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Abstract

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Morphological Dynamics of the Regenerative Hepatocyte

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Background: Malignancy of the liver is one of the primary causes of cancer-related death world-wide. Partial hepatectomy (PH) is first-line curative treatment owing to the unique ability of the liver remnant to regenerate while maintaining body homeostasis. Depending on the size and functional capacity of the liver remnant, deterioration of function can lead to post-hepatectomy liver failure. Therefore, if malignancy is widespread in the liver, the size of PH and, hence, the size of the liver remnant may limit curability. The overall aim of this study was to investigate and characterize the impact of different sizes of PH with regard to the rat liver regenerative process. Specifically, for results presented here, we wanted to investigate the morphological dynamics of the regenerating hepatocyte (hypertrophy and proliferation) assessed by stereology.

Materials and Methods: In a rat model of 30%, 70%, and 90% PH together with a group of sham-operated and non-operated rats, the following were investigated at post-operative days (POD) 1, 3, and 5: hepatocyte volume and hepatocyte proliferation together with liver-specific biochemistry and regeneration ratio.[1]

Hepatocyte volume was estimated using immunohistochemical staining for the hepatocyte cell membrane-proteine β-catenin, involved in cell-cell adhesion and localized to the cell membrane in normal cells ([1]). The object of interest (OI) was hepatocytes with a clear β-catenin-stained cell membrane and a counterstained oval nucleus. The planar rotator was used for estimation ([2]). The procedure is described briefly in Figure 1. The mean hepatocyte volume in a section (V_{mean}) was calculated using the following equation (1):

$$V_{\text{mean}} = 10^{V_{\text{mean}}(\log(V_i))} \quad (1)$$

[1] A percentage estimate of the actual size (wet weight) of the liver remnant relative to the estimated liver weight prior to PH to evaluate the increase in liver size (weight) in the postoperative period after PH.

$$V_{\text{mean}}\Big(\log(V_i)\Big) = \text{a logarithmic calibrating constant}$$
$$= \sum_i \log\Big((V_i) \times \Big(\frac{t_i/h_{\text{dis}}}{\sum_j (t_j/h_{\text{dis}})}\Big)\Big),$$
$$V_i = \text{the individual hepatocyte volume, calculated by the NewCast software,}$$
$$t_i = \text{the section thickness} = 30\,\mu\text{m},$$
$$h_{\text{dis}} = 12\,\mu\text{m}.$$

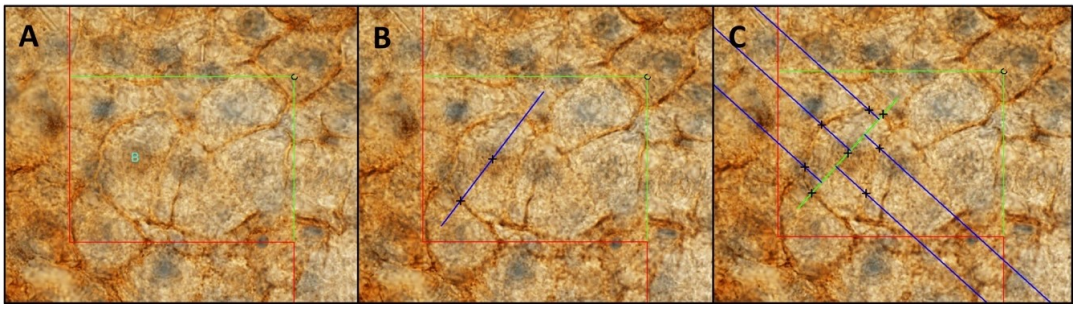

Figure 1: β-catenin stained section presenting the steps measuring the hepatocyte volume by the planar rotator [2]. CF is displayed and the above described counting rule was followed, the OI being hepatocytes with a clear β-catenin stained cell membrane (dark-red) and a counterstained (gray) oval nucleus in focus. The planar rotator was applied as follows: **A**: Through the middle of the nucleus the longest axis of the hepatocyte was marked ("B" in the picture indicating that this hepatocyte is counted). **B**: Intersections between the longest axis and the hepatocyte cell-membrane were marked. defining the cell "height". **C**: The software generated uniform, random, and parallel test-lines perpendicular to the longest hepatocyte cell axis and intersections between these test lines and the hepatocyte cell membrane were marked, defining the distance of the intercepts at both sides of the axis. In each section, a mean of 25 CFs (75 µm × 55 µm) and an average of 50 hepatocytes were sampled for hepatocyte mean volume estimation. The figure was published by M. Meier et al. [3].

Hepatocellular proliferation was estimated using immunohistochemical staining for the Ki-67 antigen. Ki-67 is a protein located in the nucleus and exclusively present in proliferating cells [4, 5]. The object of interest (OI) was Ki-67-positive stained nuclei of the hepatocytes. The optical fractionator method was used for estimation [6]. The procedure is described briefly in Figure 2. The total number of proliferating hepatocytes (N) of the posterior caudate lobe was calculated using the following equation (2):

$$N = \frac{1}{\text{SSF}} \times \frac{1}{\text{ASF}} \times \sum Q^- \tag{2}$$

$$\text{SSF} = \text{section sampling fraction} = \frac{\text{BA}}{\text{mean}(t_{\text{slab}})}$$
$$\text{BA} = \text{block advance} = \text{the height of the sampled section},$$
$$= 30\,\mu\text{m}$$

$\text{mean}(t_{\text{slab}})$ = the mean thickness of the embedded slabs after shrinkage
= 1784 µm [7],

ASF = area sampling fraction = $\frac{a(\text{frame})}{dx \times dy}$,

$a(\text{frame})$ = the area of the two-dimensional unbiased counting frame,

dx and dy = the stepping distances in the x and y directions,

HSF = height sampling fraction = $\frac{h_{\text{dis}}}{\text{mean}(t_{Q^-})}$,

h_{dis} = the disector height = 15 µm,

$\text{mean}(t_{Q^-})$ = the Q-weighted mean section thickness,

$\sum Q^-$ = the total count of Ki-67 positive hepatocytes in a section.

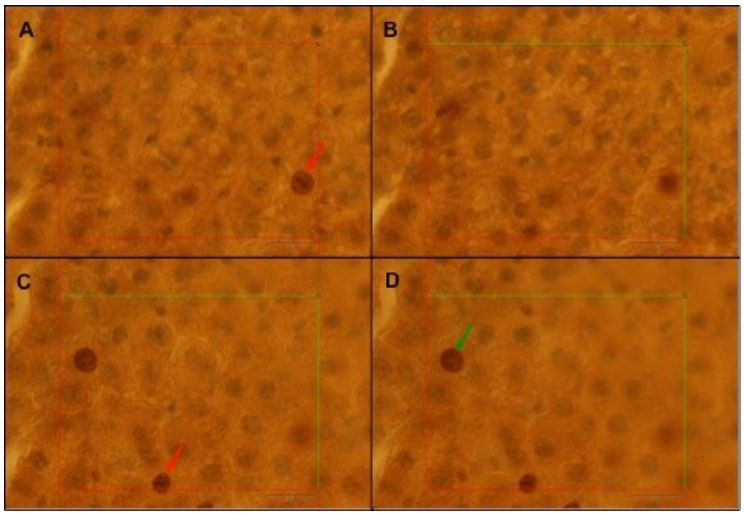

Figure 2: Figure 2 Ki-67-stained section presenting four different optical levels (A-D) within the section when focusing the microscope down through the section. The number of proliferating hepatocytes was estimated by the optical fractionator method [6]. The counting frame (CF), the red lines are exclusion lines, and green lines are inclusion lines. Following the universal counting rule [8], the objective of interest (OI) being Ki-67 positive-stained hepatocyte nuclei (Ki-67pos) were counted if they were entirely contained within the CF or if they touched an inclusion line without touching an exclusion line. **A**: Inactive CF – Ki-67pos not included (NI) (red arrow). **B**: Active CF – Ki-67pos not in focus, therefore NI. **C**: Active CF – Ki-67pos touches exclusion line, therefore NI. **D**: Active CF – Ki-67pos entirely contained within the CF, therefore included in the count (green arrow). A minimum of 100 positive cells had to be counted per section to maintain statistical significance. Therefore, prior to the counting-process each section was grouped according to an overall valuation of the number of positive hepatocytes in the section: Few Ki-67pos → number of CFs: 120. Moderate number of Ki-67pos → number of CFs: 50. Numerous Ki-67pos → number of CFs: 25. The figure was published by M Meier et al. [9].

Results: Overall, the dynamics and the molecular biological response of liver regeneration were highly dependent on the extent of the PH. Of special interest was the dynamics following PH(90%), biochemically presenting with post-hepatectomy liver failure at POD1 (very low prothrombin-proconvertin ratio and

extremely elevated bilirubin and ammonia). Although in a critical state, the regeneration rate following PH(90%) increased immediately after surgery, reaching almost 100% at POD5. The hypertrophic dynamics after PH(90%) followed that of the less extensive PHs, with an early volume increase and peak at POD1, more pronounced the more extensive the PH (Fig. 3A). Likewise, the proliferative response to PH(90%) was most pronounced, although delayed (Fig. 3B). The results are summarized in Figure 3C.

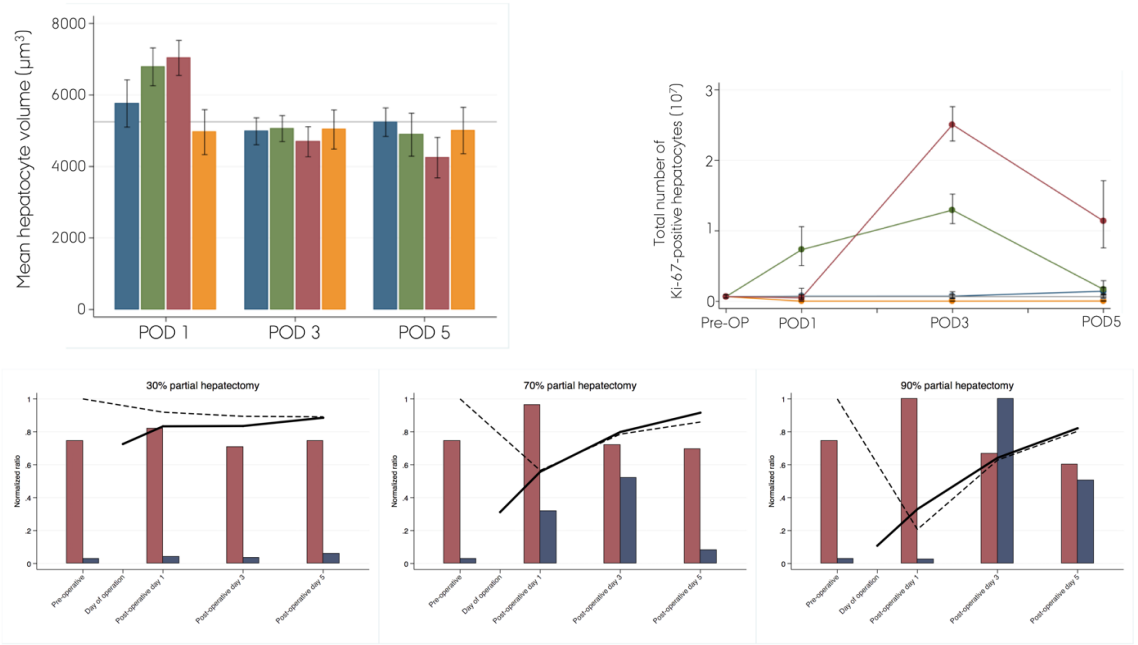

Figure 3: Figure 1 **A**: Mean hepatocyte volume by size of partial hepatectomy. **B**: Total number of Ki-67-positive hepatocytes in the posterior caudate rat liver lobe (median). Blue: PH(30), green: PH(70), red: PH(90), orange: sham, gray: baseline. 95% CI. **C**: Hypertrophy and proliferation of the hepatocytes normalized to the maximum potential (90%PH POD1 resp. POD3) in relation to the regeneration rate and liver function (PP) by size of hepatectomy pre- and postoperatively. Red bar: normalized mean hepatocyte volume, blue bar: normalized number of Ki-67 positive hepatocytes, black line: regeneration rate, dashed line: prothrombin-proconvertin ratio (normalized to baseline). The figures were published by M Meier et al. [9, 3].

Conclusion: Although in a critical state of post-hepatectomy liver failure, the hypertrophic dynamics after extensive PH(90%) follows that of less extensive PHs, with an early volume increase and peak at POD1, which is more pronounced the larger the PH. Yet, the proliferative response to PH(90%) is delayed, proposedly as a consequence of the early condition of post-hepatectomy liver failure.

The above-presented studies and results were part of the PhD thesis "The morphological and molecular biological response to partial hepatectomy and regeneration in rats" from 2019, by M. Meier.

References

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