Functional recovery after liver resection
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Hepatobiliary function assessed by Tc-\textsuperscript{99m} mebrofenin cholecintigraphy in the evaluation of severity of steatosis in a rat model
INTRODUCTION

The liver has a unique regeneration capacity after various types of liver damage (1). Therefore, the importance of liver regeneration as the basis of treatment for many liver diseases is growing (1). Basic research aims at a better understanding of the regulatory mechanisms governing liver regeneration eventually leading to new therapeutic strategies for encouraging liver regeneration and avoiding fibrosis (1). In vivo experiments however, are important because there have been found to be significant differences in physiological responses of hepatocytes in culture (2). Therefore, multiple animal models have been described (1,3,4). The ultimate choice of animal species depends on the scientific problem. However, mice and rats are frequently used because they are easy to manage and present minimal logistical problems (1).

Noninvasive evaluation of global and regional liver function in animal models remains a challenge. A real time test of liver function should give a measure of current hepatocellular capacity, rather than reflect past damage (5). Standard tests for liver enzymes and bilirubin plasma levels are not appropriate for describing actual liver function before and after surgery (6,7) or chronic liver conditions (8). Therefore, quantitative liver function tests have been evaluated (5). These tests however, show global liver function without information on regional function distribution.

Recently, hepatobiliary scintigraphy (HBS) has been shown to be able to assess total and regional liver function, both for uptake and excretion (9). Technetium-99m ($^{99m}$Tc) mebrofenin HBS has been used extensively in larger animals for the measurement of hepatocellular function. HBS has been performed successfully in rats suggesting a role for scintigraphic liver function assessment (8,10). However, these experiments were performed on routine gamma cameras without calculation of a hepatic clearance rate. The aim of this study was to validate liver function assessment with dedicated pinhole HBS in rats. To illustrate an application of this technique, liver function was assessed in 2 surgical models of liver regeneration.

MATERIALS AND METHODS

Study Design

All animal experiments were performed with approval of the Animal Research Ethics Committee of the University of Amsterdam and following its guidelines. Male specific-pathogen-free Wistar rats (250-300 g) were purchased from Harlan (Zeist, The Netherlands). The animals were acclimatized minimal 7 days to laboratory conditions at constant 24°C with a 12h light and dark cycle. The animals were fed a standard rodent chow and water ad libitum.

Characterization and Normal Values. 12 rats were anaesthetized and injected intravenously with $^{99m}$Tc-labeled (2,4,6 trimethyl-3-bromo) iminodiacetic acid ($^{99m}$Tc-mebrofenin). Upon injection, a dynamic acquisition was started. The hepatic mebrofenin uptake rate was calculated twice on separate days by the same observer to establish a normal range and the reproducibility of processing. Furthermore, a hepatic time-activity curve was made
for determination of the time at which maximal hepatic activity occurred (Tmax), as well as the time required for peak activity to decrease by 50% (T½). After scintigraphy, blood was collected by heart puncture, centrifuged (10 min, 3,000 RPM, 4°C) and plasma samples were immediately analyzed using standard laboratory methods. The degree of hepatocellular injury was assessed by serum levels of AST and ALT. For evaluation of hepatocellular synthesis function, plasma levels of albumin and prothrombin time (PT) were used. Livers were removed and weighed.

Reproducibility. 3 groups of 3 rats were anaesthetized and scanned on 3 separate days to assess the reproducibility of scintigraphic liver function testing. The hepatic mebrofenin uptake rate was calculated as well as the Tmax and T½.

**Intervention/ Surgical Procedure**

Eighteen rats were randomized into 2 experimental groups. Surgery was performed under inhalation anesthesia by a mixture of O₂/N₂O (1:1 V/V, 2 L/min) and isoflurane (1-2 % Florene, Abbott laboratories Ltd, Queensborough, UK) and pain medication buprenorphine (Temgesic i.v. 0.033 mg/ 0.1 kg).

To induce liver regeneration by hypertrophy, 2 commonly used surgical models were used. In the first group (n = 9), 70 % heptatectomy was performed to assess liver function during liver hypertrophy (11). In the second group (n = 9), a simplified and standardized method of portal vein embolization was used for evaluation of both the liver atrophy and hypertrophy. A ligation of the portal vein to the median and left lateral liver lobes was performed, comprising the portal blood perfusion of 70% of total liver mass (12). In both groups, 3 rats were anaesthetized and scanned 1, 3 and 7 days after surgery. After imagining, blood was collected by heart puncture. The degree of hepatocellular injury was assessed by serum levels of ALT. For evaluation of hepatocellular synthesis function, plasma levels of albumin were used. Livers were removed and weighed. The hepatic mebrofenin uptake rate was calculated as well as the Tmax and T½. Data was compared to measured liver weight.

**Camera Design**

For imaging of ⁹⁹ᵐTc-mebrofenin uptake in the rat liver, a gamma camera (Philips ARC 3000, Eindhoven, The Netherlands) situated in a dedicated animal care facility was equipped with a pinhole collimator fitted with a 3 mm tungsten insert. The pinhole collimator is facing up. On the detector, a mechanical support was mounted in which the animal is fixed in a perspex cylinder positioned exactly above the pinhole collimator (Fig. 1). The mechanical support was designed such that the midline of the cylinder is exactly in the middle of the pinhole. The position of the animal is adjustable in the axial dimension. Furthermore, the distance from the cylinder to the pinhole aperture is adjustable. Therefore, this gantry permits optimal pinhole scintigraphy of anterior projections of rats standardizing magnification and orientation. The gamma camera is interfaced to a NUD (Nuclear Diagnostics, Stockholm, Sweden) Hermes acquisition station.
Scintigraphy and Interpretation

The animals were sedated with ketamine/xylazine i.m. (40 mg/kg and 2 mg/kg, respectively). Once sedated, the rats were injected intravenously with a bolus of 40 MBq 99mTc-mebrofenin (Bridatec; GE-Amersham Health) in 0.3 mL saline in a tail vein. The animals were scanned upon injection of the radiopharmaceutical in anterior position with the liver and the mediastinum in the field-of-view. Dynamic images were obtained for 30 min (10 min at 5 sec per frame and 20 min at 60 sec per frame) at the 140 KeV 99mTc-peak with a 20% window in a 128x128 matrix. Data was processed on a Hermes workstation (Nuclear Diagnostics). The liver uptake was calculated based on a technique described by Ekman et al. (13) The algorithm was adapted for rat hepatobiliary scintigraphy based on the faster hepatic extraction of mebrofenin in rats (8). Regions of interest (ROI) were drawn around the liver, the heart and large vessels within the mediastinum (serving as blood pool) and around the total FOV (indicative of total activity). The liver ROI was drawn automatically on a threshold-based algorithm using 20% of the maximum liver value on a summed image of the first 2.5 min of the acquisition as cut-off. Three different time-activity curves were generated based on the liver, blood pool and total FOV. Liver uptake was calculated in %/min, based on these three parameters. Calculations of hepatic 99mTc-mebrofenin uptake were performed using scanned radioactivity values acquired between 30 and 120 sec postinjection, to make sure that calculations were made during a phase of homogenous distribution of the agent in the blood pool and before the rapid phase of hepatic excretion. Furthermore, a second liver ROI was drawn excluding large bile ducts and superimposing bowel loops. This ROI was used to create a hepatic time activity curve for calculation of the time at which maximal hepatic activity occurred (Tmax), as well as the time required for peak activity to decrease by 50% (T½).

Statistical Analysis

Commercial computer packages were used for the analysis of the data (GraphPad Prism, SPSS). Values are given as mean ± SEM. The relation between the liver uptake of 99mTc-mebrofenin and other parameters was tested using the standard Pearson correlation coefficient r. All statistical tests were 2-tailed and differences were evaluated at the 5% level of significance.

RESULTS

Dedicated animal pinhole dynamic hepatobiliary scintigraphy was very feasible in rats. Under anesthesia, intravenous injection is possible with the animal positioned before the pinhole collimator (Fig. 1). Dynamic scintigraphy shows a rapid hepatic uptake of the radiopharmaceutical, with excretion into the bowel (Fig. 2A). After partial hepatectomy, the hepatic uptake is decreased (Fig. 2B). Liver uptake can be quantified and expressed as percentage uptake per min using ROI’s encompassing the liver and the heart as blood pool correction (Fig. 3). Determination of Tmax and T½ based on the kinetics of the entire
(or remaining) liver volume was sometimes difficult due to over projection of bowel loops.
HBS caused no mortality or excessive morbidity except for the anesthesia.

**FIGURE 1.** A rat is placed in a perspex cylinder and is being injected intravenously with 99mTc-mebrofenin in a tail vein at the start of a dynamic acquisition (A). The rat is positioned with the upper abdomen before the aperture of the pinhole collimator. The transaxial position is fixed and centered in the midline of the cylinder. The axial position can be adjusted manually for optimal positioning of the target organ (B).
FIGURE 2. Dynamic pinhole HBS in a rat after intravenous injection with 40 MBq 99mTc-mebrofenin. Images are reframed to 30 sec/frame. (A) Normal HBS in a rat showing fast and homogeneous liver uptake with visible excretion into the bowel starting from 3 min post injection. (B) Abnormal HBS in a rat after partial resection. HBS shows delayed and inhomogeneous liver uptake with visible excretion into the bowel starting from 6 min post injection.

FIGURE 3. HBS in a rat. (A) Summed image from 30 – 120 sec after intravenous injection of 40 MBq 99mTc-mebrofenin. A ROI is drawn around the entire liver. A second ROI is drawn in the mediastinum.

Characterization and Normal Values
The animal characteristics and results are illustrated in Table 1. The mean body weight was 350 ± 7 g. The mean liver weight was 10.92 ± 0.44 g. The mean scintigraphic liver uptake rate was 77.29 ± 1.29 %/min. Calculation of the liver uptake percentage per min was highly reproducible, with an excellent correlation (r = 0.95, P < 0.001) between both calculations. The variation of liver uptake was low in rats assessed 3 times within 1 week (Fig. 4). All measured values stayed within the normal range. There was no influence of previous anesthesia on behavior or functional results.
### Table 1. Characterization and normal values

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<th>HBS Uptake 2 (%/min)</th>
<th>HBS Tmax (min)</th>
<th>HBS T1/2 (min)</th>
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| Mean | 350             | 10.92            | 77.29                 | 77.74                 | 2.24           | 6.19           | 69         | 67         | 33        | 16.9     |
| SEM  | 7               | 0.44             | 1.29                  | 1.23                  | 0.08           | 0.14           | 5          | 4          | 1         | 0.2      |
| SD   | 24              | 1.51             | 4.47                  | 4.27                  | 0.27           | 0.50           | 16         | 12         | 2         | 0.6      |

### Intervention / Surgical Procedure

Liver weight was decreased 1 and 3 days after resection with recuperation within the normal range 7 days after surgery (Fig. 5A). Liver weight was increased 1 and 3 days after ligation with normalization 7 days after surgery (Fig. 5A). After surgery, there was a significantly (P < 0.01) decreased liver uptake rate, with an inferior uptake rate (P < 0.05) after partial resection as compared to portal ligation (Fig. 5B). There is regeneration of liver function 3 and 7 days after surgery, which was significantly higher (P < 0.01) after resection as compared to ligation (Fig. 5B). After surgery, there is a significant (P < 0.01) increase of serum ALT. This increase is significantly higher (P < 0.01) after ligation as compared to resection (Fig 6A). Seven days after surgery, serum ALT normalizes in both groups. After resection, the albumin level in the plasma decreases, with normalization within 1 week. After portal ligation, the albumin level remains within the normal range (Fig. 6B). There is a strong positive association (r = 0.85, P < 0.001) between liver weight and the functional uptake rate after resection (data not shown). There was a good association between liver
FIGURE 5. (A) Liver weight of rats (mean ± 1 SD) at baseline and after resection (▲) or ligation (■). There is a significant reduction (P < 0.01) in liver weight 1 and 3 days after resection with recuperation within the normal range 7 days after surgery. The horizontal dashed line indicates the 95% CI based on the normal values (Table 1). (B) Scintigraphic liver uptake rate of rats (mean ± 1SD) at baseline and after resection (▲) or ligation (■). There is a significant reduction (P < 0.01) in uptake rate 1 and 3 days after resection with recuperation 7 days after surgery. The horizontal dashed line indicates the 95% CI based on the normal values (Table 1).

FIGURE 6. (A) Serum ALT of rats (mean ± 1 SD) at baseline and after resection (▲) or ligation (■). There is a significant increase (P < 0.01) in serum ALT 1 and 3 days after resection with normalization within the normal range 7 days after surgery. The horizontal dashed line indicates the 95% CI based on the normal values (Table 1). (B) Serum albumin of rats (mean ± 1SD) at baseline and after resection (▲) or ligation (■). There is a significant decrease (P < 0.01) of serum albumin 1 and 3 days after resection with recuperation 7 days after surgery. Serum albumin after ligation remains within the normal range. The horizontal dashed line indicates the 95% CI based on the normal values (Table 1).

weight and Tmax or T½ (r = 0.78, P < 0.01 or r = 0.73, P < 0.05, respectively) after resection (data not shown).

DISCUSSION

HBS has been described as a valid alternative for the indocyanine green (ICG) clearance test in the preoperative assessment of liver function (6). Iminodiacetic acid (IDA) enters the hepatocyte via a carrier-mediated non-sodium-dependent organic anion path, by a mechanism very similar to that of bilirubin or ICG (14). Both ICG and IDA-analogues are excreted in the bile by hepatocytes via the ATP-dependent export pump multidrug-resistance associated protein 1 and 2 (MRP1,2) (75). Furthermore, preoperative assessment
of remnant liver function can be performed with HBS (9). Moreover, liver function regeneration can be monitored in vivo with HBS and correlates better with quantitative liver function testing than liver volume regeneration (9). Therefore, quantitative HBS for research purpose was evaluated in rats where repeated measurement of liver function is needed for an accurate evaluation of chronic liver disease or regeneration after an acute event otherwise requiring sacrificing a number of animals per measurement time point. HBS using $^{99m}$Tc-labeled IDA-analogues has been performed in rats (8,10,16,17). However, liver function was determined by time activity curve derived parameters (8,16), invasive sampling (10) or uptake scintigraphy without correction for blood pool activity (17). The liver uptake function measured by iodida clearance rate was described by Ekman et al. (13). This technique was adapted and validated for $^{99m}$Tc-mebrofenin (6). For use in rats, the dynamic acquisition has been adapted to the faster metabolism to make sure that calculations were made during a phase of homogenous distribution of the agent in the blood pool and before the rapid phase of hepatic excretion. Calculation of the liver uptake rate is performed between 30 and 120 sec postinjection, which is before the average Tmax. Calculation of the liver uptake rate from a dynamic acquisition was highly reproducible. Furthermore, there was little inter-subject variability under standardized conditions within time. Moreover, we have demonstrated that HBS can be performed multiple times in an animal creating the possibility for repetitive measurement within an experiment increasing statistical power and possibly reducing the number of animals needed for experiments. Besides the hepatic uptake rate, Tmax and T1/2 were calculated. Unlike Tmax, which can be calculated easily for the entire liver, T1/2 was sometimes difficult to determine due to over-projection of bowel loops. This precludes characterization of the excretory phase of the entire liver or evaluation of regional excretion obscured by bowel uptake. Finally, Tmax could be influenced by intrahepatic cholestasis, which can occur initially without repercussion on the hepatic uptake function (14). Studies have been performed to estimate the hepatic functional reserve by making use of $^{99m}$Tc-DTPA-galactosyl human serum albumin ($^{99m}$Tc-GSA) liver scintigraphy in rats (18,19). $^{99m}$Tc-GSA is a liver scintigraphy agent that binds to the asialoglycoprotein receptor on hepatocytes (20). It was found that the total counts and counts per unit hepatic volume based on $^{99m}$Tc-GSA in the entire liver significantly decreased in patients with more extensive liver disease (21). The disadvantage of $^{99m}$Tc-GSA is that it does not provide any information on hepatic excretory function. Improvement of scintigraphic resolution, enabling imaging of small laboratory animals, can be achieved by using a pinhole collimator, both for planar or SPECT (22,23). Pinhole collimators are routinely available in most nuclear medicine facilities. Moreover, dedicated animal gamma cameras have become commercially available. To obtain magnified images with high spatial resolution in small-animal studies, system sensitivity is sacrificed and more radioactivity or longer acquisition time is needed. In rats, increasing the amount of radioactivity administered relative to body weight (compared with humans at a factor of 40) results in statistically useful pinhole images with a 7-fold magnification. Pilot experiments assessing the possibilities of HBS in rats using interventional models show a good correlation between liver weight and function after resection. After ligation, liver function decreases and liver weight increases, probably due to edema. Serological liver
function parameters show diverging results in function of the intervention with higher ALT values after ligation, which can be explained by increased cell damage and lower serum albumin after resection, which can be explained by a decreased functional volume. HBS shows a consistent pattern of decreased liver function, both after resection and ligation. Furthermore, HBS can be repeated to assess functional recovery within 1 animal, enabling a longitudinal study design.

CONCLUSION

Dedicated animal pinhole hepatobiliary scintigraphy using $^{99m}$Tc-mebrofenin offers a unique combination of functional liver uptake and excretion assessment with the ability to determine the liver function reserve before and after an intervention. HBS is a repeatable noninvasive imaging tool providing visual and quantitative information and enabling serial measurement within an animal potentially improving statistical power and reducing the number of animals needed for research.

REFERENCES


