Original research

The natural history of liver regeneration in rats: Description of an animal model for liver regeneration studies

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ABSTRACT

Background: Rodent models have been used to evaluate aspects of liver regeneration. The aim of the present study was to investigate the natural history of liver regeneration in healthy rats.
Methods: A 70% partial hepatectomy was performed in 64 rats. The animals were randomised into 8 groups and evaluated on postoperative days one to eight. Hepatocyte proliferation was evaluated by immunohistochemistry using unbiased stereological principles.
Results: The mean rat body weight was 238 g (211–287). The mean weight of the resected liver was 6.3 g (5.2–7.3) and the estimated mean total liver weight was 8.9 g (7.4–10.4). Both liver weight analysis and regeneration rate showed an ascending curve, with a maximum slope on postoperative days 1–4, reaching a steady state on days 5–8. Hepatocyte proliferation (positive Ki-67 cell profiles pr. mm^2) was high (250 cell profiles/mm^2) on postoperative days 1–3 and tapered off on day 5.
Conclusion: Seventy percent partial hepatectomy in healthy rats induces a rapid regenerative response and PODs 2, 4 and 8 seems optimal for assessing hepatic growth in future studies.

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1. Introduction

The liver is characterised by complex physiology and the ability to undergo rapid regeneration.[1–3] Documented evolutionary pressures have driven these unique traits, and have resulted in a phenomenal ability to recover lost functional capacity without jeopardising the viability of the entire organism. Liver regeneration is dependent on neoangiogenesis and tissue proliferation. In short, liver regeneration occurs by a compensatory hyperplasia and hypertrophy of the residual liver lobes, not involving stem cells.[4]

Animal models, particularly the rat model, are commonly used to investigate new aspects of liver regeneration. Ever since Higgins and Anderson presented a method for standardised partial hepatectomy in 1931, liver resection has been refined and used many times over.[5] Today, the most widely used models are those involving 70% and 90% resection. However, there is no consensus on how and when to assess liver regeneration in the rat model.

The aim of the present study was to evaluate the natural history of liver regeneration in healthy rats after a 70% partial hepatectomy (PHx) and to determine the optimal timing and frequency of hepatic growth assessment in this model for use in future studies.

2. Material and methods

From April 2010 to July 2010, 70% PHx was performed on 64 rats. All animal experiments were performed under the approval of the Danish Animal Research Committee, Copenhagen, Denmark, and in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health, USA. Animals were housed in standard animal laboratories, with the temperature maintained at 23 °C, an artificial 12-h light–dark cycle and free access to food and water, until the time of the experiment. All surgical procedures were performed under inhalation anaesthesia: induction was performed in a glass cylinder through which a mixture of oxygen 2.0 l/min, N$_2$O 0.5 l/min and 4% isoflurane (Forane; Abbott Laboratories, Maidenhead, UK) was blown. During surgery, anaesthesia was maintained with 2% isoflurane, oxygen and N$_2$O as above, administered through a mask covering the rat's nose.

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With the animal placed in a supine position on a heated pad, a transverse abdominal incision was made and the liver was mobilised. PHx was performed using a previously described technique.[5] Briefly, the base of the median and left lateral lobes was ligated, and the lobes were resected, resulting in 70% liver resection. The abdomen was closed with 4–0 absorbent suture in two layers single knots. At the end of all operations, the animal received a subcutaneous injection of a long lasting NSAID, 5 mg/kg Carprofen (Rimadyl; Pfizer Animal Health, Exton, USA) and 1.0 ml isotonic saline. The rats were kept in the animal facility until the end of the experiment. After the initial PHx, rats were block-randomised in groups of four for evaluation on the same postoperative day (POD), i.e., the animals in group 1 were analysed on POD 1, the animals in group 2 on POD 2 and so forth.

On the given POD, the rats were again weighed and anaesthetised, and a laparotomy was performed through the previous incision. Blood samples were collected from the heart by cannulation. All rats were subsequently sacrificed by cervical dislocation. The regenerated liver was then mobilised and removed. Liver weight was recorded, and the caudate lobe was resected and fixed in phosphate-buffered 4% formaldehyde for 24–48 h before paraffin embedding. Body weight, morbidity and mortality, was recorded daily throughout the study period. Dead rats were autopsied to establish the cause of death.

3. Biochemical analysis

Blood was sampled from the heart at sacrifice, processed and stored at −80 °C until analysis. Alanine aminotransferase (ALAT), alkaline phosphatase (ALP) and bilirubin (BR) levels were measured using the Modular P (Roche Diagnostics, Mannheim, Germany).

Rat acute phase protein α-2-macroglobulin was evaluated using a specific ELISA kit (Immunology Consultants Laboratory, Newberg, Oregon, USA) according to the manufacturer's instructions. Samples were assayed in duplicate. All assays exhibited intra- and inter-assay coefficients of variance below 5% and 10%, respectively.

Plasma IL-6 and TNF-α were measured using specific rat IL-6 (R&D Systems, Minneapolis, MN, USA) and TNF-α (R&D Systems) immunoassays, respectively. All assays used in this study have previously been validated for use in rats and were performed as specified by the manufacturers' instructions.

4. Branched-chain amino acids to tyrosine ratio

The branched-chain amino acids to tyrosine ratio (BTR), was measured using an enzymatic method (Diacolor-BTR kit; ONO Pharmaceutical, Osaka, Japan) on the Modular P (Roche Diagnostics).

5. Liver weight and regeneration rate

The preoperative total liver weight was calculated from the resected liver weight.[5] Postoperative total liver weight was measured at sacrifice. The change in liver weight was evaluated as the hepatic regeneration rate (RR). RR is defined as (liver weight per 100 g of the body weight at sacrifice/preoperative projected liver weight per 100 g of the body weight) × 100:

$$RR = \frac{(LWm/100gBW)sac}{(LWp/100gBW)pre} \times 100$$

LWm is the measured liver weight at sacrifice; LWp is the preoperative projected liver weight.

6. Immunohistochemistry

Hepatocellular proliferation was estimated using immunohistochemical staining for the Ki-67 antigen. Ki-67 is preferentially expressed during all active phases of the cell cycle (G_1, S, G_2 and M phases), but not in resting (G_0) cells.[6] Immunohistochemical staining was performed on 2 μm paraffin sections using a standard (in-house) protocol. Briefly, sections were boiled in a microwave oven in Tris-EGTA buffer, pH 9 for heat-induced epitope retrieval. Monoclonal mouse anti-rat Ki-67 specific antibody (clone MIB-5, isotype IgG1; Dako, Glostrup, Denmark)[7] diluted at 1:20 was used as the primary antibody in this study. Slides were incubated with the primary antibody overnight at 4 °C. Sections were washed, positive signals were visualized using the EnVision + horseradish peroxidase labelled anti-mouse detection system (Dako), and the slides were counterstained with haematoxylin.

7. Stereological quantitation

Systematic, uniformly random sampling was used in the preparation of histological sections as previously described.[8] Briefly, the paraffin embedded caudate lobe was exhaustively sectioned into 2 μm parallel sections. A sampling fraction of 130–250 sections, depending on the size of the caudate lobe, was used to produce approximately 10 sets of samples (range 8–14) per liver. From this set of samples, four sections were randomly selected as described previously[9] and immunostained for Ki-67.

All sections were analysed using the Olympus BH-50 microscope, modified for stereology with a motorised stage and a digital camera connected to a PC with newCAST 3.6.5.0 software (Visiopharm, Hørsholm, Denmark). The same investigator analysed all sections. An unbiased counting frame was used, as shown in Fig. 1a and b, and counting was performed using a 20× lens, for a total magnification of 700×. A mean of 75 randomly selected non-
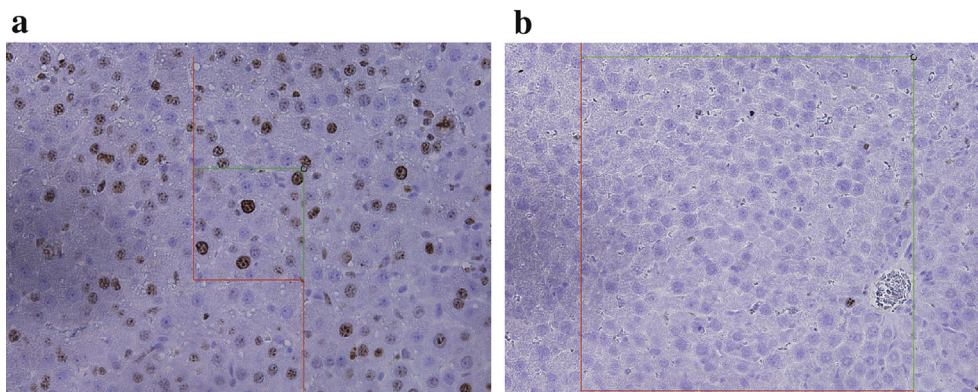

Fig. 1. 1a and 1b – Kasper Jarlhelt Andersen: Ki-67 stained liver sections from POD 3 (1a) and POD 8 (1b). The counting frames are displayed. A positive hepatocyte was counted if it was significantly stained according to a trained histologist, and if the cell was in the counting frame. The red lines (left and bottom) are exclusion lines and green lines (top and right) are inclusion lines. 1a) Proliferation Index = 256. Counting frame 100 × 100 μm Four Ki-67 positive hepatocytes are observed in the frame with numerous more outside the frame. 1b) Proliferation Index = 0.57. Counting frame 300 × 300 um One Ki-67 positive hepatocyte is observed inside the frame in the bottom right corner.

overlapping fields of view were used per section. The fraction of Ki-67 positive cell profiles Q_A was calculated using the following formula:

$$Q_A = \frac{\sum Q}{\sum A} = \frac{\sum Q(\text{Ki-67})}{a(\text{mm}^2) \cdot \sum Q}$$

Q is the number of Ki-67 positive cell profiles; a is the area of the counting frame; and P is the number of counting frames evaluated. The counting rules used are described in Fig. 1a and b.

8. Statistical analysis

Data are presented as the mean ($\pm$SEM). The reproducibility of the stereological data was tested by re-evaluation of all sections from eight randomly chosen livers. Data from the first (A_1) and second evaluations (A_2) were compared using the Mann−Whitney Rank sum test. Finally, the variability between readings was assessed by difference-average plots, as described by Bland and Altman[10]

9. Results

9.1. Mortality and morbidity

During the experiment, five animals died prior to evaluation, one of ileus, one as a consequence of suture gnawing resulting in intestinal perforation, and three of unidentifiable cause. All other rats were healthy and behaved as expected with regard to activity and food consumption.

9.2. Body weight

Preoperative mean body weight was 238 g (211−287). Weight loss was noted during the first 4 PODs, after which weight gain was observed (Fig. 2).

9.3. Liver weight

Mean weight of the resected 70% liver was 6.3 g (5.2−7.3), giving an estimated mean total liver weight (100%) of 8.9 g (7.4−10.4). Gain in liver weight during the recovery/regenerative period for each group is shown in Fig. 3a. Major growth was noted during
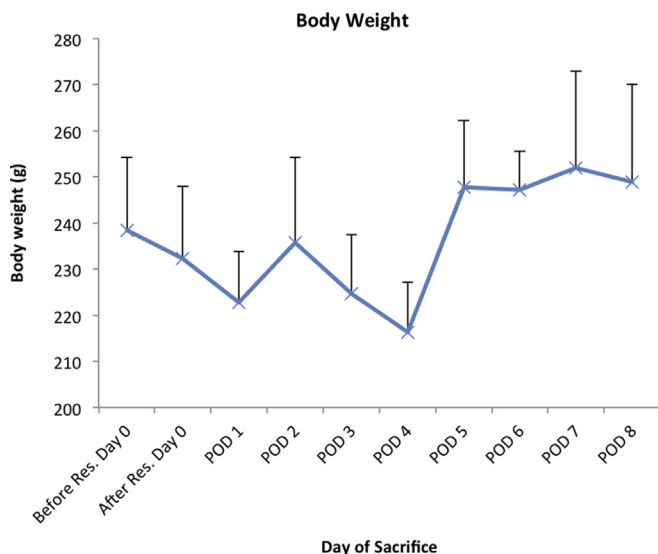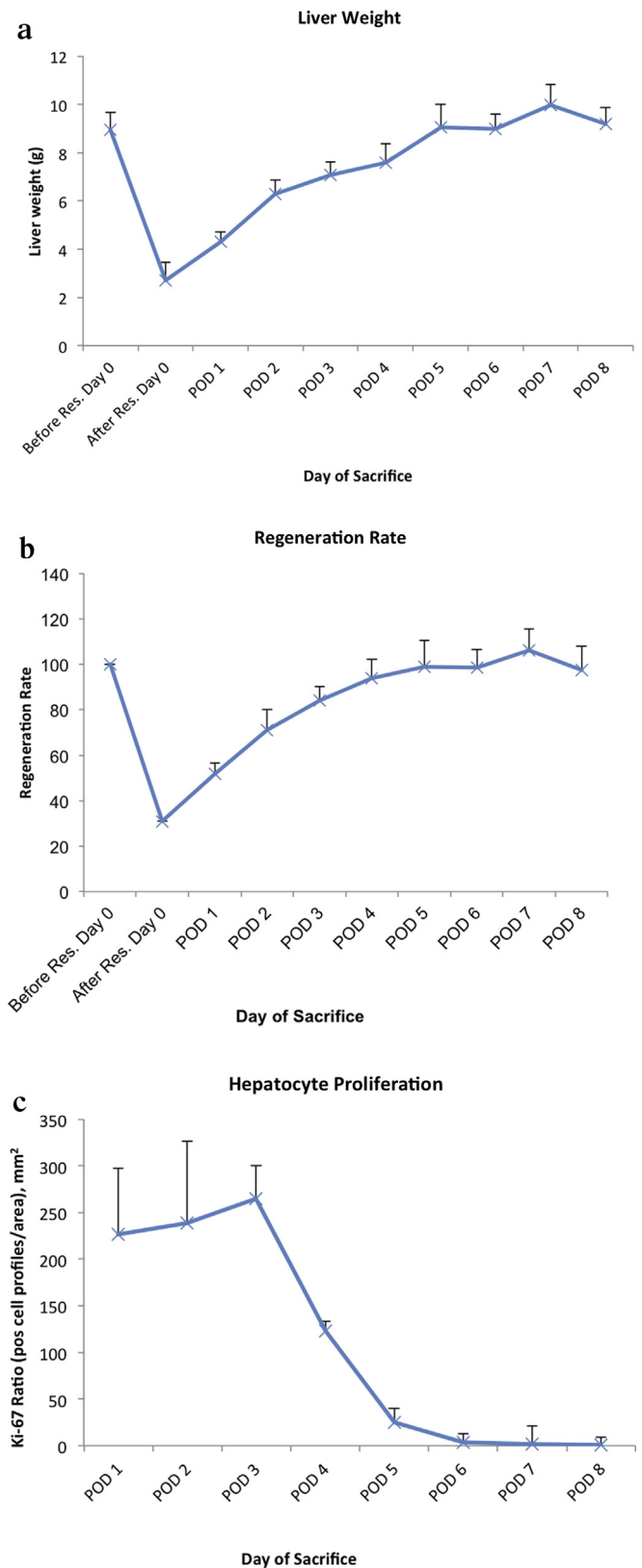
Fig. 3. 3a, 3b and 3c − Kasper Jarlhelt Andersen: Mean liver weight (3a), regeneration rate (3b), and proliferation index (3c) (+SD) for each group of animals.

Fig. 2. Kasper Jarlhelt Andersen: Mean body weight (+SD) for each group of animals.

PODs 0–5 and surpassed normal preoperative weight on POD 7 before returning to baseline on POD 8.

9.4. Liver regeneration rate

RR showed a pattern (Fig. 3b) similar to the liver weight curve, i.e., exceeding the original rate on POD 7 before reaching a steady state on POD 8. However, the RR curve was more stable and had less variation on each postoperative day than did the liver weight curve.

9.5. Hepatocyte proliferation

Proliferation was maximal on days 1–3; thereafter a decline was seen to approximately zero on POD 5 (Fig. 3c). The p-value for A_1 vs. A_2 in Ki-67 stained sections was 0.959, indicating no significant difference between the mean values. The Pearson correlation coefficient between A_1 and A_2 was 0.992.

9.6. Liver biochemistry

9.6.1. Alanine aminotransferase

High concentrations of ALAT were observed during the early part of the postoperative period with a level on POD 1 of 365 U/L. The concentration rapidly decreased during the following days before reaching a steady state of approximately 55 U/L on POD 4 (Fig. 4a).

9.6.2. Bilirubin

BR demonstrated a pattern similar to alkaline phosphatase with a peak on POD 3. The level on POD 3 was 4.50 umol/L and the steady state level was approximately 1 umol/L (Fig. 4b).

9.6.3. Alkaline Phosphatase

This parameter showed an increasing concentration during the first 3 PODs with a peak of 307 U/L on POD 3. Afterwards, a falling slope was observed, approaching a steady state of 250 U/L (Fig. 4c).

9.6.4. α-2-macroglobulin

This concentration showed an overall downward trend throughout the study period from 501 ug/mL on POD 1 to 78 ug/mL on POD 8. The slope, however, was a bit jagged, and a fairly large variation was observed for each day (Fig. 4d).

9.6.5. Interleukin-6

High concentrations were observed in the early phase after resection with 271 pg/mL POD 1. On days 4–8, very low levels were recorded (Fig. 4e).
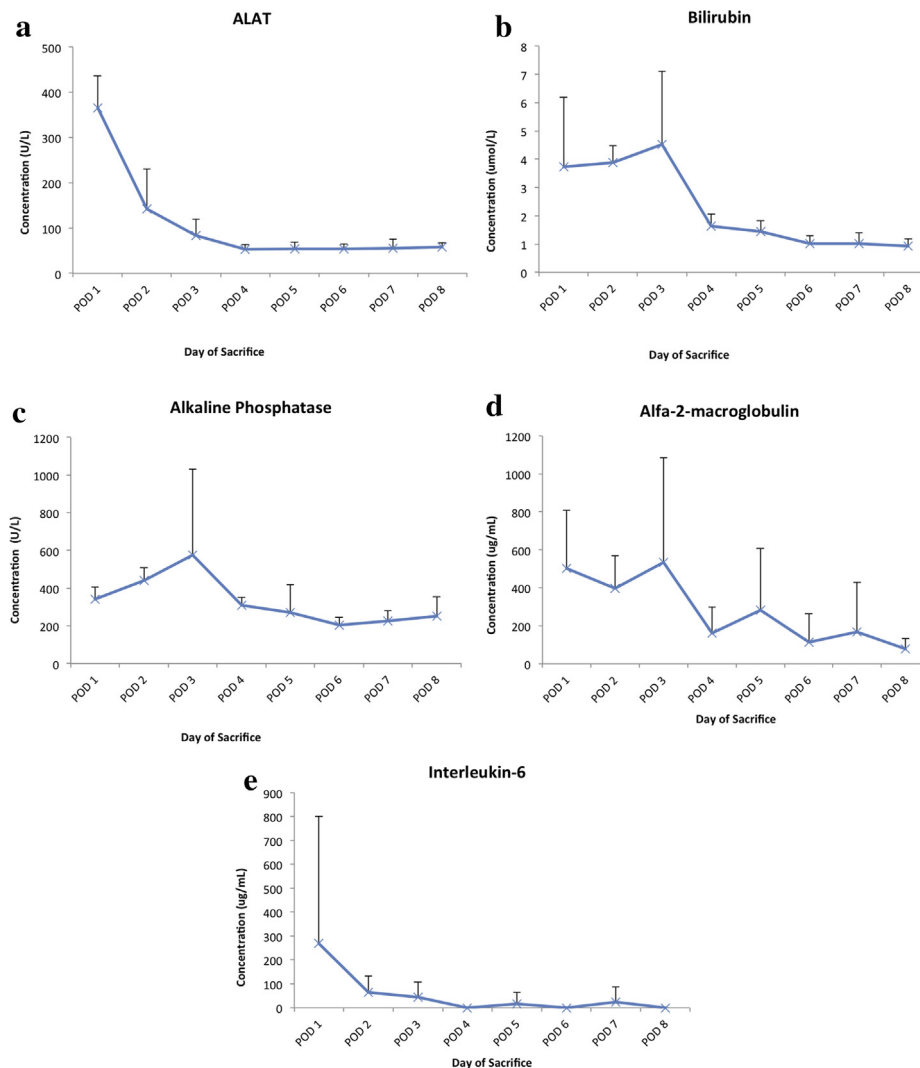

Fig. 4. 4a, 4b, 4c, 4d and 4e − Kasper Jarlhelt Andersen: Mean alanine aminotransferase (4a), bilirubin (4b), alkaline phosphatase (4c), α-2-macroglobulin (4d) and interleukin-6 (4e) (+SD) for each group of animals.

9.6.6. TNF-α

The levels of TNF-α were undetectable throughout the entire study period.

9.7. Branched-chain amino acids to tyrosine ratio

BTR analysis showed an increasing ratio throughout the study period. A rise from approximately 2.75 on POD 1 and POD 2 to approximately 4 on POD 8 was observed (Fig. 5).

10. Discussion

Rat models are widely used in the experimental investigation of liver regeneration. In spite of this, there is surprisingly no consensus as to how and when to assess hepatic regrowth in these experiments. Our study is the first to investigate systematically the natural history of liver regeneration in rats. We report that 70% PHx in healthy rats induces a rapid regenerative response with a maximum rate on PODs 1–4, liver regeneration (as judged by liver weight, hepatic regeneration rate and hepatocyte proliferation) being complete on POD 8.

Dynamics in body weight are a reliable marker of acute stress in animals and as such are crucial to studying liver resections.[11] Our study rats lost weight until POD 4, after which there was a steep increase in body weight reaching a plateau slightly above the preoperative weight on POD 5. We evaluated liver damage in the postoperative period by measuring ALAT, ALP and BR levels. Liver markers, particularly liver transaminases, have been related to the degree of hepatic injury.[12–14] In keeping with this, we found high initial levels of ALAT on PODs 1–3, consistent with resection-associated liver injury, after which levels fell to a low steady concentration. BR and ALP were elevated on POD 1 and increased until POD 3, presumably reflecting damage to the intrahepatic biliary tree. Following this, a steep decline was observed in these markers. α-2-macroglobulin, a rat acute phase protein produced by hepatocytes,[15] showed a steady decline from POD 1 to POD 8. This suggests that the initial raised α-2-macroglobulin level in our study resulted from surgical trauma and as such was not specifically related to liver regeneration.

Cytokine IL-6 and TNF-α are important mediators of injury to the liver. IL-6 has been shown to inhibit apoptosis and induce hepatocyte proliferation,[16,17] whereas TNF-α has been shown to induce hepatocellular apoptosis.[18] We observed an induction of IL-6 with high levels in the immediate period following resection. This concentration rapidly declined during PODs 2–3 before reaching a low-level steady state on PODs 4–8. Elevated IL-6 levels

immediately after resection have been reported in other studies, as addressed by Clavien PA,[19] the author concluding that IL-6 induces hepatocyte proliferation. In contrast, TNF-α concentrations were very low (undetectable) throughout the entire study period, suggesting that apoptosis is minimal during liver regeneration.

We chose BTR, a variable known to correlate with the Fisher ratio, serum albumin level, prothrombin time and retention of indocyanine green, as a marker of liver function.[20,21] BTR levels increased steadily from PODs 1–8. This suggests that liver function improved throughout the study period and was not solely related to liver weight, RR and proliferation index, since these parameters all showed maximal increases on PODs 1–3 followed by a pronounced fall. The peak in BTR observed on POD 3 could be explained by larger variations in measurements on that particular day (Fig. 5). Liver weight and RR showed nearly identical patterns, with levels increasing steeply on PODs 1–3 before reaching a plateau on POD 5. Thus, liver weight and RR were not directly related to liver function as estimated by BTR.

In tissue studies, hepatocyte proliferation can be estimated using immunohistochemistry to detect cells positive for the Ki-67 antigen. Thus, proliferation can be estimated as the number of positive Ki-67 stained liver cell profiles per tissue area high power field.[7] A variety of methods, each with inherent drawbacks, have been used in previous histological studies to evaluate hepatic injury and liver regeneration. Chief amongst these have been standard morphological assessment of routinely or immunohistochemically stained sections, and semi-quantitative systems for scoring immunohistochemically stained sections, such as described by Suzuki et al.[22] Standard morphological examination, in which an observer evaluates a representative tissue sample, is subjective, being highly dependent on the investigator and being without any component of randomisation. Furthermore, this method does not produce quantitative data, making it difficult to make statistical comparisons. We believe that these major drawbacks make this an inappropriate methodology for accurate assessment of liver growth. Similarly, semi-quantitative immunohistochemical scoring systems have inherent flaws. For example, they do not allow for the reference area or volume to be accurately estimated and, thus, run the risk of producing highly biased results. This fundamental problem can be avoided by employing histological methods based on well-established unbiased stereological principles. Although these methods are more laborious than corresponding non-stereological protocols, they generate highly accurate unbiased quantitative data.

Our studies are the first to employ stereological methods to assess hepatic growth in rats in an experimental setting. We estimated both the number of Ki-67 positive cells in study sections, and the area in which the cells were located. Fields were evaluated in a randomised manner to obtain this ratio. The reproducibility of our data was tested by comparison of means, correlation analysis and Bland–Altman plot. We found a low intra-observer variability between count A_1 and A_2. In accordance with the results of assessing liver weight and RR dynamics, we found high hepatocyte proliferation on PODs 1–3, after which levels fell to nearly zero on POD 6.

In summary, a 70% PHx in healthy rats induces a rapid regenerative response and PODs 2, 4 and 8 seems optimal for assessing hepatic growth in future studies.

Ethical approval

No Ethical Approval necessary.

All animal experiments were performed under the approval of the Danish Animal Experiment Inspectorate, Copenhagen, Denmark, under license number 2009/561-1752 and in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health.
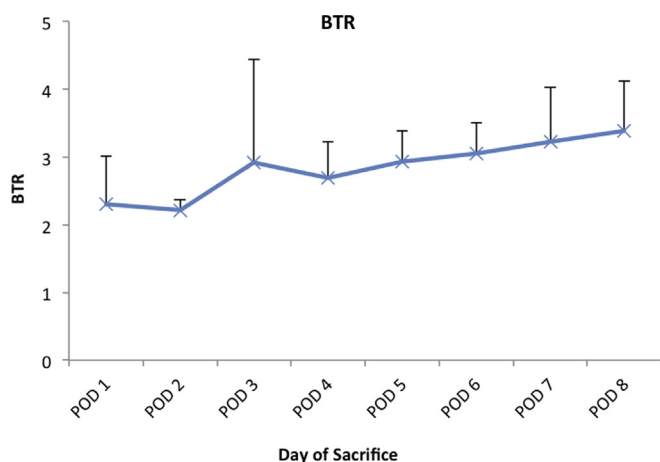

Fig. 5. Kasper Jarlhelt Andersen: Mean BTR (+SD) for each group of animals.

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