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STEREOLOGY AND MORPHOMETRY OF STEATOSIS IN HUMAN ALCOHOLIC (ALD) AND NON-ALCOHOLIC LIVER DISEASE (NALD)

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ABSTRACT

Steatosis is one of the main features of alcoholic liver disease (ALD) and may be seen in some patients with non alcoholic liver disease (NALD). In this study we have quantitatively assessed steatosis in relation to histopathological and clinical categories of ALD and NALD by stereological and morphometric techniques.

Haematoxylin and eosin (H&E) stained sections from ninety-two needle liver biopsies were analysed using the Prodit 5.2 system. All biopsies were categorised into five histopathological groups: normal, pure steatosis, steatofibrosis, pre cirrhosis and cirrhosis. The area fraction (A_A) of fibrosis, steatosis and parenchyma were assessed by stereological method. The mean diameter, area and area ratio of fat globules in zone 1 and zone 3 were determined by morphometric analysis. Stereological analysis has shown significant variation in the area fraction of fat in different histopathological categories reaching a maximum value of 54% of liver cell parenchyma. Significant differences in the areas of steatosis were observed between the clinical groups (ie ALD and NALD). Morphometric analysis showed significant differences in diameter of fat globules between zone 1 and zone 3 in the two major clinical categories. In alchoholic liver disease fat globules are mainly large and pericentral in location whereas in the non alchoholic group the fat globules are smaller and periportal in location.

It can be concluded from this study that quantitative analyses are useful techniques to differentiate between ALD and some sub-clinical categories of NALD. Stereology has the advantages of assessing steatosis in the entire liver biopsy specimen while morphometry is very useful for assessing steatosis in relation to topography.

Keywords: alcoholic liver disease, cirrhosis, liver, morphometry, steatosis, stereology.

INTRODUCTION

Chronic ethanol intake induces an injury in human liver leading to a variety of histopathological manifestations. The morphological features of alcoholic liver disease have been reviewed by many authors (Baptisto et al 1981, Harrison & Burt 1993, Popper et all 1989, Jenkins and Peter 1978). Steatosis (Auger et al 1986, Baptisto et al 1981, Chedid et al

1991, Harrison & Burt 1993, Jenkins and Peters 1978, Mark and Lieber 1988) and fibrosis (Baptisto et al 1981, Caulet et al 1989, Chedid et al 1991, De Creamer et al 1995, Ryoo et al 1989, Wormer and Lieber 1985, Rappaport et al 1983, Chevallier et al 1994, Naveau et al 1994, Rojkind et al 1979) are the main morphological features of alcoholic liver disease. These, however, are seen also in many types of non-alcoholic liver disease (Adler and Schaffner 1979, Diehl et al 1988, Klain et al 1989, Naveau et al 1994, Wanless and Leutz 1990, Nagone and Scheuer 1988).

Accumulation of fat occurs preferentially in zone III (centrolobular area), eventually involving all zones of the parenchyma. Traditionally fat globules are classified into two main forms, macrovesicular and microvesicular, the mixed form is also seen in some patients. Fat in liver disease has been assessed by semiquantitative methods (Nagone & Scheuer 1988) and by automated computerised procedures (Arnould et al 1979) and also in several animal models of fatty liver (Arnould et al 1979, Mak and Lieber 1988).

In this study, we have concentrated on the stereological and morphometric features of steatosis of alcoholic and non-alcoholic liver disease. Liver biopsies were assessed from normal controls, steatosis, steatofibrosis, pre cirrhosis and/or cirrhosis. In the order presented, the histology reflects the severity and progression of the disease process in both alcoholic and non-alcoholic liver disease. We also aimed to evaluate the morphometric subgrading of fat globules in differentiating ALD from NALD.

MATERIALS AND METHODS

Eighty seven liver biopsy specimens from patients with alcoholic (36) and non-alcoholic liver disease (51) were evaluated. All patients with alcoholic liver disease had a long history of alcohol intake with clinical and biochemical evidence of liver disease. Causes for non-alcoholic liver diseases included obesity (7), diabetes (5), drug-induced hepatitis (7), primary biliary cirrhosis and hepatitis C (6), others included pregnancy associated steatosis (9) and unknown mechanisms (13). In control patients (5) biopsies were performed during the laparoscopic excision of gallbladder. These patients had no history of excess alcohol intake and they had normal liver function tests.

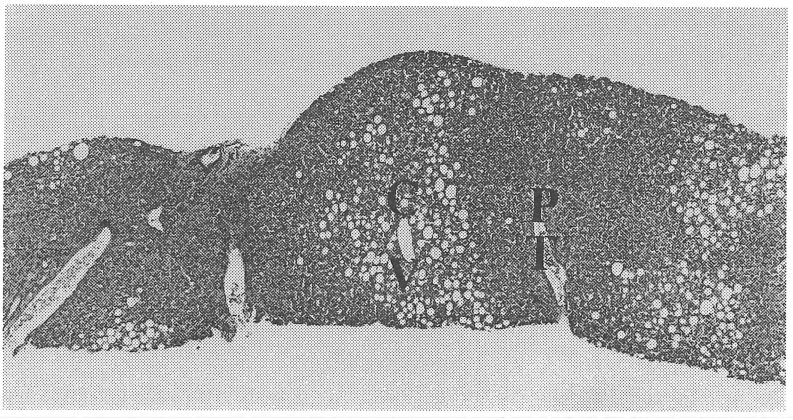

Fig 1. Histology section of Liver biopsy from patient with ALD showing fat globules mainly in zone 3 and some in zone 2. H&E X40. Central vein (CV) and portal tract (PT) are seen.

Liver biopsy specimens were fixed in 4% buffered formal saline and embedded in paraffin. Sections cut at 5μm thickness were stained with haematoxylin and eosin (H&E), Picro Mallory, reticulin and other stains. Haematoxylin and eosin stain was performed at two levels. The specimens were assessed for the presence of fat and were sub-divided into five histopathological categories: normal control, pure steatosis, steatofibrosis (steatosis with portal fibrosis or pericellular fibrosis), pre cirrhosis (bridging fibrosis with incomplete nodules) and established cirrhosis. The morphological changes of steatosis in relation to topography and grade of fat globules in ALD and NALD are shown in Figures 1-4. Quantitative assessment was performed without prior knowledge of the clinical condition or diagnosis. Steatosis was assessed in H&E stained sections by stereological and morphometric analysis.

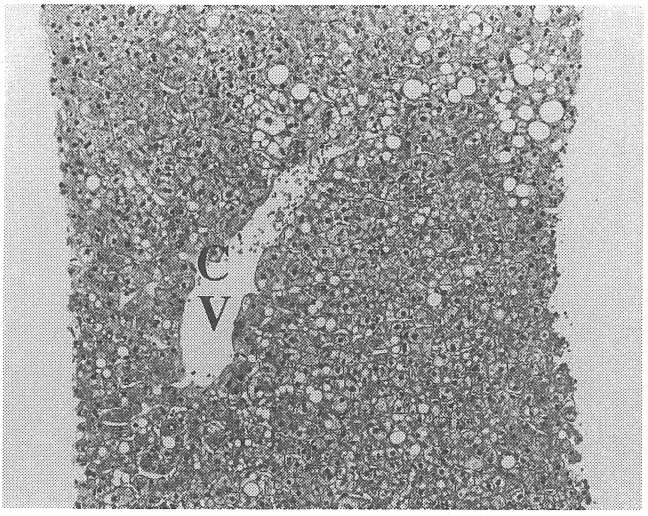

Fig. 2. Histology section of Liver biopsy from patient with ALD showing mixed macro and micro vesicular streatosis around the central vein area (CV). (H&E X100)

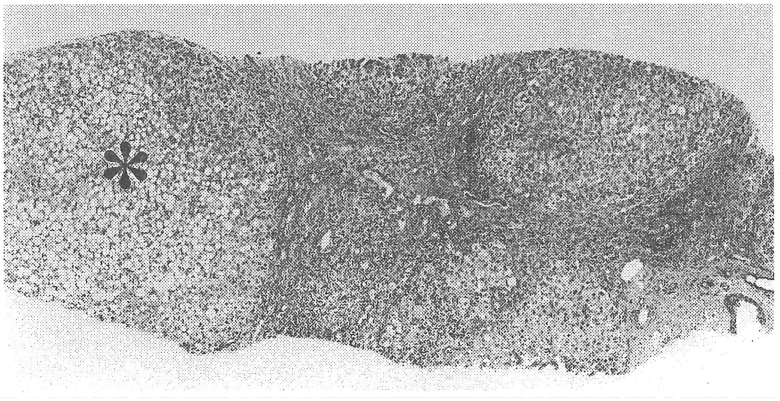

Fig. 3. Histology section of Liver biopsy from patient with alchoholic cirrhosis showing steatosis within the nodule(*). (H&E X40)

Stereology

The area fraction (A_A) of steatosis, unchanged parenchyma, portal tract, central veins and fibrosis in all zones were assessed by stereological analysis using objective at magnification of X10 Nikon microscope. Stereology assessment was carried out using a computer programme by image analysis system (Prodit 5.2, BMA, The Netherlands). A monitor screen with the Weibel grid overlay on fat globules, unchanged parenchyma, portal tract areas, central veins and fibrosis was used. The parallel Weibel grid (72 points at a distance of 31.82μm) was used assuming that the liver tissue was isotropic and the changes were uniform and randomly orientated. At least 1,000 points covering the above features were counted in each liver biopsy specimen. The whole biopsy area was covered at one level of sectioning.

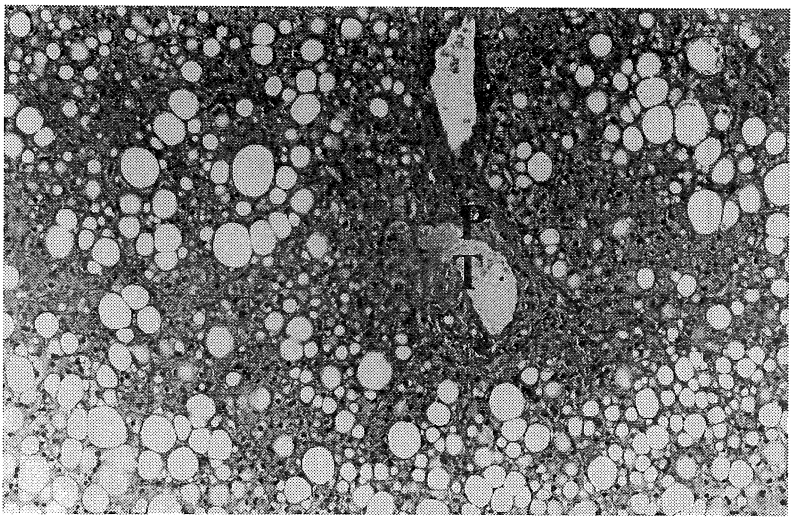

Fig 4. Histology section of Liver Biopsy from patient with NALD showing mixed micro and macro vesicular steatosis within zone one portal tract is shown (PT). (H&E X100)

Morphometry

Steatosis was assessed in two areas: centrilobular (zone III) area and periportal area (zone I). A special computer programme was used to assess the area of parenchyma and steatosis. This computer program allowed the measurement to be done in two phases. In the first (phase 1), we draw an outline around the liver parenchyma and then around the area of fatty changes (phase 2). Some areas contained no fat globules, therefore phase 2 was 0. For each zone at least ten areas were measured. The following morphometric parameters were obtained (all measurements are in micrometers for length and micrometers squared for area):

- Mean area of parenchyma
- Mean area of steatosis
- Mean diameter of fat globules
- Other parameters were calculated including:
  - Mean area of fat globules/10000μm^2 (equal to 0.01mm^2) of parenchyma
  - Mean number of fat globules /10000μm^2 (equal to 0.01mm^2) of parenchyma
  - Fat globules/parenchyma ratio.

Grading of fat globules

In this study fat globules were graded quantitatively as follows:

1. Microvesicular steatosis (microglobules): The mean diameter of fat globules plus the standard deviation is less than 15μm. (M + SD <15μm)
2. Macrovesicular steatosis (macroglobules). The mean diameter of fat globules minus SD is equal or larger than 15μm. (M-SD≥15μm)
3. Mixed macro and microvesicular steatosis: The mean diameter of fat globules plus SD is more than 15μm or the mean minus the SD is less than 15μm (M + SD >15μm or M-SD<15μm).

Definitions

Parenchyma: is defined by the area occupied by hepatocytes, Kupffer cells, sinusoids with fatty changes (unchanged parenchyma without fat globules). Areas of fibrosis, portal tract and central vein are excluded.
Fibrosis: any areas of septal, pericellular, periportal or perivenular fibrosis.
Steatosis: is represented by the fat globules within the cytoplasm of hepatocytes.
Area fraction of fat globules (A_A): is the fraction of the area occupied by fat globules to the area of parenchyma.
Normal control: patients underwent cholecystectomy operation, who had no history of alcohol intake and their liver function tests were normal.

Statistical analysis

Data were analysed using a software programme EP1 INFO Version 6. The ANOVA test was used to derive the p value for normally distributed data. When Bartlett's test showed the variances in the samples to differ, the non parametric Kruskal-Wallis H (equivalent to chi squared) was used to obtain the p value (two tailed probability). Statistically significant level was set at $p<0.05$.

The intraobserver reproducibility for assessing the stereological technique was tested in two ways. Firstly the intrafield variation was tested by measuring five fields from one patient and each was measured five times. Secondly a single field from five patients was measured twice. The coefficient of variation (CV) of the intrafield variation was 1.6 and the intraobserver CV between patients was 3.8. Reproducibility is equal to 100 minus CV.

RESULTS

Stereological changes

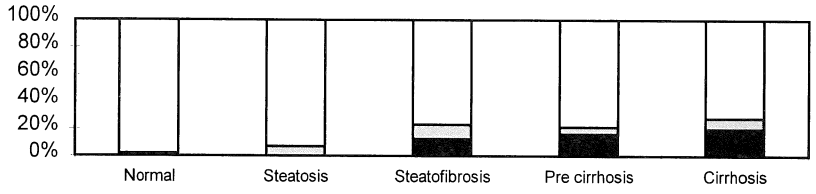

Fig 5. The area fraction of fat ▨, fibrosis ■, and the remaining liver tissue □ (unaffected parenchyma, portal tract area and central vein area).

Figure 5 and Table 1 show the area fraction (AA) of fat cells/parenchyma obtained by stereological analysis in all patients in relation to the pathological categories. There was significant variation in the area fraction between the groups (p=0.049). In some patients with steatofibrosis and cirrhosis, the maximum values exceeded 54%.

Table 1. The area fraction (A_A) of fat / parenchyma (%) in different histopathological categories. Both cases with ALD and with NALD are included.

Pathological category	Number of cases	Mean	Minimum	25 %ile	75 %ile	Maximum
Normal	5	2.03	0.10	0.64	3.22	5.41
Steatosis	19	16.93	2.77	4.47	26.32	33.03
Steatofibrosis	38	20.85	0.81	6.75	27.41	54.22
Pre cirrhosis	9	10.73	2.95	9.44	13.37	14.83
Cirrhosis	21	21.97	2.83	10.25	21.56	53.97

Table 2 shows the stereological analysis of area fraction of fat/parenchyma in relation to the clinical categories. The difference between the clinical groups was highly significant (p=0.00003). The highest values were seen in ALD and obesity induced disease. The lowest values were seen in drug induced disease and the group of PBC and hepatitis.

Table 2 Fat area fraction(A_A) in clinical categories obtained by the stereological technique.

	Normal	ALD	Obesity	Diabetes	Drug induced	PBC & Hepatitis	Unknown	Others
Num. of Patients	5	36	7	5	7	6	12	9
Mean	2.03	14.04	24.48	13.98	5.58	4.24	17.27	12.12
SD	1.98	8.96	14.47	11.14	6.61	5.05	11.36	10.26
Minimum	0.09	0.90	5.42	3.66	0.36	0.56	1.50	2.93
75%ile	2.95	19.54	35.46	14.31	7.63	5.87	24.90	22.63
Maximum	4.98	42.17	49.16	32.32	19.49	17.46	36.04	27.05

p Value (between the groups)= 0.00003.

Morphometric changes

Table 3 shows the mean diameter of fat globules in zone 3 and zone 1 in all patients in the histopathological categories. In zone 3 there was a significant difference between the pathological and normal groups (p=0.0038). In zone 1 the changes were not statistically significant.

Table 3. The mean diameter, SD and the variation in the diameter of of fat globules in zone 3 and zone 1 in relation to the pathological categories obtained by morphometric analysis.

	Pericentral (Zone 3)		Periportal (Zone 1)	
Pathological Categories	Mean	SD	Mean	SD
Normal	10.33	6.31	9.65	5.51
Steatosis	17.84**	4.44	15.57*	4.01
Steatofibrosis	17.33**	4.39	16.38*	3.91
Pre cirrhosis	15.04**	2.15	18.99*	12.44
Cirrhosis*** (within nodules)	17.79	3.33		

***figures for cirrhosis **p= 0.0038 *p= NS

p value (with each zone) do not relate to any given zone.

Table 4 shows the morphometric features of fat globules in ALD and all combined groups of NALD. Statistically significant differences were found in the number of fat globules in zone 3 (p=0.031) and zone 1 (p=0.004). There was no significant difference in the diameter and area between the two major clinical groups in both zones.

Table 4. Morphometric findings in ALD and the combined groups of NALD (including unknown cases). Number of fat globules/10,000μm² of parenchyma (0.01mm²). NS: not significant.

		ALD (36)	NALD(51)	p value
Diameter	zone 3	17.92 ± 3.19	16.88 ± 4.45	NS
	zone 1	16.42 ± 3.16	16.54 ± 6.63	NS
Number of globules /parenchyma	zone 3	2.73 ± 1.72	2.00 ± 1.55	0.031
	zone 1	2.05 ± 1.33	1.24 ± 1.11	0.004
Area ratio of fat/parenchyma	zone 3	6.03 ± 4.92	5.22 ± 5.93	NS
	zone 1	3.24 ± 2.77	2.03 ± 2.74	NS

Table 5 shows the mean value, minimum, 25%-ile, 75%-ile and maximum of fat globules/parenchyma ratio in both zones 3 & 1 in different histopathological categories. There was significant difference in the area ratio in zone 3 (p=0.0141) and zone 1 (p=0.0176).

Table 5. Area ratios of fat globules/parenchyma in pericentral and periportal zones in different histopathological categories obtained by morphometric analysis.

Histopathological category		Mean	Minimum	25%ile	75%ile	Maximum
Normal controls	Zone 3	0.29	0.00	0.16	0.44	0.47
	Zone 1	0.27	0.00	0.19	0.24	0.69
Steatosis	Zone 3	4.92	0.30	0.67	10.00	15.60
	Zone 1	1.78	0.14	0.37	2.95	6.54
Steatofibrosis	Zone 3	6.45	0.11	0.71	12.87	18.87
	Zone 1	2.89	0.15	0.43	4.44	15.27
Pre cirrhosis	Zone 3	2.99	0.20	0.79	3.16	9.62
	Zone 1	1.60	0.34	0.54	2.95	3.63
Cirrhosis (within nodules)	Not related to any zone	5.59	0.21	0.91	9.93	13.61

p value for zone 3 = 0.0141; p value for zone 1 = 0.0176.

Figure 6 shows the relationship between the grades of fat globules assessed by morphometry and topography in both groups of ALD and NALD. The percentages of macroglobules and mixed macro and micro (globules) were higher in patients with ALD than NALD in zone 3. In zone 1 the percentage of microglobules was higher in NALD than ALD.

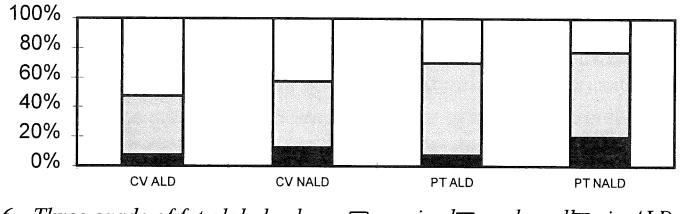

Fig. 6. Three grade of fat globules: large □ , mixed ▨ and small ■ in ALD and NALD in relation to togography of pericentral (CV) and periportal (PT) areas.

DISCUSSION

Steatosis of the liver is not a disease entity but a histological feature that may have clinical association. It is the most common morphological feature that is seen in patients with ALD and some patients with NALD. In this study we described a quantitative method to assess steatosis in all histopathological stages of liver disease and in some main clinical conditions that are known to be associated with steatosis. Semigrading of steatosis has been described by many authors (Adler and Schaffner 1979, Auger et al 1986, Nagone & Scheuer 1988). Semiquantitative and fully automated methods have also been decribed (Auger et al 1986). This, however, requires a special staining with high quality of slides. In semiautomated interactive methods H&E stained sections prepared for routine diagnosis can be used. We have assessed steatosis by two quantitative methods. The advantage of stereology is the assessment of steatosis in relation to all morphological features in the biopsy specimen. Two phase morphometry is an accurate method for assessing steatosis in relation to topography and size and area percentage of fat globules.

The current study has shown significant variation in area fraction (A_A) of steatosis between different histopathological groups. In some patients, the area fraction exceeded 54% of the area of biopsy. The highest values were obtained in patients with steatofibrosis and in cirrhotic nodules. The overall area fraction of steatosis also varied significantly between clinical groups ($p=0.0003$) being lowest in patients with drug-induced hepatitis and the group of PBC and hepatitis and the highest figure found in patients with ALD, diabetes and obesity. Confident distinction between patients with ALD and NALD on histological ground is difficult (Harrison & Burt 1993,) but some differences have emerged using quantitative techniques (Nagone & Scheuer 1988).

The study has shown significant differences in the diameters of fat globules in centrilobular zone 3 in the patient groups compared to controls. This may be because in humans, fat globules initially formed in zone 3 coalesce to form large droplets. This may explain the variation in the diameters of fat globules in zone 3. The morphological findings were that steatosis is more commonly seen in zone 3 than in zone 1 in all clinical groups. However, in diabetes, the area ratio of fat in zone 1 was higher than that of patients with ALD. This is in agreement with the study reported by Nagone & Scheuer (1988). The current study has shown that the number of fat globules was significantly higher in patients with ALD in comparison with the combined groups of NALD in both zone 3 ($p=.031$) and zone 1 ($p=0.004$) and also the area fraction of steatosis increases with the increased severity of steatosis.

The study has shown that in ALD the percentage of macroglobules and mixed macro and micro globules in zone 3 were higher than that of patients with NALD. The number of microglobules in zone 1 was higher in patients with NALD than ALD.

The goal of this study was to obtain a correlation between the morphometry and the morphological grading of fat globules. This system can be easily used by pathologists when assessing liver biopsy specimens with steatosis.. Stereology offers a means of assessing steatosis in the entire biopsy specimen while morphometry is very useful in assessing steatosis in relation to topography. Our method is simple and can be performed by any image analysis system. The results of this study have shown significant differences in the area fraction (A_A) of steatosis between patients with ALD and some groups of NALD. The number, diameter and area ratio of fat globules can facilitate differentiation between ALD and some groups of NALD.

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