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MORPHOMETRY AND RECONSTRUCTION OF HEPATIC LOBULES IN PIG BASED ON SERIAL HISTOLOGICAL SECTIONS

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Abstract: *Aims of our study were as follows: 1) to perform a 3D reconstruction of parts of the adjacent classical hepatic lobules, 2) to compare the histomorphometric techniques available for estimating the volume of the lobules using 3D reconstruction and interactive stereological techniques, 3) to assess the volume fraction of liver parenchyma, connective tissue, and blood vessels, 4) to quantify the shrinkage in tissue blocks porcine liver. The morphometric parameters were assessed using serial histological sections and stereological methods. Volume of the sampled portions of the lobules ranged between 0.282-0.498 mm^3. The volume fraction of parenchyma in liver was 0.867, the volume fraction of connective tissue was 0.077and the fraction of blood vessels was 0.026. The mean volume shrinkage of porcine liver was 55.23%. This value can be used for correcting the data based on paraffin-processed histological sections. For further quantitative studies, we suggest the nucleator technique as a fast, robust and reliable method for estimating the volume of the lobules. For biomechanical modelling, the absolute volumes resulting from histological studies have to be corrected due to the tissue shrinkage affecting the paraffin-processed tissue samples.*

Keywords: Liver, Stereology, Volume, Point grid, Nucleator.

1. Introduction

Advanced biomechanical models of biological organs should be based on statistical morphometry of their microscopic architecture. Quantitative description of the microscopic properties of real tissues sample is an advantage when devising computer models that are statistically similar to biological tissues under physiological or pathological conditions (Králíčková, 2013). Biomechanical models of liver have already proved to be useful when clarifying biological problems such as liver perfusion, ontogenetic growth, fibrosis and cirrhosis, metastatic spread, and remodeling or regeneration after surgical resection (Debbaut et al, 2014; Marcos et al., 2012).The research of the liver is often done using porcine model (Ekataksin and Wake, 1991; Králíčková, 2013; Lehmann et al., 2008) due to its similarity with human liver.

Description of hepatic blood circulation gives an insight into liver pathology and may be used to inspire or modify surgical techniques as well. Corrosive vascular casts and micro-computed tomography are used for three-dimensional reconstruction of the hepatic circulation (Debbaut et al., 2014). A simplified liver microstructure may be characterized by periodically repeating morphological units known as representative volume elements (REVs). Naturally occurring REVs in liver are the classical polygonal hepatic lobules surrounding the central veins. In rat, three-dimensional (3D) reconstruction of these units revealed their volume to range between 0.094 and 0.621 mm^3 (Teutsch et al., 1999).

Another morphometric parameter of liver tissue is the ratio between the functional parenchyma and the structural supporting connective tissue. This ratio may greatly vary, e.g., in hepatic fibrosis or cirrhosis. However, all 3D reconstructions and morphometric studies based on routine formalin-fixed and paraffin-embedded histological sections are biased by a significant and hardy predictable tissue shrinkage (Dorph-Petersen et al., 2001).Therefore the aims of our study were as follows: 1) to perform a 3Dreconstruction

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of parts of the adjacent classical hepatic lobules as REVs, 2) to compare the histomorphometric techniques available for estimating the volume of the lobules using 3D reconstruction and interactive stereological techniques, 3) to assess the volume fraction of liver parenchyma, connective tissue, and blood vessels, 4) to quantify the shrinkage in tissue blocks representing porcine liver.

2. Methods

Four tissue blocks (approx. 1 × 1 × 1 cm) were taken from the liver of a healthy pig (age 14 weeks, weight 25 kg). The animal received humane care in compliance with the European Convention on Animal Care and the whole project was approved by the Faculty Committee for the Prevention of Cruelty to Animals.For 3D reconstruction, one sample was fixed in 10% formaldehyde, rinsed in 70% ethanol, and dehydrated. The cutting plane was randomized and the blocks were embedded in paraffin. The block was cut into a series of consecutive 3-µm-thick 50 histological sections, every second of them was stained with aniline blue and nuclear fast red. All sections were photographed using a 2× objective. The images were registered in order to correct the shift and rotation deviation between the serial sections using the Imagreg software (Jiri Janacek, The Academy of Sciences of the Czech Republic, Prague). Using the Polygon tool of the Ellipse software (ViDiTo, Košice, Slovak Republic), five classical lobules as well as the central veins were outlined (Figs. 1a and 1b) and their areas were recorded.Using the Surface module of the Ellipse software, a 3D model connecting the contours was rendered and used for both visualization (Figs. 1c and 1d) and quantification of the reconstructed parts of lobules.

Next, profiles of the same lobules used for reconstruction were measured using the Nucleator plugin in the Ellipse software. The nucleator method has been devised as a local stereological probe to estimate volume of objects provided that each object has a unique arbitrary point and the sections are either isotropic uniform or vertical uniform (Gundersen et al., 1998; Marcos et al., 2012). Briefly, in any n-dimensional space, the distance l between an arbitrary fixed point measure and the object boundary in any isotropic direction provides an unbiased estimate of the object content as follows:

$$content = c \cdot \overline{l^n} \tag{1}$$

where for n = 1, 2, 3, the content is length, area, or volume and c = 2, π, or 4π/3 (Gundersen et al. 1998). In our case, a two-dimensional (2-D) nucleator was used. Therefore n=2 for a sample of two-dimensional isotropic uniform sections and content gives an estimate of mean area of the profile of morphological lobules. We used a 2-D nucleator probe with four isotropically oriented rays (Fig. 2c). Next, we estimated the volume of the sampled portions of classical lobules using the Cavalieri principle (Mouton, 2002) by multiplying the sum of there are as from the serial sections by the distance between the sections. We used the stereological point grid (Mouton, 2002) to quantify the volume fractions of the parenchyma, connective tissue and the blood vessels (Figs. 2a and 2b).The variation between the serial sections was assessed with use of the coefficient of error CE calculated with the quadratic approximation formula of Matheron, modified for use in a stereological context (Gundersen& Jensen, 1987).

Three tissue blocks were taken using punch biopsy (10-mm diameter) to estimate the tissue shrinkage. The dimensions of each biopsy was precisely measured using a caliper and their volume was calculated and labelled as *V(primary volume)*. The samples were fixed in 10% formaldehyde, rinsed in 70% ethanol, dehydrated and routinely embedded in paraffin. Tissues blocks were exhaustively cut into series of consecutive 5-µm-thick histological sections, every tenth section was stained with hematoxylin and eosin. The volume of each processed tissue block *V(volume_after_processing)* was estimated using the stereological point grid (Fig. 2d) and Cavalieri principle. The volume shrinkage was calculated using the formula *1- V(volume_after_processing)/V(primary).*

3. Results and Discussion

Volume of the sampled portions of the classical lobules ranged between 0.282 mm^3 and 0.498 mm^3.

The volume fraction of parenchyma in liver was 0.867 (CE = 0.009), the volume fraction of connective tissue in liver was 0.077 (CE = 0.021) and the fraction of blood vessels in liver was 0.026 (CE = 0.021).

The volume shrinkage was 56.8% in sample #1, 59.4 in sample #2, and 49.5% in sample #3. The mean volume shrinkage of porcine liver was 55.23%. Shrinkage of hepatic tissue was substantial, probably due to the large blood supply. This value can be used for correcting the data based on paraffin-processed

histological sections. The final volume of the structures before histological processing *V(final)* can be calculated as *V(final)=V(primary volume)*(1-volume shrinkage).*

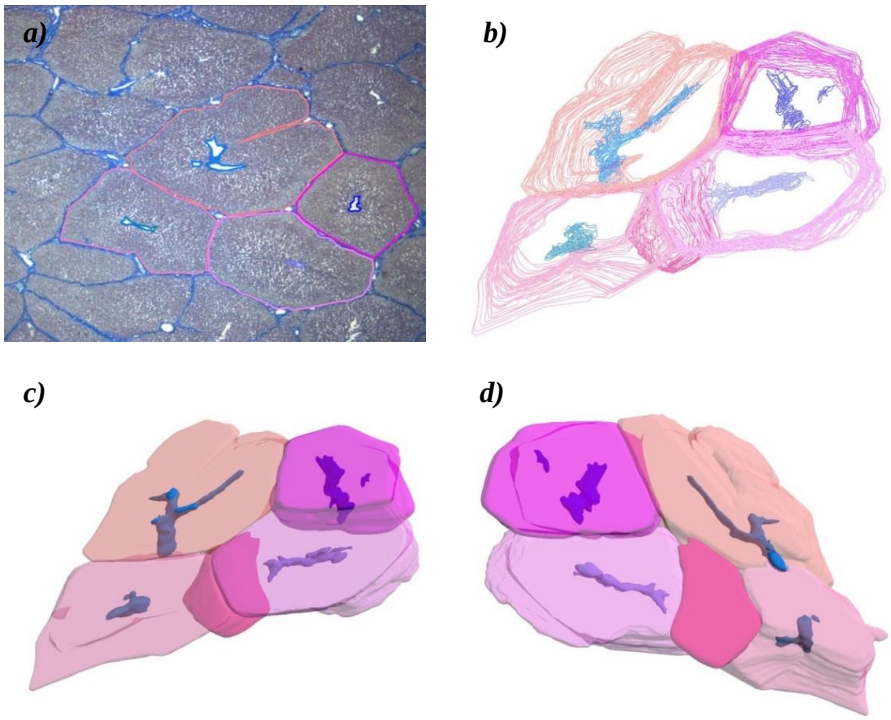

Fig. 1: Three-dimensional reconstruction of hepatic lobules: a) Tracing the contours; b) 3D view of the contours; c), d) 3D reconstruction of classical lobules and their central veins.

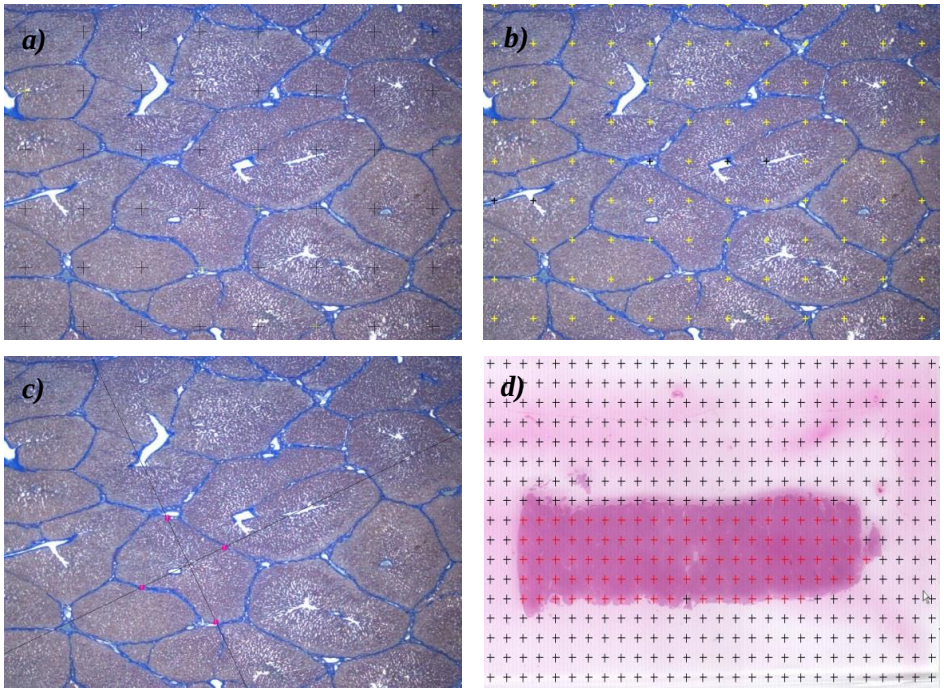

Fig. 2: Estimating volumes and areas using stereological point grid (a),b),d)) and the nucleator technique (c)): a) – Points hitting the interlobular connective tissue were marked yellow and used for estimating the volume fraction of the connective tissue. Each point represents the same predetermined area. B) Points hitting the functional parenchyma were labelled yellow. c) The sectional area of a lobule quantified using a two-dimensional nucleator probe with four isotropically oriented rays. The intersections of the probe rays with the borders of the lobule are marked. d) Using point grid for quantifying the volume after histological the processing to quantify the tissue shrinkage.

Tab. 1: Volume of sampled portions of hepatic lobules assessed by three methods: planimetry (V(contours)), 3D reconstruction (V(3D)) and the nucleator method (V(nucleator)). The variability of the volume estimates is characterized by the coefficient of error (CE).

	V(contours) [mm^3]	**CE (contours)**	**V(3D) [mm^3]**	**V(nucleator) [mm^3]**	**CE (nucleator)**
lobule#1	0.498	0.008	0.493	0.491	0.017
lobule#2	0.282	0.009	0.279	0.275	0.017
lobule#3	0.372	0.008	0.366	0.363	0.014
lobule#4	0.305	0.008	0.299	0.297	0.010
lobule#5	0.050	0.020	0.046	0.472	0.037

4. Conclusions

Using 3D reconstruction and stereological methods, we visualized portions of five adjacent classical hepatic lobules and the corresponding central veins, thus partially demonstrating their spatial relations. We compared three techniques for estimating the volume of the lobules. For further quantitative studies, we suggest the nucleator technique as a fast, robust and reliable method. Next, we quantified the volume fractions of the major constituents of the liver, namely the functional parenchyma, the connective tissue, and the blood vessels. For biomechanical modelling, the absolute volumes resulting from histological studies have to be corrected due to the tissue shrinkage affecting the paraffin-processed tissue samples.

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