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Morphological changes in the isolated rat liver perfused in a non-recirculating system: scanning and transmission electron microscopy

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Summary. Isolated perfused rat livers have been used for various studies, but detailed investigation into the structural integrity of hepatocytes of this system is lacking. In this study, isolated rat livers were perfused in vitro with oxygenated Krebs-Ringer bicarbonate buffer solution, for 2 minutes and 1, 2, 3, and 4 hour (s) at 37°C, using a non-recirculating perfusion system. The perfused livers were processed for semithin section light microscopy, transmission electron microscopy, and scanning electron microscopy. Sectional areas of cell deaths were measured by a camera-tracing assembly from 1.5 μm thick Araldite sections stained with toluidine blue. Progressive nuclear and cytoplasmic changes, leading to cell death, occurred in the hepatocytes of the centrilobular zone, during the 2nd, 3rd, and 4th hour of the perfusion at a rate of 9.03% ±1.5%, 38.7% ±2.7%, and 55.1% ± 5.9% (mean ± standard deviation) of the total sectional areas respectively. Midzonal hepatocytes showed normal basophilic staining but exhibited loss of glycogen granules, loss of microvilli, development of aqueous vacuoles and formation of blebs. The fine structures of cell organelles, glycogen granules, microvilli and plasma membrane of the cells in the periportal zone were well preserved throughout the experimental period. For further quantitative, metabolic and functional studies using isolated rat liver perfused with Krebs-Ringer solution, it is evident from the present investigation that the periportal zone represents the functional region of the hepatic lobule. Whilst progressive changes, leading to cell death, occurred in the centrilobular zone.

Key words: Isolated liver - Perfusion - Electron microscopy - Rats

Introduction

Isolated, perfused rat liver preparations have been extensively used to study the hepatic metabolism (Buschiazzo et al., 1970; Keiding et al., 1980), hormonal effects such as thyroid hormones (Moller and Seitz, 1980; Bartels and Sestoft, 1980; Hassan and Ramsden, 1981), insulin (Misbin et al., 1976; Weiland et al., 1979), and the effects of vasoactive hormones (Hems et al., 1976). More recently, isolated rat liver has been used for radiotracer studies (Scheffel et al., 1984, 1985, 1986), and transplantation investigations (Kamada et al., 1980; Otto et al., 1986). The viability of hepatocytes, of isolated perfused livers, was evaluated mainly by biochemical methods. The detailed studies of structural changes in different zones of the hepatic lobules of this system are lacking. The present study describes the findings of the structural and ultrastructural changes in the hepatocytes of the isolated rat liver perfused in vitro with oxygenated Krebs-Ringer bicarbonate buffer solution at 37°C for 2 minutes and 1, 2, 3, and 4 hour(s) in a non-recirculating system.

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Materials and methods

Twenty-two male Wistar white rats, weighing 200-250 g were used. They were housed in airconditioned quarters with regulated light and temperature. The rats were fed standard laboratory feed ad libitum.

Surgical procedure

Under sterile conditions, the rats were anaesthetized with an intraperitoneal injection of 1 ml of 3.5% chloral hydrate per 100 g body weight. 5000 unit heparin in 1 ml was injected into the penile vein one minute prior to the ligation of the abdominal inferior vena cava. The portal vein and the thoracic inferior vena cava were cannulated. The liver was immediately transferred to the perfusion chamber and immersed in the perfusion medium at 37°C. The total time elapsed between the cessation of the portal blood circulation and the initiation of the perfusion was less than two minutes.

Perfusion medium

The perfusion medium was freshly prepared oxygenated Krebs-Ringer bicarbonate buffer solution containing NaCl (6.48 g/l), KCl (0.34 g/1), $CaCl_2$ (0.28 g/1), MgS04-7H_20 (0.21 g/l), KH_2Po4 (0.12 g/1), NaHC03 (2.1 g/l), glucose (3 g/l), and supplemented with bovine serum albumine Cohn fraction V, (Sigma), (2 g/l). The pH and the Po_2 readings of the medium, after oxigenation, were 7.4 and 293 mm Hg respectively, measured by an Automatic Gas Check AVL 940. The total osmolarity of the medium was 290 m0sm measured by and Advanced Digimatic Osmometer 3DII. The temperature of the perfusion medium was kept constant at 37°C.

Perfusion technique

The perfusion system was modified from the one originally described by Miller (1973). The influent medium was perfused through the cannulated portal vein, and the effluent perfusate was drained by the cannulated thoracic inferior vena cava. The perfusion flow rate was kept constant at the normal physiological rate of 10 ml/minute, using a gravity feed with a head of 10 cm. The perfusion experiments lasted for 2 minutes and 1, 2, 3, and 4 hour(s). Four livers were perfused for each of the experimental periods.

Fixation

At the end of each experimental period the influent perfusion medium was replaced by the fixative solution for the perfusion fixation. The fixative solution consisted of 1.5% glutaraldehyde in 0.12 M phosphate buffer (pH 7.4), with 0.02 mM CaC12. The total osmolarity of the fixative solution was 420 m0sm. The fixative solution was perfused for 2 minutes, then the liver was immersed in the same fixative solution for 2 hours. After fixation, 200 µm thick liver slices were cut with an Oxford vibratome from the right, left and median lobes. The liver slices were postfixed in 1% buffered osmium tetroxide for 1 hour.

Electron microscopy

The liver slices were dehydrated and embedded in Araldite; ultrathin sections were cut, stained with uranyl acetate and lead citrate and examined under a Jeol 1200 EX transmission electron microscope. For scanning electron microscopy the liver slices were dried by the critical point method, coated with gold and examined under a Jeol GSM 840 scanning electron microscope.

Measurements

1.5 µm thick Araldite sections were cut and stained with toluidine blue. The sectional area was drawn using a camera tracing assembly attached to a Leitz microscope, and measured with a planimeter. The sections were cut from the main three lobes of the liver. For each of the experimental periods, 12 sections from four livers were cut and measured. The total measured sectional area, and the area of cell death, are expressed as mean ±standard deviation (Table 1).

Histochemistry

Liver slices from same regions of the three lobes of the liver, were processed and embedded in paraffin wax blocks. Sections were cut and stained with sudan black and oil red 0 for lipids, and with periodic acid Schiff stain for glycogen granules.

Control experiments

Two unperfused liver fixed only by immersion fixation, and four livers perfused with the perfusion medium for 2 minutes followed by perfusion fixation, served as control experiments.

Results

The tissue of the control experiments after 2 minutes of perfusion, showed that the liver lobules were uniform in the fixation and staining. The portal veins, liver sinusoids and central veins were clear of blood. Nuclei, cytoplasm, cell organelles, glycogen contents, plasma membrane, and microvilli of the hepatocytes were all well preserved in the centrilobular zone (around the central vein) and in the periportal zone (around the portal vein). There was no evidence of cellular injury in the briefly perfused livers attributable to the surgical procedure or perfusion technique. The fine-structural morphology of the liver perfused with the medium for 2 minutes, followed by perfusion fixation was more uniform in the fixation and staining than unperfused liver preserved by immersion fixation.

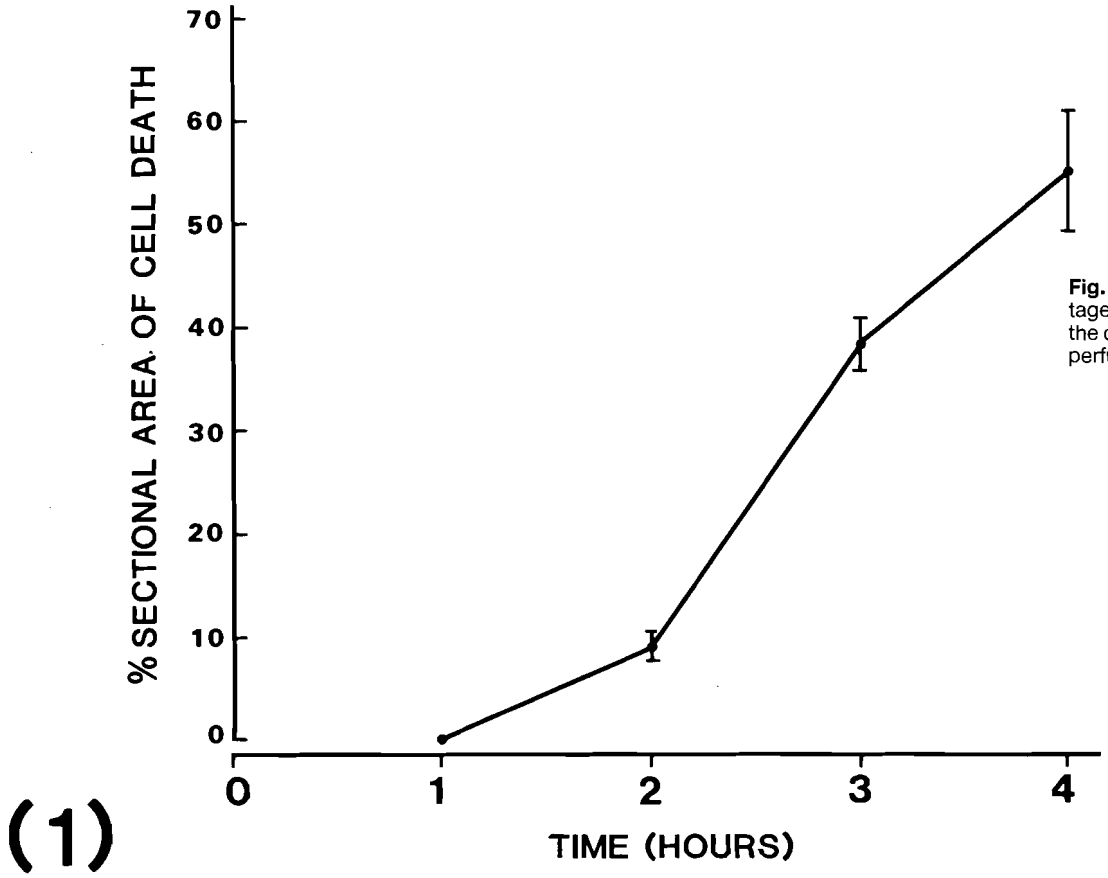
After 1 hour of perfusion, hepatocytes in a narrow zone around the central vein showed a very slight reduction in their basophilia with minimal cell death of 0.3% ±0.18% (Table 1, Figs. 1, 2). The liver architecture and the ultrastructural morphology of the hepatocytes, in the periportal zone, were similar to the control experiments.

After 2 hours of perfusion, the centrilobular zone exhibited cell deaths and the area of cell death was 9.03% ± 1.5% of the total sectional area (Table 1, Fig. 1). The ultrastructural morphology of hepatocytes in the periportal zone was similar to the control experiments.

After 3 hours of perfusion, the area of cell death in the centrilobular zone was increased to 38.7% ±2.7% of the total sectional area (Table 1, Figs. 1, 3). The cells in this zone displayed cumpling of the nuclear chromatin, the cytoplasm was occupied by round and vacuolated mitochondria, other cell organelles were lost, and the cytoplasmic membrane showed loss of villi and became disintegrated (Fig. 4). A narrow «intermediate» zone or «midzonal region» was localised between the centrilobular and periportal zones (Fig. 3). The hepatocytes in this zone showed basophilic staining, similar to the periportal

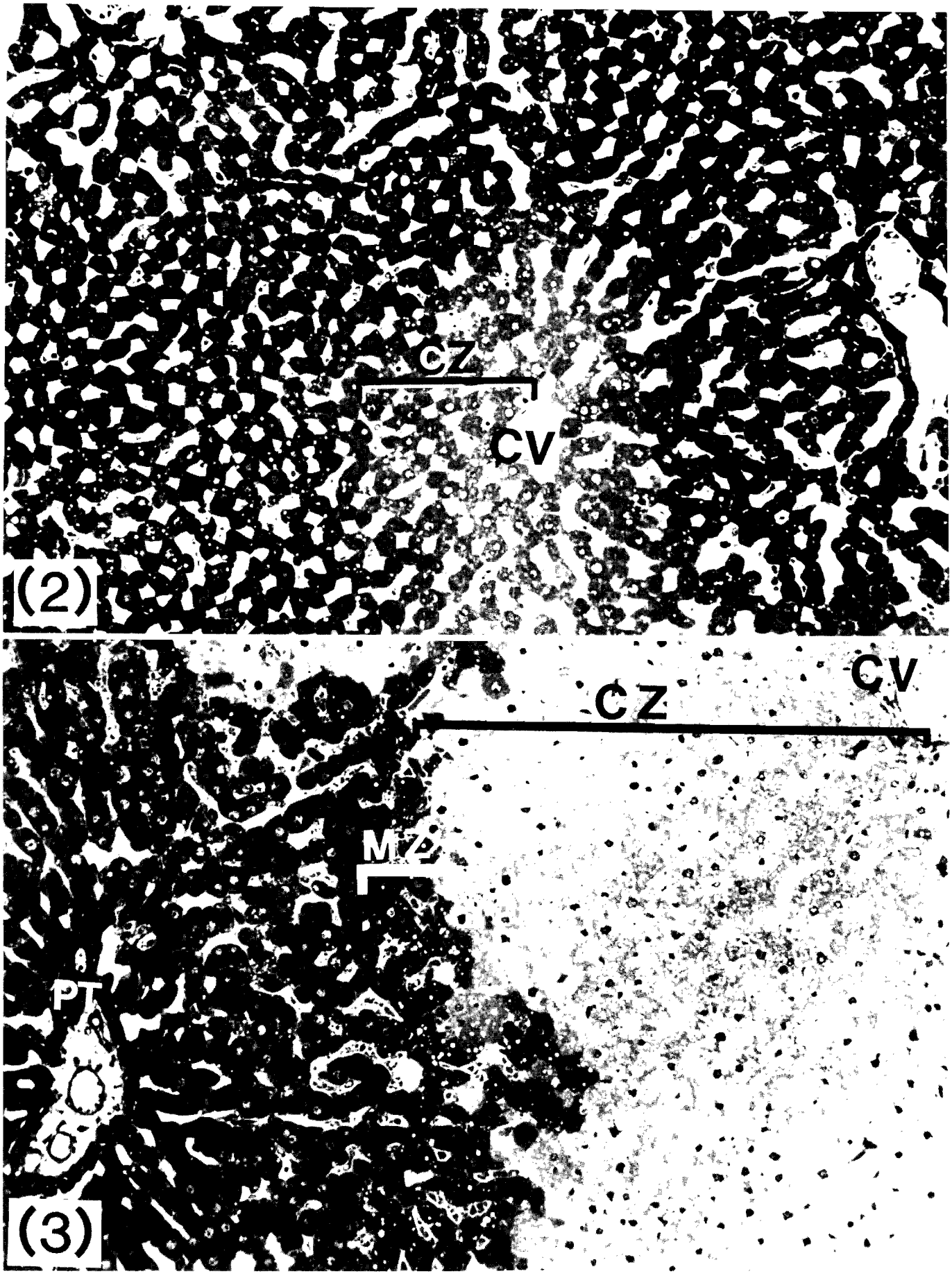
Table 1. Sectional area of cell death in isolated liver in relation to the perfusion time

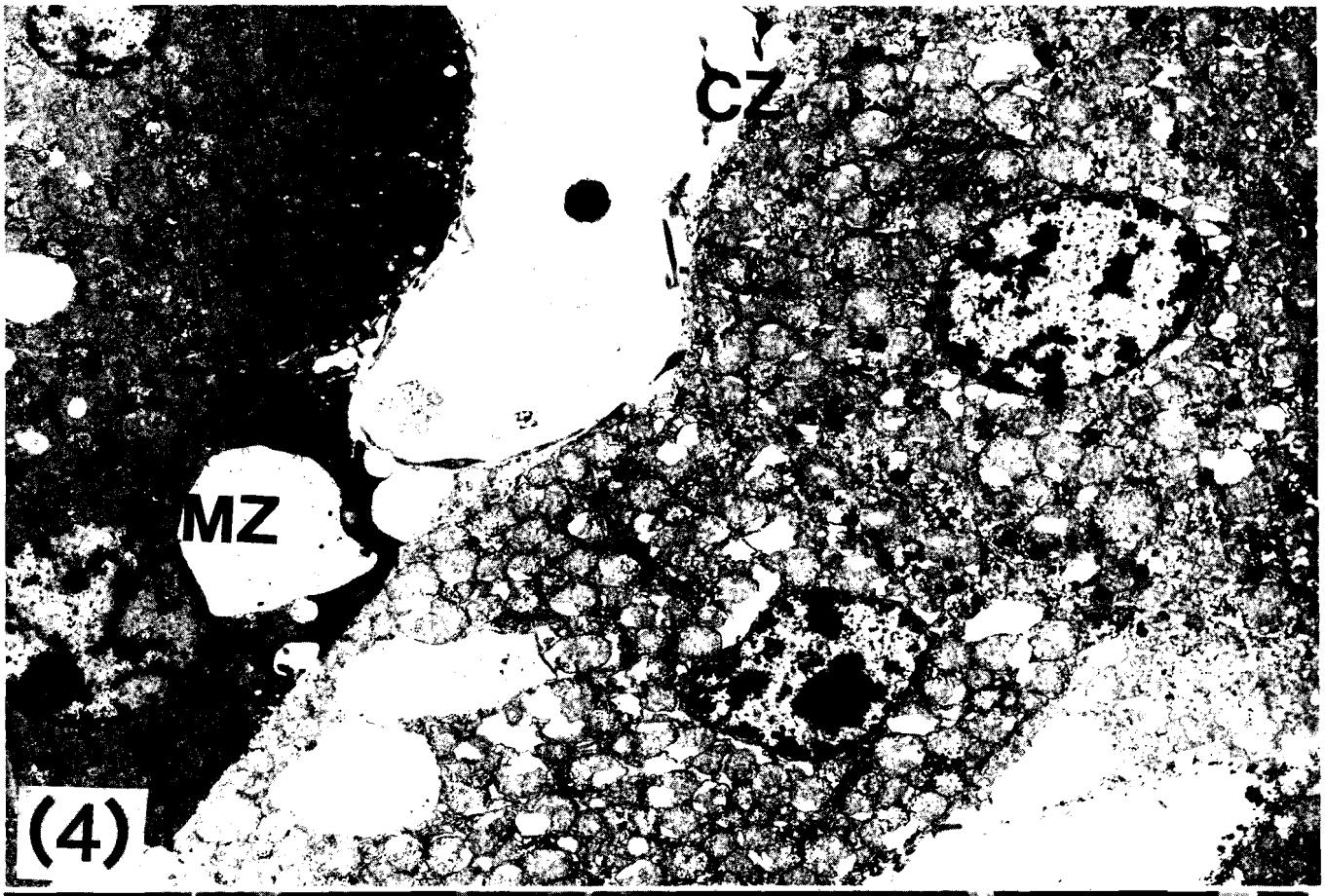
Perfusion time	1 hour				2 hours				3 hours				4 hours			
Sectional area	EXP. No.	Total area (mm2)	Death area (mm2)	% of death	EXP. No.	Total area (mm2)	Death area (mm2)	% of death	EXP. No.	Total area (mm2)	Death area (mm2)	% of death	EXP. No.	Total area (mm2)	Death area (mm2)	% of death
	1.	12.4	0.024	0.19	5.	22.07	1.77	8	9.	13.8	5.47	39.6	13.	13.44	5.9	43.9
Sections	2.	12.69	0.032	0.25	6.	16.58	1.55	9.3	10.	15.1	5.82	38.5	14.	13.7	7.1	51.8
from left	3.	11.34	0.015	0.13	7.	25.7	1.48	5.76	11.	10.5	4.27	40.6	15.	13.63	7.9	57.9
lobe	4.	10.56	0.012	0.11	8.	23.6	2.36	10	12.	9.25	3.28	35.5	16.	12.46	7.8	62.6
	1.	8.54	0.06	0.70	5.	16.66	1.83	11	9.	8.22	3.06	37	13.	10.84	7.05	65
Sections	2.	6.76	0.044	0.56	6.	15.8	1.58	10	10.	8.64	3.74	43	14.	8.56	5.15	60.1
from right	3.	7.76	0.031	0.3	7.	14.9	2.23	10.5	11.	22.82	7.64	33.5	15.	13.78	7.5	54.4
lobe	4.	8.11	0.012	0.14	8.	18.8	1.5	8	12.	10.56	4.1	39	16.	12.98	6.62	51
	1.	6.65	0.032	0.48	5.	10.8	0.97	9	9.	9.68	3.96	40.9	13.	8.56	4.63	54.1
Sections	2.	7.14	0.021	0.29	6.	15.6	1.71	11	10.	8.96	3.76	41.9	14.	7.41	3.81	51.4
from med-	3.	5.92	0.018	0.30	7.	14.8	1.11	7.5	11.	7.76	2.95	38	15.	11.3	5.75	50.8
ian lobe	4.	6.14	0.014	0.22	8.	13.6	1.14	8.4	12.	10.55	3.9	36.9	16.	9.66	5.63	58.3
Mean		8.66	0.026	0.305		17.41	1.602	9.03		11.32	4.33	38.7		11.36	6.24	55.1
SD		±2.45	±0.014	±0.18		±4.3	±0.42	±1.5		±4.22	±1.35	±2.7		±2.3	±1.3	±5.9



(1)

Fig. 1. Illustrates the percentage of the sectional areas of the cell death in relation to the perfusion times.


CZ
CV
(2)
CZ
CV
MZ
PT
(3)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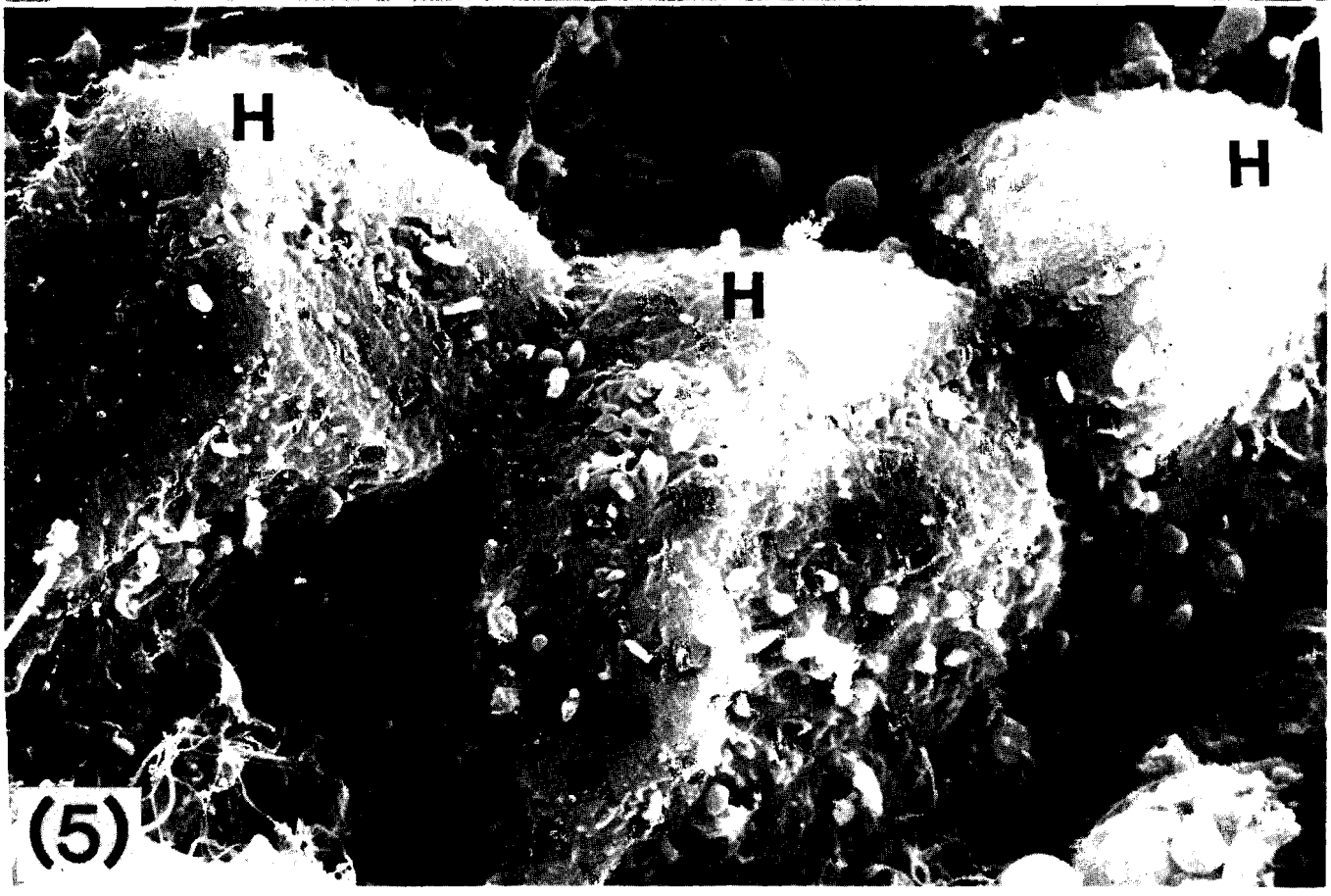
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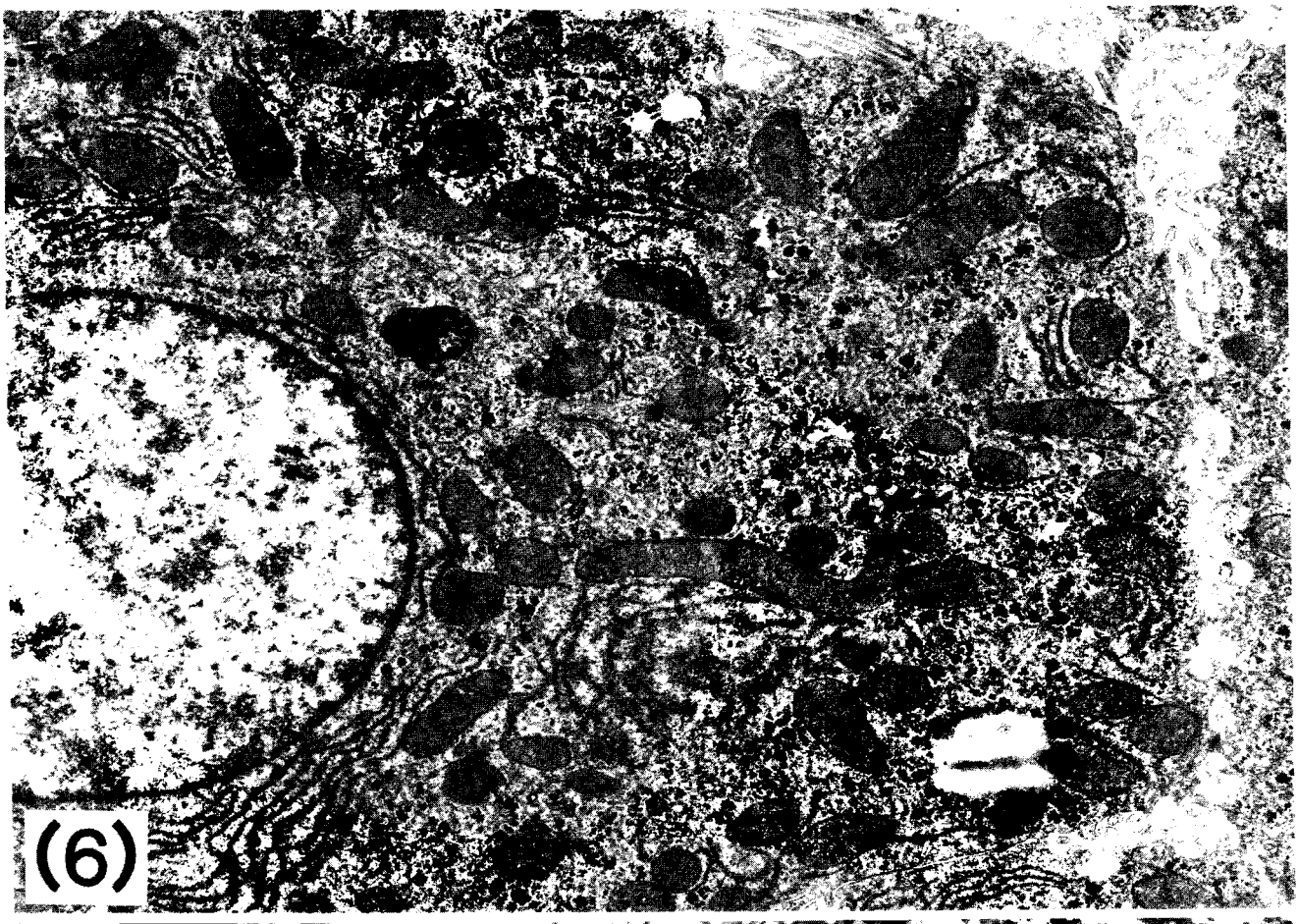

(6)

(7)

Fig. 2. After 1 hour of perfusion, the hepatocytes in a narrow centrilobular zone (CZ) exhibited a slight reduction in the basophilia and a few vacuoles were formed. CV central vein. LM ×150

Fig. 3. After 3 hours of perfusion, the area of cell death was increased, the hepatocytes of the centrilobular zone (CZ) exhibited pyknotic nuclei and total loss of the cytoplasmic basophilia. Hepatocytes in the midzonal region (MZ) were vacuolated, but with normal basophilic staining. Hepatocytes in the periportal zone were normal. CV central vein; PT portal tract. LM ×150

Fig. 4. After 3 hours of perfusion, the cells of the centrilobular zone (CZ) showed clumping of the nuclear chromatin and rarefaction of euchromatin. The cytoplasm exhibited loss of electron density and contained vacuolated mitochondria; other cell organelles were lost. Midzonal cells (MZ) showed normal electron density but vacuolated cytoplasm. TEM ×6,200

Fig. 5. Hepatocytes (H) of the midzonal region marked in Fig. 3. The plasma membrane showed formation of blebs and loss of microvilli. SEM ×5,120

Fig. 6. Hepatocyte in the periportal zone after 4 hours of perfusion. The nucleus, cytoplasm, cell organelles and glycogen granules were all well preserved. TEM ×11,550

Fig. 7. After 4 hours of perfusion, a hepatocyte in the periportal zone showing well preserved plasma membrane and microvilli. SEM ×10,700

cells, but exhibited loss of glycogen granules and development of large clear vacuoles (Fig. 4). The vacuoles did not take up lipid stains. The cell surface showed loss of microvilli and formation of blebs (Fig. 5). In the periportal zone the fine-structural morphology of the cells and liver architecture were comparable with the control experiments.

After 4 hours of perfusion, the ultrastructural changes were very much similar to the previous period, but the area of the cell death in the centrilobular zone was slightly larger and was equal to 55.1% ± 5.9% of the total sectional area (Table 1, Fig. 1). The periportal zone remained intact and cellular ultrastructural features of the hepatocytes, cell organelles, glycogen granules, plasma membrane and microvilli were remarkably well preserved (Figs. 6, 7).

Discussion

In vitro, hepatocytes can be studied either by tissue culture or liver perfusion techniques. The liver is an excellent system for organ perfusion studies, because a perfusion medium can be delivered to the liver through the portal vein, and extensive fenestrated sinusoidal capillaries, then the effluent perfusate can be collected by a cannula placed in the thoracic inferior vena cava for analysis. Hepatocyte tissue culture lacks the normal intercellular coordination and it is further removed from the physiological environment when it is compared with the isolated, perfused liver system. Whereas, in vivo investigations, the circulating blood contains variable amounts of hormones and secondary metabolites which alter or modify the effects of a specific substrate under investigation. Perfusion of isolated livers with a solution of known composition offers the best compromise, as long as the viability of the cells and the hepatic architecture can be adequately maintained and morphologically evaluated.

In this report, oxygenated Krebs-Ringer bicarbonate buffer solution was used as the perfusion medium. Although various perfusion media have been used, which varied from one laboratory to another, oxygenated Krebs-Ringer bicarbonate buffer solution remained principally the most widely used perfusion medium for isolated livers (Terris and Steiner, 1975; Misbin et al., 1976; Bartels and Sestoft, 1980; Scheffel et al., 1984, 1985). Isolated rat livers were perfused with either a recycled perfusion medium «recircultaing system» (Misbin et al., 1979; Scheffel et al., 1985) or with a non-recycled perfusion medium «non-recirculating system» (Mondon et al., 1975; Bartels and Sestoft, 1980; Keiding et al., 1980; Hassan and Ramsden, 1981). In the present investigation, a non-recirculating perfusion system was employed because substances of cellular products were formed, liberated and accumulated in the perfusion medium. When the perfusion medium is recycled these substances may exert harmful effects on cells as they emerge in excess into the extracellular environment.

Structural changes in the isolated liver, perfused in vitro, may arise as a result of the surgical procedure or improper perfusion technique. These difficulties were overcome by carrying out pilot experiments to attain the optimum conditions for the subsequent experiments. Liver cell damage due to the interruption of portal circulation was relatively mild when the tissue was fixed immediately after ischaemia, but the cellular damage became accentuated when the liver was recirculated with blood or perfusion medium after an interruption of 10 minutes. These changes consisted of gross vacuolation, formation of blebs and disintegration of plasma membranes. These changes depend on the free availability of water in the recirculated blood or perfusion medium for hydrolytic catabolism of phospholipids in the plasma membrane as well as for the entry of water by osmosis (Dixon, 1982). The time elapsed, therefore, between the arrest of the portal circulation and initiation of the perfusion was kept to the minimun of less than two minutes. The other problem was the pressure exerted by the perfusion medium on the hepatic cells. The pressure was kept within the physiological range, because it was noticed that during the initial stage of the perfusion and the flush out of blood with high flow pressure, the cells developed peripheral vacuolations followed by mass cell death not related to the zones of liver lobules. For the same reason the outlet of the effluent perfusate was free and at the lowest level to avoid the build up of back pressure.

The cells of the centrilobular zone showed progressive changes leading to cell death. The highest rate of cell death occurred between the secon and third hours of perfusion. The results showed cellular changes in the midzonal region composed of cell swelling, aqueous vacuolation, loss of villi and formation of blebs. Cell swelling and vacuolation developed when the cellular semipermeability was increased, but not totally destroyed. Once the injury to a cell is severe enough to cause disintegration of the plasma membrane and escape of

macromolecules, cell swelling and gross vacuolation does not continue to develop, but cell death follows immediately (Dixon, 1982). Damage to the midzonal cells apparently was not sufficiently severe to cause cell death, and/or the cells received enough oxygen and nutrients to stay alive at the moment of fixation, but in an attenuated form. The results showed that the periportal zone, about 40% of the total sectional area, remained morphologically intact and the cellular fine-structures of the hepatocytes were remarkably well preserved throughout the experimental periods. The heterogeneity of the reactions of hepatocytes of the isolated perfused liver is very much similar to reactions of hepatocytes in many pathological conditions in humans, for example ischaemic injuries to the hepatic cells resulting from progressive heart failure. The heterogeneity in the reactions of the hepatocytes may be due to the differences in locations of cells with relation to the hepatic vasculature, differences in the homeostasis of hepatic cells, and/or due to lack of substrates in the perfusion medium essential for the survival of centrilobular hepatocytes. This investigation showed that the periportal zone of the liver remained morphologically intact for fours hours perfused in vitro at body temperature. The periportal zone represents the functional region of the hepatic lobule in this model system.

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