

Early stereological changes in liver of Sprague-Dawley rats after streptozotocin injection

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Background: Diabetes mellitus is associated with biochemical, physiological and pathologic alterations in the liver. We measured changes in structure of rat liver after streptozotocin injection, using stereology.

Methods: Livers of 36 streptozotocin-injected rats were removed after 4, 8 and 12 weeks. Liver volume and weight were measured, and volume-weighted mean volume of hepatocytes and their nuclei were estimated in periportal (Z1), interstitial (Z2) and perivenous (Z3) zones of liver acini. Volume of liver sinusoids was also estimated. **Results:** Mean volume and weight of the liver were reduced by 15% and 12%, respectively at 4 and 8 weeks after injection. Mean hepatocyte volumes were reduced by approximately 30%, 31% and 24% in Z1, Z2 and Z3 at 4 weeks, 19% and 24% in Z2 and Z3 at 8 weeks, and 14% in Z1 at 12 weeks. Mean volume of hepatocyte nuclei was reduced by approximately 18% and 20% in Z2 and Z3 at 4 weeks, 23% in all three zones at 8 weeks, and 18%, 15% and 13% in Z1, Z2 and Z3, respectively, at 12 weeks. The absolute volume of the sinusoids decreased by 16.5% only at 4 weeks. **Conclusion:** Streptozotocin injection leads to early reduction in volume of hepatocytes, their nuclei and sinusoids in rat liver.

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Most research on liver in diabetes mellitus has focused on biochemical, physiological and histological changes; in comparison, quantitative aspects of liver structure have received less attention. Also, reports on pathology have focused mainly on late changes in diabetes, viz., glycogenosis, fatty liver and cirrhosis of the micronodular variety;¹⁻⁴ early quantitative structural changes in the liver have not been studied well.

Streptozotocin injection is a well-established means of inducing diabetes in the animal model, by destruction of B cells of the pancreas.⁵ We studied quantitative changes in liver volume, volume-weighted mean volume (sampling and measurement of a particle with a chance proportional to the individual volume) of the hepatocytes and their nuclei in various zones of the liver, and the proportional and absolute volume of liver sinusoids at early stages after streptozotocin injection.

Methods

Seventy-two male rats (Sprague-Dawley) with mean (SD) weight of 250 (30) g were used. All animals received care as recommended in the "Guide for Care and Use of Laboratory Animals".⁶ They were housed in an isolated caging system under controlled conditions and fed pellet, vegetables and tap water throughout the experimental period. Serum glucose levels were measured in tail vein blood obtained before streptozotocin injection, and at 2 days and 2 weeks after injection.⁷

Preparation of tissue

Thirty-six rats (experimental group) were injected intraperitoneally with a single dose of 65 mg/Kg of streptozotocin,⁷ dissolved in distilled water just before use. Both the experimental and control animals were dissected after perfusion fixation. Briefly, in a deeply anesthetized rat, a wide-bore needle was inserted to the left ventricle (inflow) of the animal and the right atrium was incised (outflow). The saline solution was run through first, until the effluent fluid was clear. The fixative (neutral buffered formaldehyde) was then perfused until the whole body of the animal was hard and inflexible.

The liver was dissected at 4, 8 and 12 weeks after streptozotocin injection. Liver weight and volume were measured using immersion method and fixed in neutral buffered formaldehyde for one week. The liver was then divided into bars; five randomly selected bars from each liver were studied.

Isotropic uniform random sections⁸⁻¹² were obtained using the orientator method. Briefly, each liver bar was placed on a circle that was divided into 36 equal pies using radial lines, and was sectioned along the line bearing a randomly selected number. The sectioned surface of the bar was placed on the 0-0 direction of the circle with 97 unequal sinus-weighted divisions and the second cut was done. The new surface was embedded in paraffin, sectioned (4 μ m thickness), and stained with Feulgen-PAS, which stains sharply the borders of hepatocytes, their nuclei and liver sinusoids. In brief, the sections were treated with diastase to prevent the reaction of glycogen,

Table 1: Mean body weight (g), liver weight (g), liver volume (mL), and sinusoidal absolute volume (mL) in control and experimental rats

Parameter	4 weeks		8 weeks		12 weeks	
	Controls	STZ	Controls	STZ	Controls	STZ
Body weight (g)	255.7(4.1)	215.5(3.7)*	289.2(5.5)	276.8(7.8)	338.7(3.4)	288.6(5.1)*
Liver weight (g)	9.7(0.2)	8.3(0.2)*	10.6(0.5)	9.4(0.3)‡	11.2(0.6)	10.5(0.4)
Liver volume (mL)	9.0(0.2)	7.7(0.1)*	9.6(0.3)	8.4(0.3)†	10.1(0.5)	9.5(0.4)
Absolute sinusoid volume (mL)	2.1(0.2)	1.8(0.1)*	2.0(0.4)	1.8(0.4)	2.0(0.3)	2.3(0.6)

STZ: Streptozotocin treated rats. Data are shown as mean (SD)

*p<0.001, ‡p<0.02, †p<0.007, STZ vs. corresponding controls

hydrolyzed in hydrochloric acid, transferred into periodic acid solution, stained with Schiff's reagent, and counterstained with fast green.

Morphometric measurement

A light microscope equipped with a morphometry attachment (Visupan, Austria) with an oil immersion lens (final magnification 1250X) was used. Approximately ten (8-12) microscopic fields were selected randomly in each liver section. In brief, an area was randomly selected outside the tissue section; the microscope stage was then moved a pre-fixed interval along the X and Y directions every time till the entire section had been studied. Three zones can be distinguished in the portal acinus; zones 1 (Z1; periportal), 2 (Z2; interstitial) and 3 (Z3; perivenous) form roughly concentric layers. In every selected field of vision, the histological zones were determined and the field was superimposed with a square test frame with equidistant points. The length of intercepts of the cells and their nuclei (approximately 100 cells in each zone) that had been hit with the points of the frame were measured using a ruler in the horizontal direction, and represented as l_0 .^{10,12} An unbiased estimate of volume^{8,9,10,12} was then calculated as $\bar{v}_v = \frac{\pi}{3} \bar{l}_0^3$. This formula includes a correction for the fact that the hepatocytes and their nuclei are sampled with a chance proportional to their individual volume, i.e., the mean volume measured is volume-weighted. An estimate of volume density (V_v) or the fraction of liver tissue that is occupied by the sinusoids was obtained by point-counting method as follows:⁹ $V_v = P(\text{sinusoids})/p(\text{reference})$ where P(sinusoids) and p(reference) are the number of points falling on the sinusoid profiles and the

liver tissue, respectively. Absolute amount of sinusoids was estimated by multiplying sinusoidal volume density by the liver volume to prevent the "reference trap".⁹

Statistics

Data were expressed as mean (SD), and inter-group comparisons were done using ANOVA and Student's *t* test; *p* values <0.05 were taken as significant.

Results

Blood glucose levels varied between 75 and 128 mg/dL in the control animals and 395 and 500 mg/dL in the experimental animals at 2 weeks.

Body weight, liver weight and volume

Experimental rats lost weight (mean loss 12% as compared to the control group; Table 1). Mean liver volume and liver weight in experimental rats were 15%, 12% and 7% lower, respectively, at 4, 8 and 12 weeks after streptozotocin injection, than the control rats (Table 1). The relative liver weight ([liver weight/body weight] ×100) was ~3% in both the groups.

Volume-weighted mean hepatocyte volume

Mean hepatocyte volume was significantly reduced by approximately 30% in Z1, 31% in Z2 and 24% in Z3 at 4 weeks after streptozotocin injection (Table 2). The corresponding reductions in the three zones at 8 weeks were 14%, 19% and 24%, respectively. At 12 weeks, there was a reduction of 14% in Z1, a reduction of 5% in Z2, and an increase of 2% in Z3 (Table 2).

Volume of the hepatocytes progressively decreased from zone 1 to zone 3 in both control and experimental rats (Table 2).

Table 2: Mean hepatocytes volume (mm3) in three zones of the liver in control and experimental rats

Liver zone	4 weeks		8 weeks		12 weeks	
	Controls	STZ	Controls	STZ	Controls	STZ
Zone 1	5731.6(243.3)	3977.6(130.6)†	5677.2(558.2)	4880.7(212.5)	6142.8(215.6)	5311.3(188.1)#
Zone 2	4074.2(158.1)	2796.9(128.0)†	4362.5(294.8)	3545.5(171.5)†	4012.9(122.3)	3803.6(82.5)
Zone 3	2501.6(134.3)	1900.6(102.9)*	3363.2(262.9)	2573.3(86.8)‡	2666.6(76.7)	2710.3(280.9)

STZ: Streptozotocin treated rats. Values are mean (SD)

†p<0.001, ‡p<0.01, *p<0.002, #p<0.008, †p<0.02, STZ vs. corresponding controls

Table 3: Mean hepatocyte nucleus volume (mm³) in three zones of liver in control and experimental rats

Liver zone	4 weeks		8 weeks		12 weeks	
	Controls	STZ	Controls	STZ	Controls	STZ
Zone 1	238.9 (12.1)	216.8 (25.4)	249 (9.6)	191.8 (9.6)†	254 (9.1)	209.8 (7.2)†
Zone 2	203.7 (15.6)	166.5 (6.5) [#]	209.0 (9.4)	162.5 (5.7)†	212.7 (12.4)	181.2 (5.1) [*]
Zone 3	143.6 (6.6)	115.8 (9.6) [*]	181.2 (7.8)	140.1 (4.7)†	156.8 (8.0)	136.5 (4.8) [#]

STZ: Streptozotocin treated rats. Values are mean (SD)

[#]p< 0.04, ^{*}p< 0.02, †p< 0.001, STZ vs. corresponding controls

Volume-weighted mean nuclear volume

At 4 weeks after streptozotocin injection, mean volume of hepatocyte nuclei decreased by 9%, 18% and 20% in Z1, Z2 and Z3, respectively (Table 3). The mean volumes significantly decreased approximately 23% in all three zones at 8 weeks, and by 18%, 15% and 13% in Z1, Z2 and Z3, respectively, at 12 weeks (Table 3).

Volume of the hepatocytes nuclei progressively decreased from zone 1 to zone 3 in both control and experimental rats (Table 3).

Volume of sinusoids

The absolute volume of the sinusoids decreased by approximately 16.5% (p<0.007) only at 4 weeks after injection (Table 1).

Discussion

The present study, using unbiased stereological methods, showed that liver volume, proportional and absolute volume of the sinusoids, and volume-weighted mean volume of hepatocytes and their nuclei in rats are changed after streptozotocin injection. Streptozotocin has a transient toxic effect on liver function but this disappears by day 15-30.^{13,14,15} Also, repeated administration of streptozotocin in patients with pancreatic neoplasms induces hepatotoxicity only occasionally.⁵ We therefore believe that the changes that we observed were due to induction of diabetes and not due to toxic effect of streptozotocin.

Streptozotocin treatment led to a failure to gain body weight that became more pronounced with the passage of time; this finding has been reported previously.¹⁵ However, the relative liver weight did not change, suggesting that the reduction in liver weight and volume was related to reduction in total body mass. In the chronic phase of diabetes, an increase in liver weight or hepatomegaly might occur due to glycogen deposition or fatty metamorphosis.^{1,16-19} Herrman *et al*¹⁵ noted that hepatomegaly observed in streptozotocin-induced experimental diabetes might be due to hyperplasia in the early phase, and decreased apoptosis in the later stages.

We also studied liver structure early after

streptozotocin injection. We found that the volume of hepatocytes and of their nuclei progressively decreased from zone 1 to zone 3 in both control and experimental rats. After streptozotocin, hepatocyte volume initially decreased in all the three zones; however, by week 12, the reduction was apparent only in zone 1. Zone 1 (periportal) cells are important for glycogen synthesis and glycogenolysis. This is also the main area for protein metabolism and formation of plasma proteins. The cells of zone 3 (perivenous) are associated with glycogen storage, lipid and pigment formation. Zone 2 (interstitial) cells share functions with other zones.¹⁷ The maximum effect of streptozotocin was observed in the cells of zone 1, which are the first to come in contact with blood flow and thus glycogen synthesis. At the end of the experiment, cells in zones 2 and 3 did not show significant difference from controls. This could be due to glycogen deposition or fatty metamorphosis, which occur later in diabetes.¹⁶⁻¹⁹ Herrman *et al*¹⁵ previously reported smaller-sized hepatocytes in diabetic liver; however, they did not measure the hepatocyte volume and based their conclusion on presence of more cells per unit area.

In addition to cytoplasmic changes, hepatocyte nuclear volume also decreased after streptozotocin injection. This was observed in zones 2 and 3 at the end of week 4, but the maximum decrease was seen later in all three zones. Nuclei are responsible for production of RNA and consequent protein synthesis. In non-mitotic cells, nuclear volume had been shown to be closely related to DNA content and the level of activity.^{12,20,21} Even in cells in which mitosis is a normal occurrence, such as rat hepatocytes, changes in nuclear size without change in the ploidy have been related to changes in protein synthesis and nuclear activity.^{19,20} Therefore, decrease in nuclear volume observed by us may represent a reduction of metabolic activity of liver cells, due to reduction in insulin and glucagon. Both hormones appear to stimulate hepatic RNA synthesis and protein synthesis. A basal insulin level is also necessary to maintain the state of aggregation of the endoplasmic reticulum-bound polysomes for secretory protein synthesis. In insulin-deficient animals, loss of rough endoplasmic

reticulum, reduced amino-acid incorporation into protein, and a decrease in rough endoplasmic reticulum-bound ribosomes occurs.^{22,23,24} This suggests that patients with diabetes mellitus predominantly have a defect in secretory protein synthesis. Therefore, reduction in nuclear or cell volume may be due to inadequate levels of insulin. However, some studies² have reported an increase in the nuclear surface area in the later stages of diabetes, due to the appearance of intranuclear inclusions.

Another parameter we studied was absolute volume of sinusoids. At the end of week 4, this volume decreased. We believe that changes in sinusoid volume are related to changes in the volume of hepatocytes. Because the sinusoid walls are made by hepatocytes, the decrease in volume of the cell may be a cause for reduction in sinusoid volume.

In conclusion, streptozotocin injection and the resultant diabetes mellitus lead to a reduction in volumes of hepatocytes and their nuclei. These changes may help in understanding the pathogenesis of liver pathology in patients with diabetes mellitus.

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