significant.

For the correlation studies between HEF and BrdU incorporation or LW/AW ratio after the partial hepatectomy, Pearson correlation was applied and evaluated at 5% significant level.

Results

Animal studies

Correlation between post-operative HEF, T1/2, T_{max} and liver/bodyweight ratio

We verified a positive correlation between HEF kinetics and LW/AW ratio after the hepatectomy, and a negative correlation between T1/2 or T_{max} and LW/AW ratio after hepatectomy (Fig. 1a–c).

Correlation between post-operative HEF, T1/2, T_{max} and BrdU incorporation

A positive correlation between hepatocyte proliferation evaluated by BrdU staining and HEF after hepatectomy was obtained. Similarly, we observed a negative correlation between hepatocyte proliferation and T1/2 or T_{max} after surgery (Fig. 2a–d).

Patient studies

Correlation between pre- and post-operative HEF, T1/2 and T_{max}

Overall patient population. The mean HEF values in our series were $97 \pm 10\%$ for T0, $98 \pm 8\%$ for T5 and $99 \pm 4\%$ for T30 ($P = 0.694$). The mean T_{max} values were 16 ± 8 min for T0, 14 ± 6 min for T5 and 14 ± 5 min for T30 ($P = 0.073$). The mean T1/2 values were 36 ± 22 min for T0, 86 ± 105 min for T5 and 40 ± 19 min for T30 ($P = 0.0001$).

Subgroup of patients treated by major resection. For the 35 patients who underwent MAHR, the HEF values were $97 \pm 5\%$ (T0), $96 \pm 13\%$ (T5) and $99 \pm 2\%$ (T30) (0.472); the mean T_{max} values were 17 ± 7 min for T0, 14 ± 6 min for T5 and 15 ± 5 min for T30 (0.073); and the mean T1/2 values were 27 ± 26 min for T0, $89 \pm$

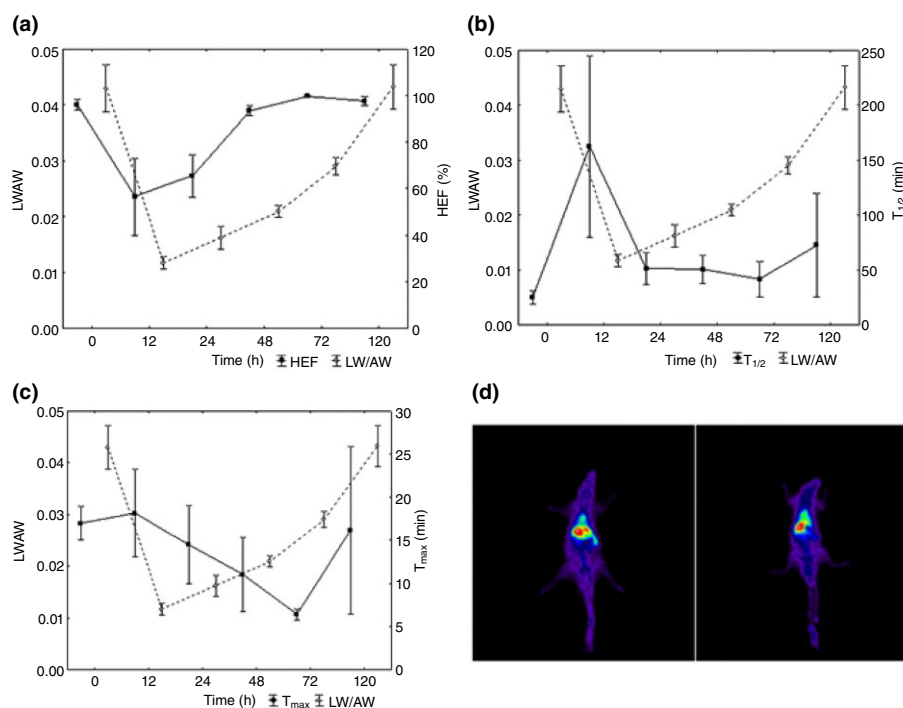


Fig. 1. Correlation between post-operative hepatic extraction fraction (HEF), T1/2, Tmax and rat liver/bodyweight. (a) Positive correlation between HEF kinetics and liver/bodyweight ratio after the 85% partial hepatectomy. (b) Negative correlation between T1/2 and liver/bodyweight ratio (LW/AW) after 85% hepatectomy. (c) Negative correlation between Tmax and liver/bodyweight ratio (LW/AW) after 85% hepatectomy. (d) 99mTc-mebrofenin scintigraphic static image in animal models before and 24 h after 85% partial hepatectomy.

74 min for T5 and 42 ± 9 min for T30 ($P = 0.001$). There are significant differences only for the values of T1/2 in T5 when compared with T0 ($P = 0.035$) (Fig. 3a,b).

Subgroup of patients treated by minor resections. For the 60 patients who underwent MHR, the HEF values were $97 \pm 11\%$ (T0), $99 \pm 2\%$ (T5) and $99 \pm 5\%$ (T30) ($P = 0.879$); the mean Tmax values were 15 ± 9 min for T0, 14 ± 6 min for T5 and 14 ± 6 min for T30 ($P = 0.098$); and the mean T1/2 values were 39 ± 19 min for T0, 85 ± 122 min for T5 and 39 ± 22 min for T30 ($P = 0.001$). There are significant differences only for the values of T1/2 in T5 when compared with T0 ($P = 0.04$) (Fig. 3a,c).

Discussion

During the last two decades, there have been major advances in the understanding of the mechanisms of liver regeneration. This knowledge, obtained from laboratory research using animals, alongside with the use of data from solid organ transplantation in human, has been applied in different experimental studies as well as certain clinical situations.³⁻⁶ The research conducted so far has resulted in the identification of a number of factors, such as HGF, transforming growth factor- α , and heparin-binding epidermal growth factor-like.^{1,3,4,6} Indeed, these factors play a very important role in enhancing and accelerating liver regeneration after liver resection or transplantation, and could positively influence livers affected by small-for-size syndrome or in injured livers secondary to trauma.

The induction of liver regeneration by partial hepatectomy depends on the reduction of functional liver mass. The model of 2/3 hepatectomy in the rat devised many years ago by Higgins and Anderson has found broad acceptance.¹³ Therefore, the increment of the portion of liver resected increases the stimulus to liver regeneration, so this is the reason why a hepatectomy of 85% was used. Increasing the proportion of liver volume resected would increase the stimulus to liver regeneration, and this was the rationale why a hepatectomy involving up to 85% of liver parenchyma was performed in our experimental arm. On the other hand, 85% hepatectomy may mimic the changes of hepatic perfusion (e.g. portal hyperperfusion) associated with reduced-size livers that can be a source of complications as the small-for-size syndrome.⁹ This is characterized by severe liver dysfunction or even liver failure in the first post-operative week, and is consequently associated with high mortality rate.⁹

Our experimental results show that immediately after hepatectomy in the rat, the liver function decreases, with the lowest values being at 24 h. After this time, the liver function recovers and returns to normal. These results suggest a correlation between liver function evaluated by 99mTc-mebrofenin and liver regeneration evaluated with BrdU staining and LW/AW ratio quantification. Our results are in line with the results published by others where no significant differences were found between 99mTc-mebrofenin uptake and conventional liver volume on days 3, 5 and 7 after 70% hepatectomy was performed in same animal model.^{19,21} Also, in this study, the authors demonstrated a good correlation between the

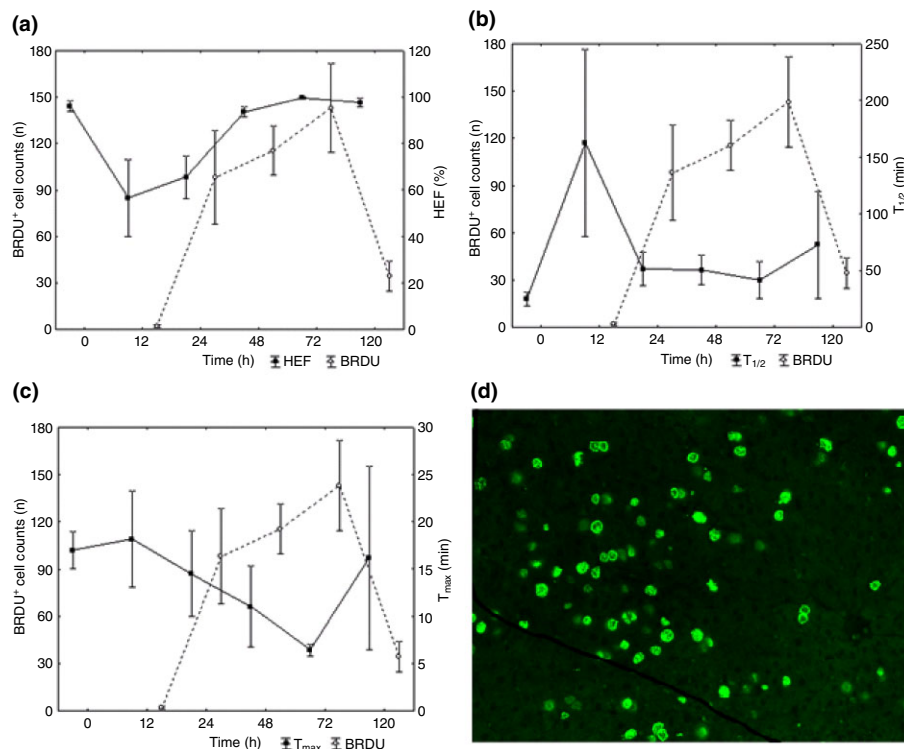


Fig. 2. Correlation between post-operative hepatic extraction fraction (HEF), T_{1/2}, T_{max} calculation after ^{99m}Tc-mebrofenin administration and bromodeoxyuridine deoxyribonucleic acid (BrdU) incorporation after rat 85% hepatectomy. (a) Positive correlation between hepatocyte proliferation evaluated by BrdU staining and HEF after rat 85% hepatectomy. (b) Negative correlation between hepatocyte proliferation evaluated by BrdU staining and T_{1/2} after rat 85% partial hepatectomy. (c) Negative correlation between hepatocyte proliferation evaluated by BrdU staining and T_{max} after rat 85% partial hepatectomy. (d) Hepatocyte proliferation evaluated by BrdU staining 24 h after rat 85% hepatectomy.

results of scintigraphic study with ^{99m}Tc-mebrofenin and the evaluation of hepatocellular proliferation by immunohistochemistry using monoclonal anti-Ki67, which is a marker of cell proliferation.²² Moreover, the authors also found that the ^{99m}Tc-mebrofenin uptake was the most sensitive of all functional methods to evaluate hepatic function during liver regeneration in standardized rat model.²¹

However, despite attempts, the obtained experimental data have not been much applied or used in clinical practice (new treatment strategies), and it was this motive that led us to conduct this clinical research. The two questions we addressed in this study are the following: First, do the same features of liver regenerations observed in animals apply to the regeneration of the human livers? Second, can our results be applied in our daily clinical practice?

Concerning the above, our study demonstrated a surprising capacity of human liver cells to regenerate, resulting in an early liver regeneration. This regeneration occurs early enough to normalize hepatic function (within 5 days after minor or major liver resection). Nevertheless, hepatic deuration needs more time in the earlier post-operative period (T₅), which improves later reaching almost normal values at T₃₀. The delay on T_{1/2} can be explained by the non-anatomic liver regeneration associated with the latter cholangiocyte regeneration compared with the hepatocyte regeneration verified in animal models of liver regeneration.⁵ In fact, the latter cholangiocyte regeneration can be the cause of the longer T_{1/2},

despite the fact that the hepatocyte regeneration is already done. Liver regeneration is difficult to study in humans, and despite extensive research on the mechanisms responsible for the complex recovery of hepatocellular volume and function that occur during liver regeneration, they are not completely understood.

CT volumetry is considered the gold standard method to evaluate liver volumetry. However, performing a CT volumetry study 24 h or 48 h after surgery is not easy considering patient's condition after complex liver surgery. Volumetric tests (e.g. CT volumetry), however, has some limitations because it evaluates the volume but does not distinguish functional from non-functional hepatic 'volume'. Moreover, CT liver volume is an indirect measurement of hepatic function because the method assumes a homogeneous distribution function, a reality that is not valid particularly in patients with cirrhosis.²⁰⁻²² Additionally, there is a poor correlation between the volumetric regeneration and functional regeneration, particularly during the first days after hepatectomy, and in our study it was essential to use functional evaluation tests to assess the recovery process of liver.^{20,22}

The most frequently used test for evaluating preoperative liver function is the indocyanine green (ICG) clearance test, which has been shown to be a better indicator of liver function than the Child-Pugh classification. ICG is a tricarboyanine dye, exclusively removed by the hepatocytes and excreted into the bile. Alternatively, ^{99m}Tc labelled iminodiacetic acid analogues, transported in blood

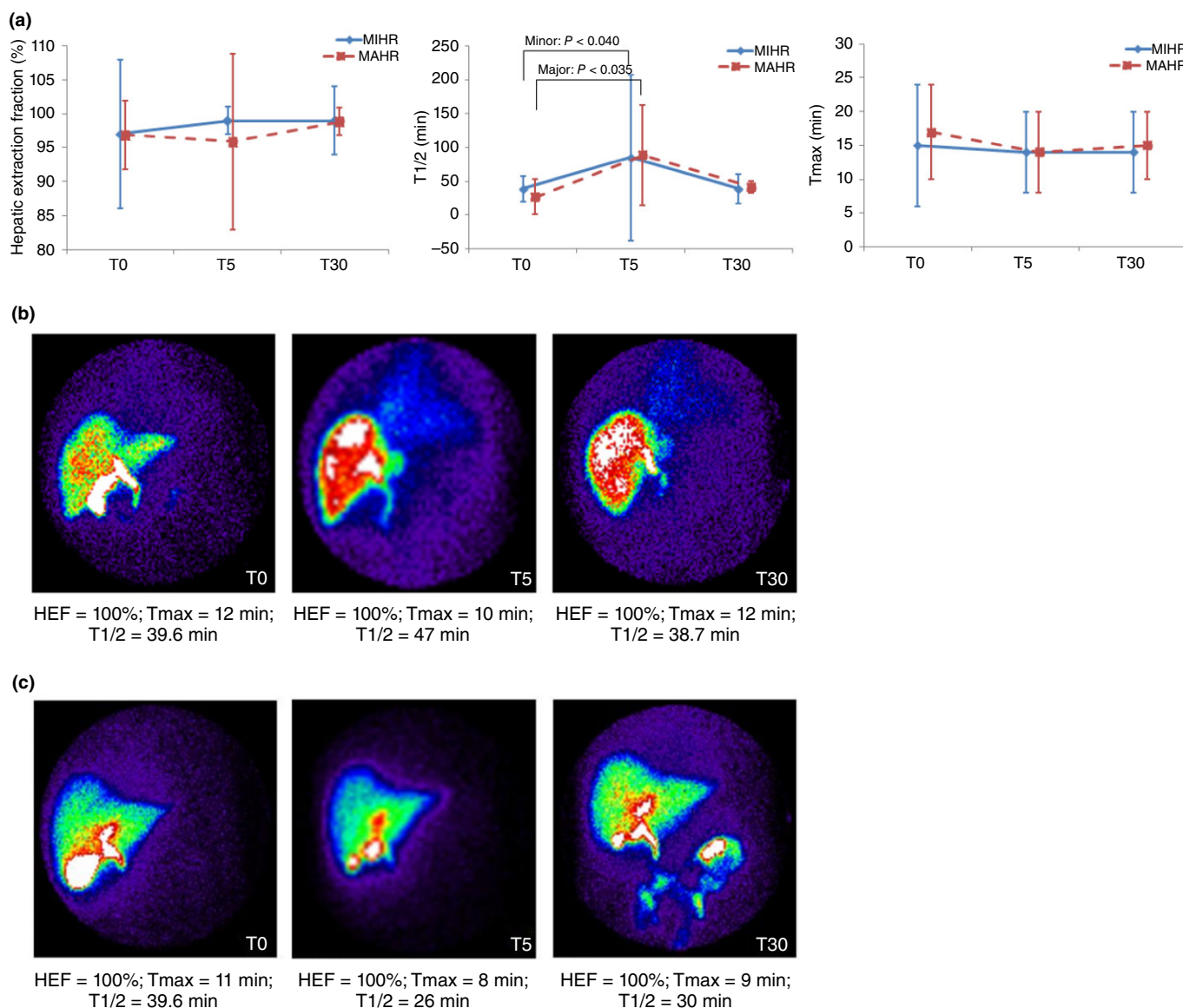


Fig. 3. Study of dynamic human liver regeneration. (a) Hepatic extraction fraction (HEF), T_{max} and T_{1/2} in each one of the evaluations (T0, T5 or T30 days after hepatectomy) as showed graphically. Time made no differences on HEF and T_{max}; however, it had a significant statistical effect on T_{1/2} (P value < 0.001), which is visible graphically, in individuals submitted to minor hepatic resection (MIHR) ($P < 0.040$), as well as those who underwent major hepatic resection (MAHR) ($P < 0.035$). (b) ^{99m}Tc-mebrofenin scintigraphic images obtained on a patient with colorectal liver metastases submitted to a major hepatic resection (MAHR) 1 day before hepatectomy (T0), 5 days (T5) and 30 days after hepatectomy (T30). (c) ^{99m}Tc-mebrofenin scintigraphic images obtained on a patient with colorectal liver metastases submitted to a minor hepatic resection (MIHR) 1 day before hepatectomy (T0), 5 days (T5) and 30 days after hepatectomy (T30).

by binding to albumin in the same manner as ICG, can be used for hepatobiliary scintigraphy in the assessment of liver function. Both ICG and ^{99m}Tc-mebrofenin are excreted in bile by the hepatocytes by the ATP-dependent export pump multidrug resistance-associated protein 2, without undergoing biotransformation during their transit through the hepatocyte. Therefore, these agents are well suited for the study of hepatic transport. In fact, ^{99m}Tc-mebrofenin uptake rate assessed by scintigraphy is a valid method for determining liver function, and correlates well with ICG clearance.²³ On the other hand, a correlation between liver function evaluated by ICG clearance and hepatic parenchymal cell volume was also demonstrated.²⁴

During the regeneration process of the liver, a strong correlation between conventional liver volume and liver functional volume was demonstrated.^{1-9,20} As liver regeneration is associated with liver volume and/or liver weight increase, what we observed with the proposed study is that hepatocellular function recovery occurs before the liver regeneration process. Our results coming from animal studies by analysing DNA synthesis using BrdU incorporation and the liver/bodyweight ratio also support the same conclusion. Additionally, these results correlate with those observed in human patients, showing that the human liver regeneration is fast, non-anatomic and certainly complex.

How could the new knowledge be applied in clinical practice?

The surprising replicative capacity of human hepatocytes has a very important clinical significance as it has allowed us to address the second question – the clinical applicability in real life of such observation. To begin with, the implications of the observed results can greatly influence the ongoing oncological practices of adjuvant chemotherapy, possibly resulting in treatments administered much earlier after surgical resection than the 3-week interval currently applied by most oncologists. Similarly, the same applies to liver resection following portal embolization. With a regeneration taking place in the first 5 days, the planned liver resection can be performed much earlier also. Furthermore, several conditions can influence liver regeneration, including the extent of resection, diabetes and the presence of underlying liver disease.^{25,26} In case of liver transplantation, the duration of the cold ischemic period and the presence of organ rejection are additional risk factors. The HEF calculated with ^{99m}Tc-mebrofenin can give a reliable assessment of liver function during regeneration, and is therefore important after transplantation or partial hepatectomy.

Conclusions

During the last few years, there have been major advances in understanding the mechanisms of liver regeneration. These advances derive, to a great extent, from the studies of liver regeneration conducted in animal models, as the results shown by us concerning the positive correlation between HEF kinetics and LW/AW ratio after hepatectomy, or the positive correlation between hepatocyte proliferation evaluated by BrdU staining and HEF after partial hepatectomy.

In parallel with the experimental work, human liver transplantation, such as cadaveric split grafts and living donors, continues to increase, stimulating hepatologists and surgeons to learn more about the mechanisms that regulate and promote regeneration. Our data based on animal and clinical results support the view that human liver regeneration is early, fast, non-anatomical and functionally complete 5 days after hepatectomy. The findings have an important clinical impact as they can lead to a strategy change of the oncological therapeutic approaches like the early start of adjuvant chemotherapy after hepatectomy.

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