

## **Ultrastructural changes in rat livers perfused *in vitro* and *in vivo* with a high dose of methotrexate**

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**Summary.** Methotrexate is an antifolate that is widely used in the treatment of malignant tumours and other diseases. The present study was undertaken to examine the short-term effects of high doses of methotrexate (HD-MTX) on the ultrastructure and metabolic activity of isolated rat livers. The authenticity of the drug-induced changes was substantiated by the concomitant use of *in vivo* experiments.

Isolated rat livers were infused with HD-MTX via the portal vein for 3 hours (total dose for each liver 2000 mg). For *in vivo* experiments, each rat received a single intravenous injection of a maximum tolerated dose of MTX (100 mg/kg body weight) that allowed the animals to survive for 3 days. At the end of each experimental period, MTX-treated and control livers were processed for light microscopy (LM), scanning (SEM) and transmission electron (TEM) microscopy. Oxygen consumption and thyroxine metabolism were measured in treated and control isolated livers.

With the exception of a few minor differences, the structural changes in the hepatocytes after MTX treatment *in vitro* and *in vivo* were similar. There were focal changes consisting of disruption of normal hepatic plates and swelling and vacuolation of the hepatocytes, with no clear evidence of restriction to a specific hepatic zone. SEM revealed striking changes in the plasma membrane, the microvillar system, intercellular junctions and the sinusoidal endothelium. TEM revealed disorganized endoplasmic reticulum, dispersion of the polyribosomes, a variety of mitochondrial changes, and glycogen redistribution. In MTX-treated isolated rat livers, the uptake of tetraiodothyronine (T4) was not affected, but triiodothyronine (T3) release was impaired. Oxygen consumption was increased in livers treated with MTX.

Employing an organotypic liver perfusion model in conjunction with the *in vivo* experiment and the use of

SEM, TEM and hepatic thyroxine measurements, this investigation revealed that infusion of HD-MTX induced early ultrastructural changes in cell membrane, intercellular junctions and cell organelles and disturbance in the functional integrity of the hepatocytes in isolated rat liver.

**Key words:** Methotrexate, Livers, Perfusion, EM, Rat

### **Introduction**

Since its introduction as an antifolate agent more than fifty years ago, methotrexate (MTX) remains a widely used drug in medical, surgical, dermatological and pediatric specialties. MTX and its active metabolites have made a significant impact on the treatment of a wide range of diseases such as neoplasms, rheumatoid arthritis and psoriasis as well as being used as immunosuppressive agents in organ transplantation (Wyatt et al., 2001; Ros et al., 2002; Abrey et al., 2003; Schmiegelow et al., 2003; Weiss et al., 2003; Aithal et al., 2004; Bamias et al., 2004; Kaplan et al., 2004; Cosnes et al., 2005; Matsui et al., 2005; Scott, 2005). MTX is metabolized by the liver cells to a polyglutamate derivative that is stored in the cells. The intracellular MTX polyglutamate derivatives bind to dihydrofolate reductase, thereby eventually reducing cellular folate stores and consequently inhibiting DNA synthesis. MTX does not react chemically with DNA but it accumulates at the expense of the cellular folate pool (Scheufler, et al., 1981; Gewirtz et al., 1984; Hendel et al., 1985; Flynn and Treves, 1987). A more recent development has been the use of high doses of methotrexate (HD-MTX), as either monotherapy or in combination therapy, in order to achieve an excess of intracellular MTX and its metabolites (Luyckx et al., 1985; Abrey et al., 2003; Poortmans et al., 2003; Schmiegelow et al., 2003; Fukuda et al., 2005; Hoekstra et al., 2005; Meyers et al., 2005). Although guidelines concerning HD-MTX therapy have been recommended (Bremnes et al., 1991; Smeland et al., 1994; Poortmans et al., 2003), the direct

and short term effects of HD-MTX on the hepatocyte cell membrane and organelles have not been clearly elucidated in either human or experimental animals.

The liver is the principle organ responsible for MTX metabolism and, given the diversity and extent of today's use of MTX therapy, conflicting reports on the effects of MTX on the liver are not unexpected. Some investigators have found that changes in the liver following MTX administration are minimal, inconsistent and clinically insignificant (Laine and Chojkier, 1986; Kremer and Kaye, 1989; Smeland et al., 1994; Kremer et al., 1995; Bessler et al., 1996; Ros et al., 2002; Baughman et al., 2003; Aithal et al., 2004). Others have implicated MTX in hepatotoxicity, including structural changes, in both human and in experimental animals (Nyfors and Hopwood, 1977; Beck and Foged, 1983; Hendel et al., 1985; Bjorkman et al., 1988; Bremnes et al., 1991; Kremer et al., 1995; Fuskevåg et al., 2000a,b; Fathi et al., 2002; Batchleor et al., 2003; Maiti and Chen, 2003). However, other factors influencing the course of hepatotoxicity cannot be excluded such as age, nutritional status, pre-existing diseases before MTX treatment (such as obesity and diabetes), steroids, oral contraceptives and alcohol intake (Grossie et al., 1982; Leszczynska-Bisswanger 1985a,b; Kremer and Kay, 1989; Ros et al., 2002). Moreover, liver diseases are so common in patients that it is difficult to attribute the alterations entirely to the effects of a drug. Liver function tests alone are not a sufficiently reliable indication of MTX damage, since disparities have been seen between liver biopsies and biochemical liver function tests (Lawrence et al., 1983; Hendel et al., 1985; Kremer et al., 1995; Zimmerman and Ishak, 1995; Fathi et al., 2002). Furthermore, in small laboratory animals, the tests are of even more limited value (Hinton and Grasso, 1999; Fuskevåg et al., 2000b).

The liver is a unique organ in terms of its haemodynamics and vascularity, approximately 70% of its blood supply is provided by the portal vein (Crawford, 2005). The portal blood containing drugs and other substances percolates through fenestrated hepatic sinusoids to come in direct contact with the hepatocytes before it drains into the inferior vena cava. This pattern of vascularity conveniently allows investigators to perfuse isolated livers via the cannulated portal vein. The perfusate can then be collected by the cannulated thoracic inferior vena cava for biochemical analyses. This *in vitro* model has been widely used for various biochemical and hormonal assays (Lemberg et al., 1967; Ross, 1972; Miller, 1973; Weiland et al., 1979; Merker et al., 1983; Scheffel et al., 1986), and we have previously optimized the conditions for maintaining the structural and functional integrity of the isolated liver model (Al-Ali et al., 1987; Hassan et al., 1989, 1990).

This investigation describes, for the first time, the short-term effects of HD-MTX on the ultrastructural morphology of the isolated rat liver maintained *in vitro*. This study employed an organotypic liver perfusion model for which fully controlled conditions could be

used. In conjunction with the *in vitro* experiments, rats injected with HD-MTX were used for an *in vivo* study to substantiate the authenticity of the drug-induced changes following the infusion of HD-MTX. Survey low magnification and high power scanning electron microscopy (SEM) complemented with transmission electron microscopy (TEM) were used. SEM was the appropriate tool to visualize the surfaces of the hepatocytes and endothelial lining of the sinusoids. TEM was used for detecting the ultrastructural changes in the hepatocyte cell organelles. In addition to the morphological evaluation, this study examined the peripheral thyroxine metabolism to evaluate the functional integrity of isolated liver following HD-MTX perfusion since the liver is normally the main site for peripheral thyroid hormone metabolism (Hassan et al., 1989).

## Materials and methods

### *Animals and anesthesia*

Male Wistar rats (n=32) weighing 220-350 g were maintained at a constant temperature (22°C) and fed standard laboratory chow *ad libitum*. Guidelines of the animal ethics committee were strictly followed (project KU MA 025). The animals were anaesthetized by intraperitoneal injection of chloral hydrate (1 ml of 3.5% chloral hydrate per 100 g body weight), and were operated on under deep anaesthesia, being insensitive to deep pain pressure. As an anticoagulant, 1 ml heparin (500 units) was injected into the penile vein prior to perfusion.

### *In vivo* MTX-treated rats (Group 1)

Each rat (n=12) received a single intravenous injection of 100 mg MTX / kg body weight (Scheufler, 1982; Kamen et al., 1984). The dosage was chosen in an attempt to induce maximal toxicity without mortality within the first 3 days of MTX administration. The animals usually die after 4 days from severe hemorrhagic enteritis. The rats were sacrificed 3 days after MTX injection. For control experiments MTX was replaced with normal saline. The livers were fixed by portal perfusion with 1.5% glutaraldehyde in 0.1 M phosphate buffer and processed for microscopy as described below.

### *In vitro* untreated control livers (Group 2)

Isolated rat livers (n=10) were maintained *in vitro* for 3 hrs at 30°C by a non-recirculating perfusion system (Fig. 1), previously described (Al-Ali et al., 1987, Hassan et al., 1989, 1990). The influent (input) medium was perfused through a cannula in the portal vein and effluent (output) perfusate was drained by a cannula in the thoracic inferior vena cava. The perfusion flow rate was kept within the normal physiological range at 12-15

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ml/min. The perfusion medium (PM) consisted of oxygenated Ham's F10 tissue culture medium 4.95 g/l, sodium bicarbonate (1.5 g/l), bovine serum albumin 2 g/l (Sigma), D+ glucose (dextrose) 10 g/l, and Dextran-40. Thyroxine (T4), (Sigma), used in the medium, was prepared from stock solution (77.7  $\mu\text{g/l}$ ). The osmolarity of the medium was 390 mosm. The pH and  $p\text{O}_2$  readings of the perfusion medium (after oxygenation) were 7.4 and 300 mm Hg respectively and were kept constant throughout the perfusion time. For T3 and T4 measurements 6 rat livers were used from this group.

#### *In vitro* MTX-treated livers (Group 3)

Isolated rat livers (n=10) were perfused, as described above for group 2, with the perfusion medium (PM) containing MTX. MTX was continuously infused through a needle in the cannulated portal vein at a rate of 11.11 mg MTX/min for 3 hours, using a syringe pump (Sage Instruments). The total infused dose was 2000 mg MTX. For T3 and T4 measurements 5 rat livers were used from this group.

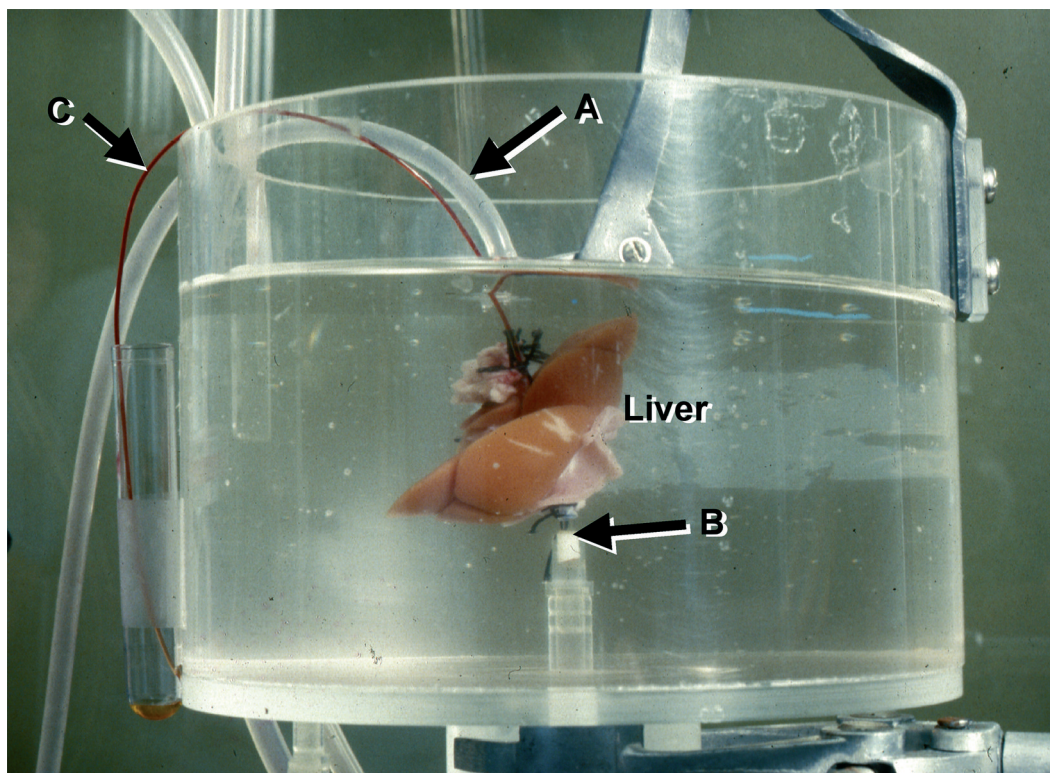
#### Microscopy

At the end of each experimental period, the livers were perfused with fixative via the portal vein and drained via the thoracic inferior vena cava using a

gravity feed system with a head of 10 cm, followed by immersion fixation in the same fixative overnight. The primary fixative solution consisted of 1.5% glutaraldehyde in 0.1 M phosphate buffer. For TEM, vibratome tissue slices (300  $\mu\text{m}$ ) were cut and postfixed in 1% buffered osmium tetroxide. The slices were dehydrated in ascending series of ethanol, treated with propylene oxide and embedded in epoxy resin 'Araldite'. Semi-thin resin sections (1-2  $\mu\text{m}$ ) were cut and stained with toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Jeol 1200 EX transmission electron microscope. For SEM, the livers were sliced with a scalpel blade, dried by the critical point method, coated with gold and examined with a Jeol GSM 840 scanning microscope. Manually sliced livers were used for SEM because they showed more surface details than vibratome sectioning.

#### HPLC

The concentration and chemical stability of MTX in the perfusion medium was measured at several intervals over 3 hrs by high pressure liquid chromatography (HPLC). MTX was stable in the perfusion medium, and no MTX metabolites were detected up to 8 hours after preparation of the standard medium. Similar results were obtained when the samples were measured by a UV



**Fig. 1.** Liver perfusion system. Cannulas are in the portal vein (A), in the thoracic inferior vena cava (B), and in the common bile duct (C). The excreted fluid in the bile cannula is concentrated perfusion medium (dark red), demonstrating the preservation of biliary function in this system.

spectrophotometer (Fuskevåg et al., 2000a).

#### Oxygen consumption

The partial pressure of oxygen ( $pO_2$ ) and the pH were assessed in the influent medium and effluent perfusate at 0 time and every 30 minutes for 3 hrs using an ABL Radiometer. Eleven rat livers were used (6 livers from group 2 and 5 livers from group 3).

#### Measurements of thyroxine metabolism by isolated perfused liver

After 10 minutes of washing with thyroxine free perfusion medium, tetraiodothyronine (T4) and triiodothyronine (T3) were measured in the influent and effluent samples collected every 15 min. T4 and T3 were assayed using the RIA kit (Amersham). Standards and samples were assayed in duplicate, and all tubes were counted until 10,000 counts had accumulated in the maximum binding tubes.

Because the flow rate remains constant for each experiment, it is assumed that the amount of T4 entering the liver is directly proportional to the number of effluent fractions and to the length of time of perfusion. Total input of T4 at any given time was calculated by multiplying the T4 concentration in the perfusion medium by the volume of fluid, which passes through the liver at that time. The amount of T4 not taken up by the liver at any given time was calculated from the measured hormone concentration in the effluent medium by the fraction volume and the time at which the fraction was collected. Net T4 taken up by the liver was therefore the difference between the input and output. Cumulative T3 production was calculated in the same fashion as T4. The perfusion medium contained a small amount of T3 introduced as a contaminant. Net T3 release was calculated as the difference between T3 input and output. Net T4 uptake and net T3 release were measured in  $\mu\text{g/g}$  of wet liver. The T4 to T3 conversion rate is expressed as  $[\text{net T3 release (mg)}/\text{net T4 uptake (mg)} \times 100]$ . This experimental model has been previously described (Hassan et al., 1989).

Calculations were performed using the following equations.

Cumulative thyroxine output =

$$= \sum_1^n z \left[ (x_2 - x_1) \left( \frac{y_2 - y_1}{2} \right) + (x_2 - x_1) y_1 \right]$$

$$= \sum_1^n \frac{z}{2} [(x_2 - x_1)(y_2 - y_1)]$$

Where,

n = Total number of fractions.

z = Fraction volume.

$x_1, x_2$  = Fraction numbers.

$y_1, y_2$  = Thyroxine concentration in fraction and respectively.

## Results

The fine structural morphology of the isolated rat livers maintained at 30°C in vitro by perfusion in control livers is represented in Fig. 2.

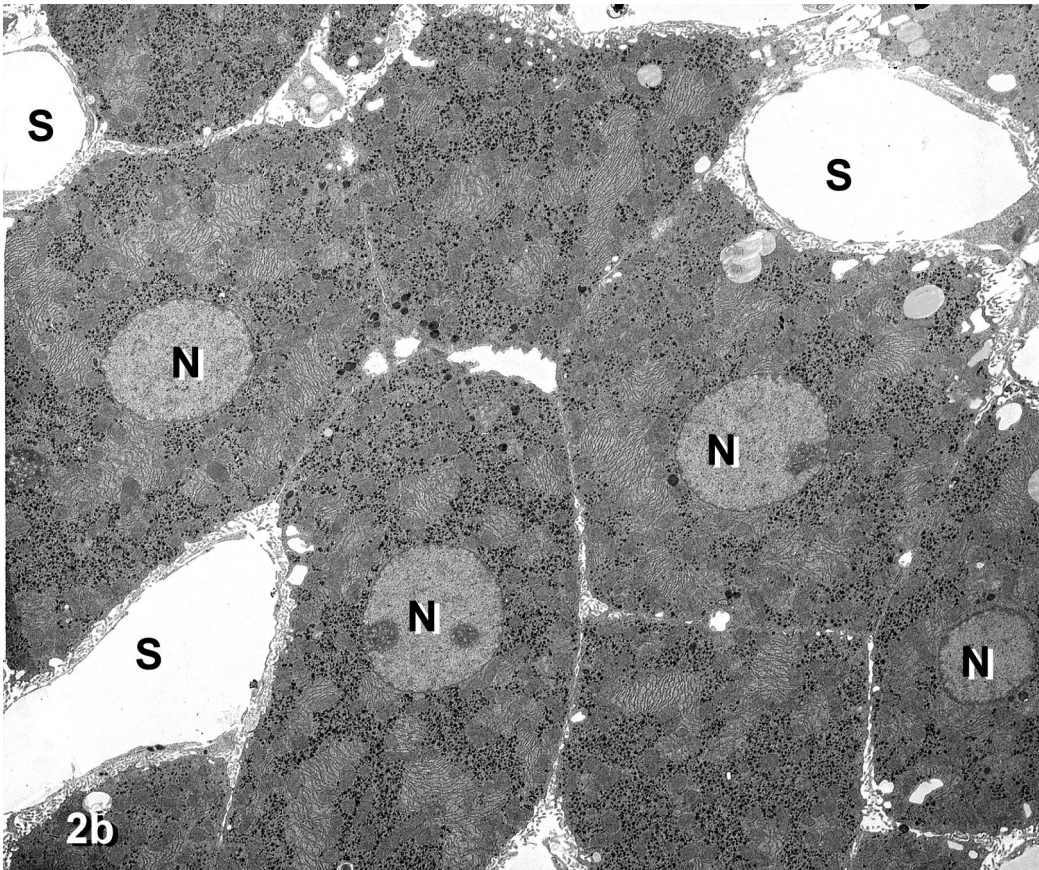
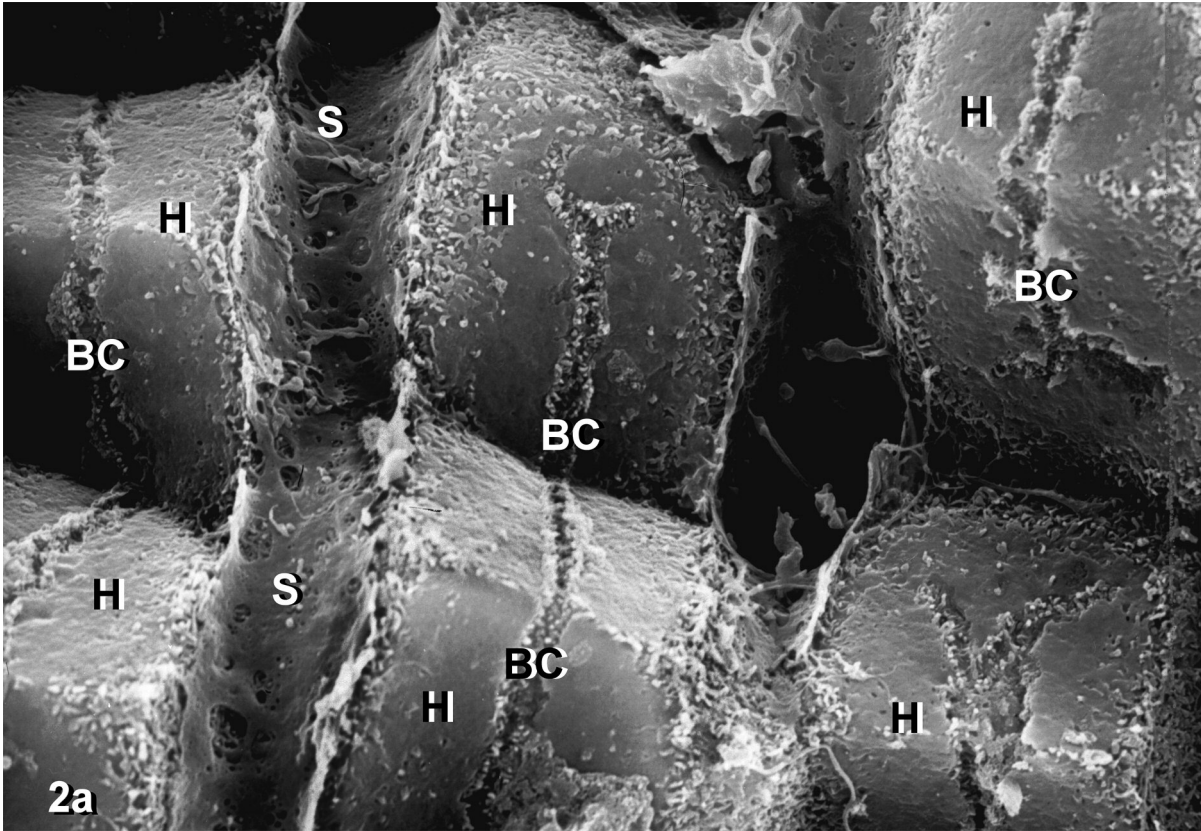
The structural changes in the hepatocytes following the administration of HD-MTX were, with the exception of a few minor differences, similar in both the in vivo (group 1) and in vitro (group 3) experiments. In both groups light microscopy and low power survey scanning electron microscopy revealed focal changes where there were disruption of liver plates, loss of intercellular junctions, swelling, rounding, and vacuolation of hepatocytes. These changes were not restricted to a specific hepatic zone. Normal looking areas and different degrees of structural changes were seen in the same lobule (Fig. 3a,b). Although cell debris was seen, cell death, however, was not a prominent feature.

### SEM

The cells in the affected zones were distinguished by marked changes of the surface of the cell membrane as revealed by SEM (Figs. 3, 4). Swelling and rounding of the cells and loss of intercellular junctions were seen (Fig. 3b-d). The canalicular surface of the hepatocyte exhibited short, stubby and globular microvilli, giving a cobblestone (nodular) appearance to the surface (Fig. 3e). Bile canaliculi were either dilated or occluded with swollen and fragmented microvilli (Fig. 3e-g). The sinusoidal surface of the hepatocytes exhibited striking increase in number and size of microvilli, which were tightly packed and pleomorphic (Fig. 4a,c,d). Bleb formation and shedding of membrane-bound cellular fragments into the sinusoids were seen (Fig. 4c). Enlarged sinusoidal fenestrae, detachment of sinusoidal endothelium, and protrusion of cytoplasmic blebs through enlarged fenestrae were seen (Fig. 4).

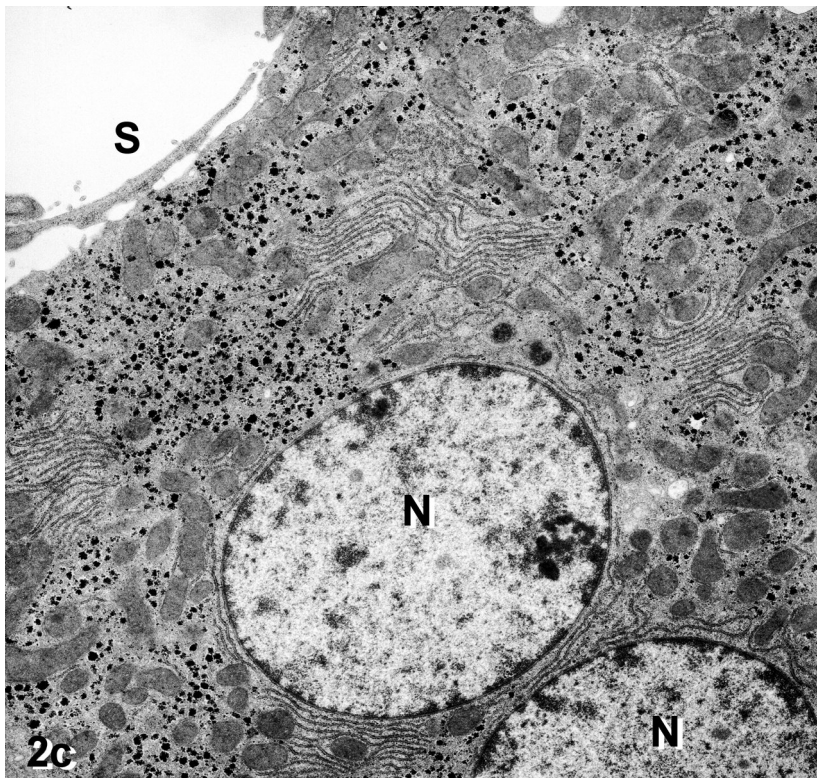
### TEM

The changes in the cytoplasmic membrane seen by SEM were confirmed by TEM (Fig. 4b). Cells in the affected zone showed fragmentation and partial degranulation of the granular endoplasmic reticulum (GER). Dispersion of polyribosomes and formation of monoribosomes was seen. Increase in number of profiles of the smooth endoplasmic reticulum (SER) and transformation into either tubular or vesicular cisternae was seen (Fig. 5a-c,e,f). A variety of mitochondrial abnormalities was present including pleomorphism, proliferation of mitochondria, swelling, and increase or decrease in the density of matrices (Fig. 5 a-c,g). The cytoplasm was packed with altered mitochondria. Fragmentation, peripheral displacement or partial lysis



**Fig. 2.** Hepatocytes from livers maintained by perfusion with the culture medium (group 2) for 3 hrs at 30°C. BC: bile hemicanaleculus; H: hepatocyte; N: hepatocyte nucleus; S: hepatic sinusoid. a, SEM x 3900; b, TEM x 3000; c, TEM x 7860

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**Table 1.** Oxygen consumption by isolated rat livers perfused in vitro for 3 hours.

LIVER/GROUPS	WEIGHT OF LIVER (g)	FLOW RATE (ml/min)	pH OF INFLUENT PERFUSION MEDIUM	pH OF EFFLUENT PERFUSION MEDIUM	OXIGEN COSUMPTION ( $\mu\text{mol O}_2/\text{min/g}$ wet liver)
Group 2 (control) n = 6	11.18 $\pm$ 0.80	14.5 $\pm$ 0.80	7.38 $\pm$ 0.05	7.27 $\pm$ 0.13	1.18 $\pm$ 0.14
Group 3 (MTX 2000 mg) n = 5	10.71 $\pm$ 1.48	12.1 $\pm$ 0.22	7.4 $\pm$ 0.07	7.21 $\pm$ 0.03	4.18 $\pm$ 1.01

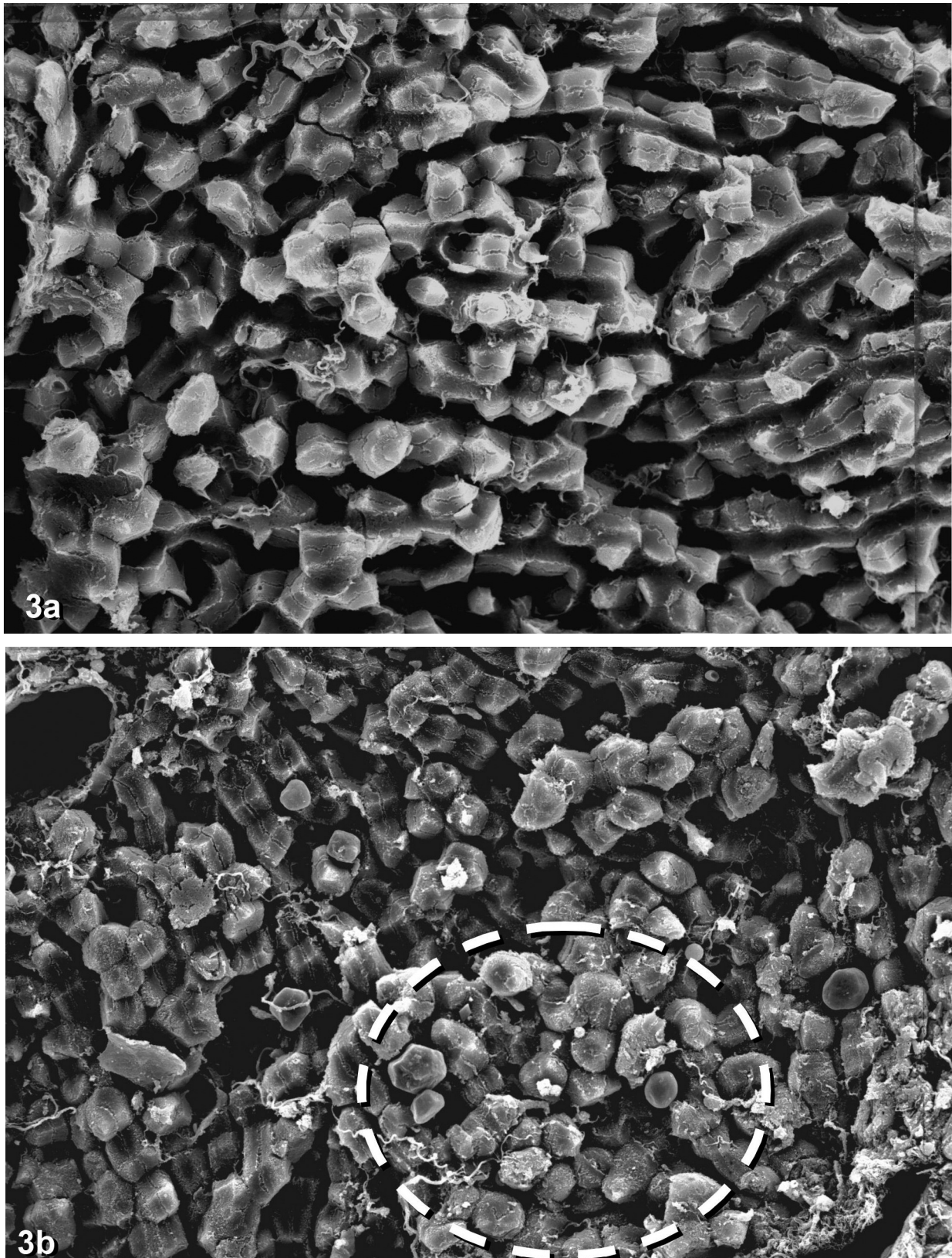
**Table 2.** T4 uptake and T3 output for 6 livers perfused without MTX for 3 hours (Group 2).

EXP. No.	T4 CONC. IN PM ( $\mu\text{g/ml}$ )	FLOW RATE (ml/min)	ACTUAL T4 INPUT ( $\mu\text{g}$ )	% T5 UPTAKE	NET T4 UPTAKE ( $\mu\text{g}$ )	NET T3 OUPU ( $\mu\text{g}$ )	T3/T4 (%)	RAT Wt. (gms)
1	82.4	15.0	222.2	49.3	109.6	3.2	2.9	300.0
2	67.0	14.0	176.2	39.9	70.2	1.5	2.1	265.0
3	62.2	15.0	167.8	45.8	76.8	1.1	1.4	254.0
4	82.4	13.0	192.7	52.4	101.0	1.7	1.7	308.0
5	84.7	15.0	328.7	55.1	125.8	1.2	1.0	270.0
6	58.3	15.0	157.3	62.1	97.7	0.9	0.9	280.0
Mean $\pm$ SD	72.8 $\pm$ 11.7	14.5 $\pm$ 0.8	207.5 $\pm$ 63.6	50.8 $\pm$ 7.7	96.9 $\pm$ 20.6	1.61 $\pm$ 0.84	1.68 $\pm$ 0.76	279.5 $\pm$ 20.9

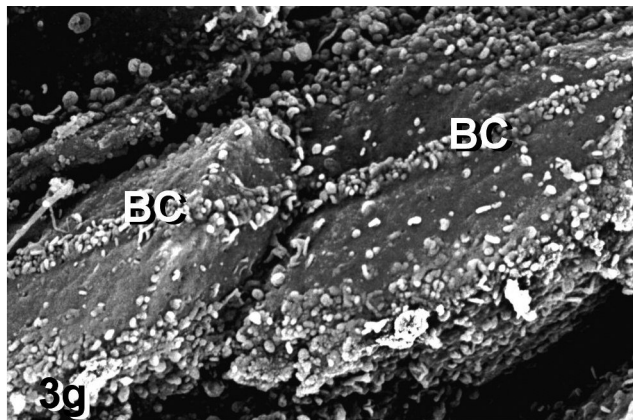
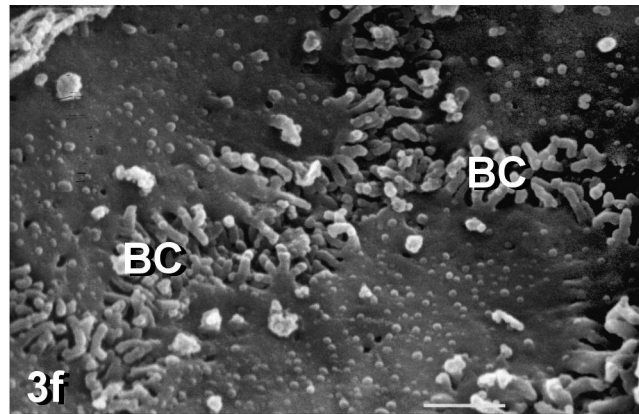
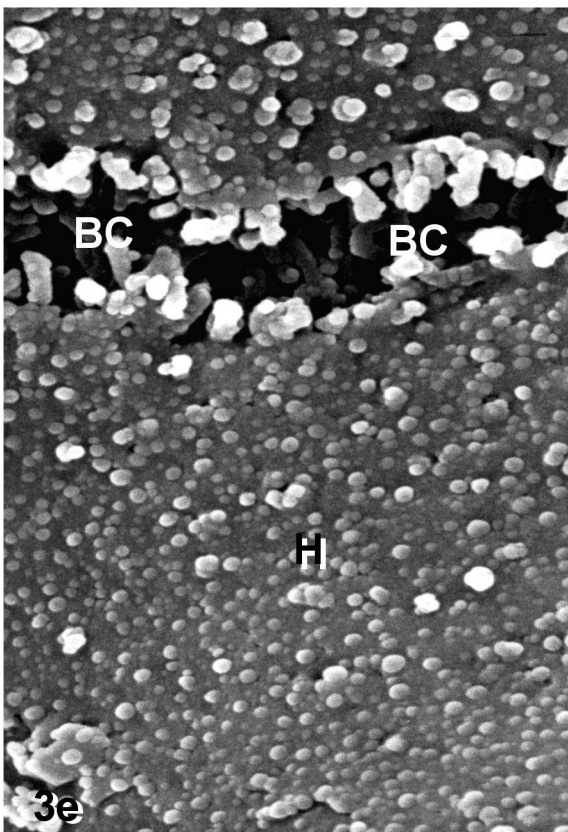
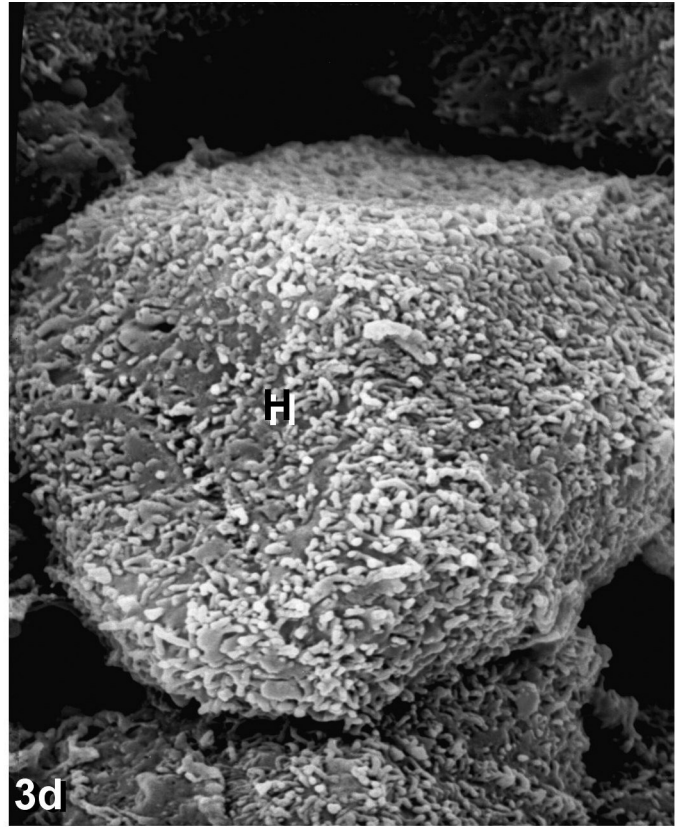
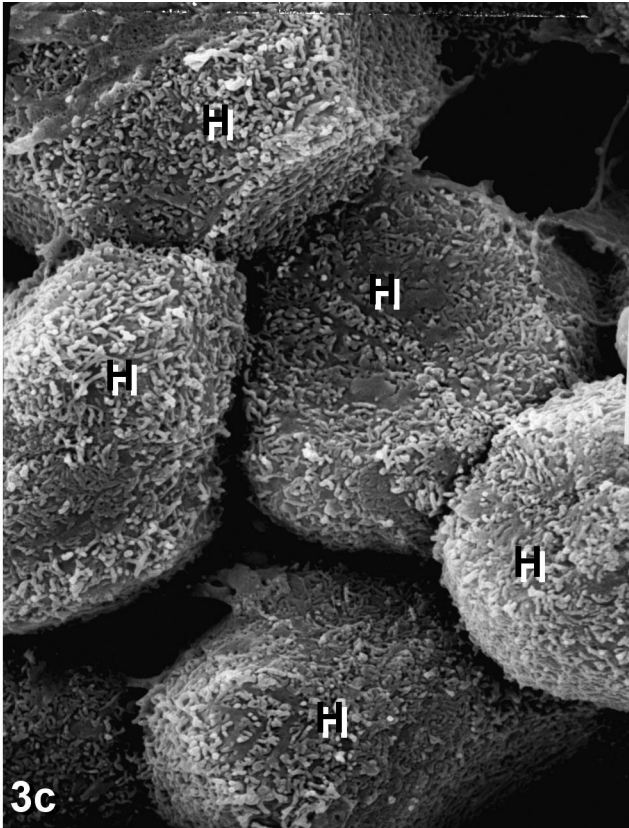
**Table 3.** T4 uptake for 6 livers perfused with MTX for 3 hours (Group 3).

EXP. No.	T4 CONC. IN PM ( $\mu\text{g/l}$ )	FLOW RATE (ml/min)	ACTUAL T4 INPUT ( $\mu\text{g}$ )	% T4 UPTAKE	NET T4 UPTAKE ( $\mu\text{g}$ )	RAT Wt. (gms)
1	74.4	14.5	194.2	43.1	83.7	280.0
2	77.2	15.0	208.4	40.5	84.4	275.0
3	85.4	14.0	215.2	42.8	92.1	285.0
4	84.5	13.0	197.7	49.5	97.9	305.0
5	86.6	12.0	187.1	42.8	80.1	279.0
Mean $\pm$ SD	81.6 $\pm$ 4.9	13.7 $\pm$ 1.1	200.5 $\pm$ 10.1	43.7 $\pm$ 3.0	87.6 $\pm$ 6.5	284.8 $\pm$ 10.6

## Isolated rat liver perfused with methotrexate

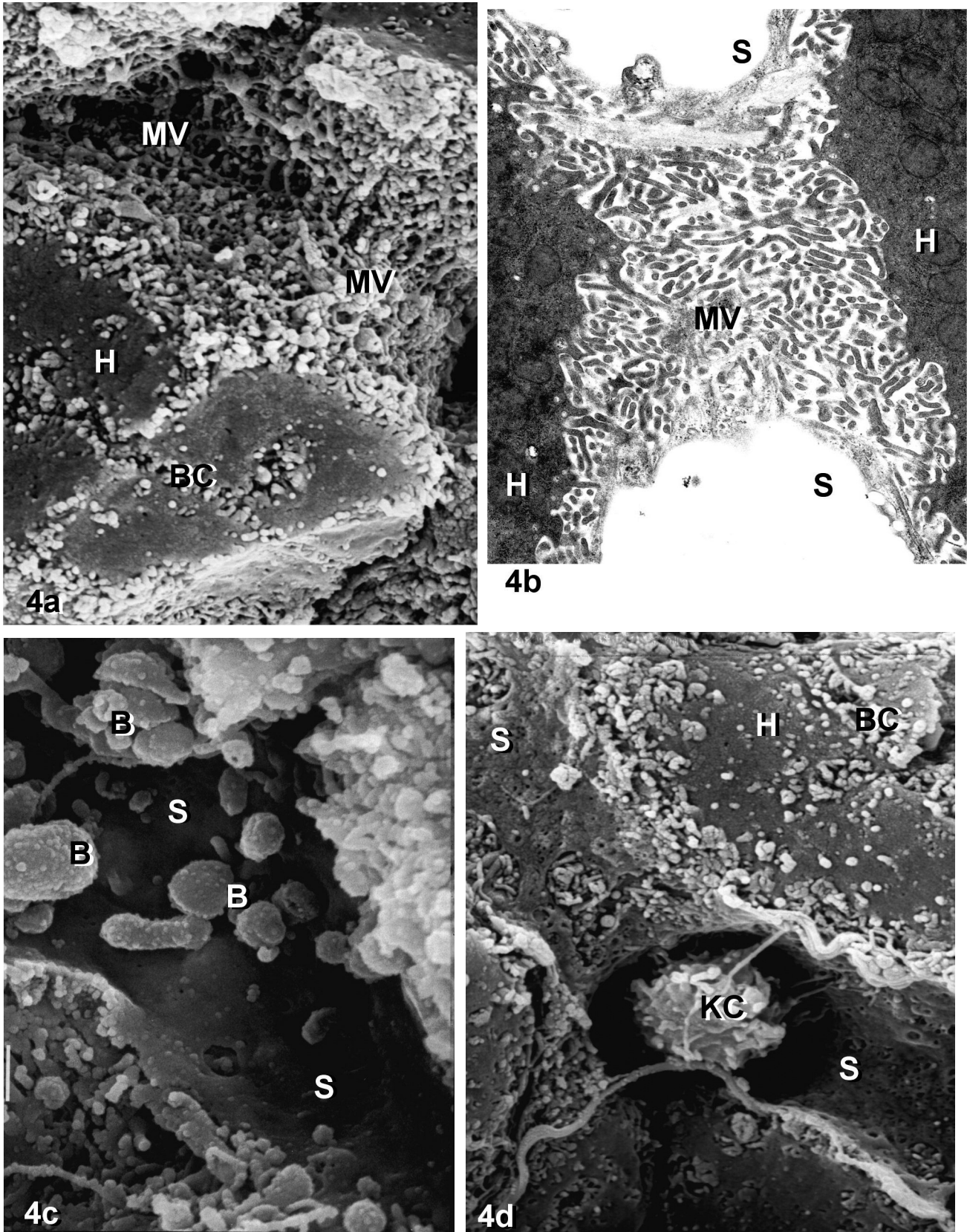


**Fig. 3.** Survey SEM (a, b) of livers treated with HD-MTX showing normal appearance of hepatic plates (a), and focal disruption of the plates (encircled in b). At a higher magnification of the affected area, the hepatocytes (H) were round, the intercellular junctions were enlarged and the surfaces were covered with a large number of microvilli (c, d). The canalicular surfaces of the hepatocytes were nodular giving a cobble-stone appearance (e). Bile hemicanaliculi (BC) were either dilated or obstructed with enlarged microvilli (f, g). SEM. a (in vivo, group 1), x 660; b (in vivo, group 1), x 500; c (in vitro, group 3), x 3500; d (in vitro, group 3), x 5000; e (in vitro, group 3), x 15000; f (in vitro, group 3), 11000; g (in vivo, group 1), x 3600

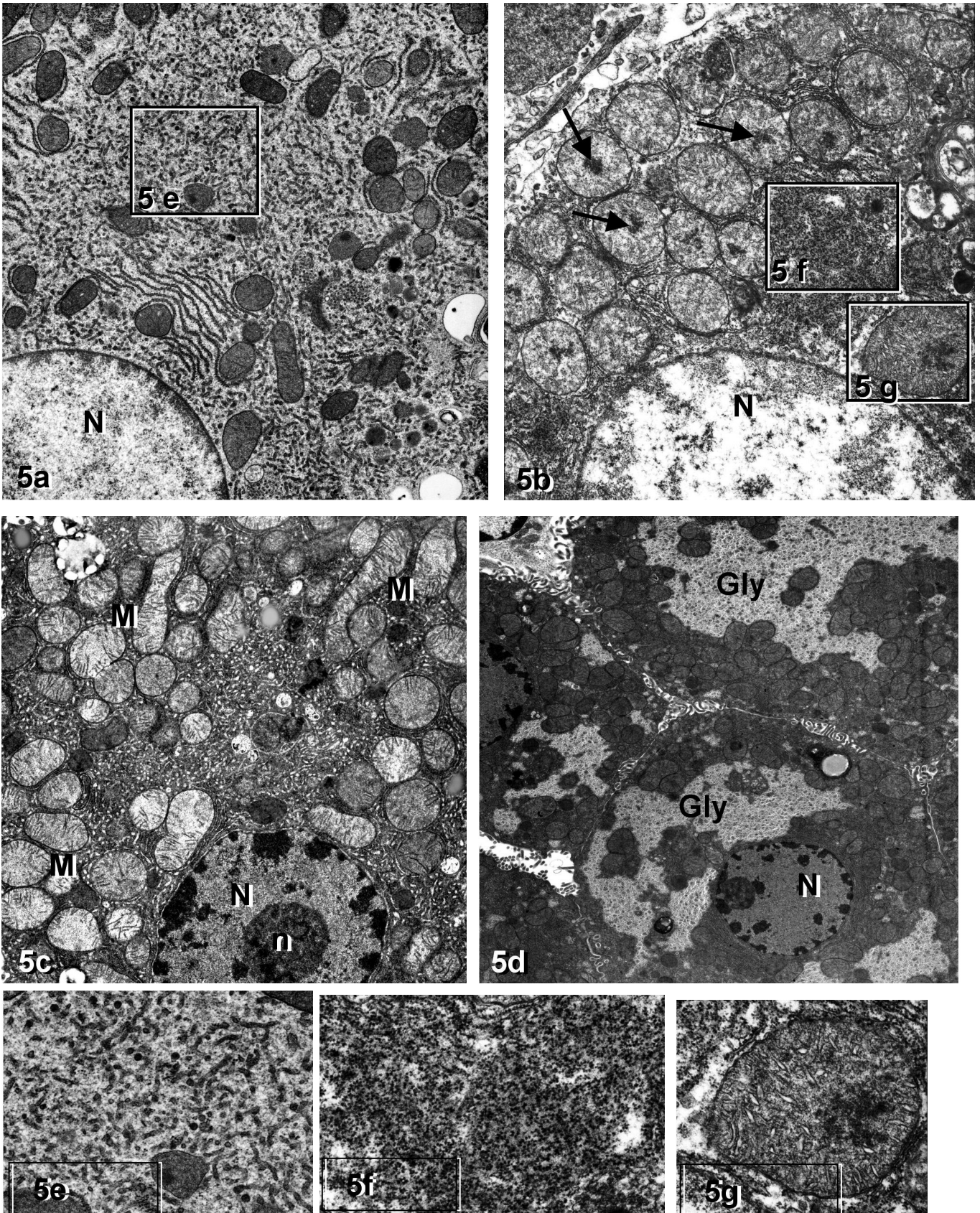




## Isolated rat liver perfused with methotrexate



**Fig. 4.** In vitro group 3. The sinusoidal surfaces of the hepatocytes (H) exhibited marked increase in the number of the microvilli (MV) and loss of the endothelial lining (**a, b**). The sinusoidal surface showed protrusion of blebs (B) through enlarged endothelial fenestrae and in some places loss of the sinusoidal (S) endothelial lining (**c, d**). Kupffer cell (KC). SEM. a, x 5800; c, x 10650; d, x 6000. TEM. b, x 12500



**Fig. 5.** TEM changes (a-g). Hypertrophy and tubulovesiculation of the smooth endoplasmic reticulum (a, c, e). Paucity and disorganization of the granular endoplasmic reticulum, formation of monoribosomes (b, f). Accumulation of mitochondria (M), fragmentation of cristae, and development of flocculant woolly focal densities (arrows) (b, c, g). Accumulation of glycogen granules (Gly) (d), which are finer and less electron dense than normal, into cytoplasmic pools. Condensation of nuclear (N) chromatin and hypertrophy of the nucleolar (n) elements (c, d). a (in vitro, group 3), x 9690; b (in vivo, group 1), x 11000; c (in vitro, group 3), x 8450; d (in vitro, group 3), x 4590; e (in vitro, group 3), x 17500; f (in vivo, group 1), x 24000; g (in vivo, group 1), x 24000

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of mitochondrial cristae was seen (Fig 5b,c,g). The mitochondria developed flocculent woolly focal densities. These focal mitochondrial inclusions were present in group 1 and 3 but were more evident in the in vivo, group 1, (Fig. 5a,b,e,g). Glycogen granules were either depleted or accumulated in large pools within the cytoplasm. The granules were largely of the beta type, fine and less electron dense than in control livers (Fig. 5d). The cytoplasm contained inclusions of various sizes and densities, clear vacuoles and lipid droplets. Osmiophilic lipid droplets were seen in the in vivo (group 1) but not in vitro (group 3) experiments. The

nuclei exhibited clumping and margination of the nuclear chromatin, and hypertrophy of nucleolar elements (Fig. 5 c,d).

#### Oxygen consumption

There was a marked increase in  $O_2$  consumption by MTX-treated livers, group 3 ( $4.18 \pm 1.01 \mu\text{M } O_2/\text{min/g}$  wet liver) as compared with untreated (group 2) livers ( $1.18 \pm 0.14 \mu\text{M } O_2/\text{min/g}$  wet liver) (Table 1).

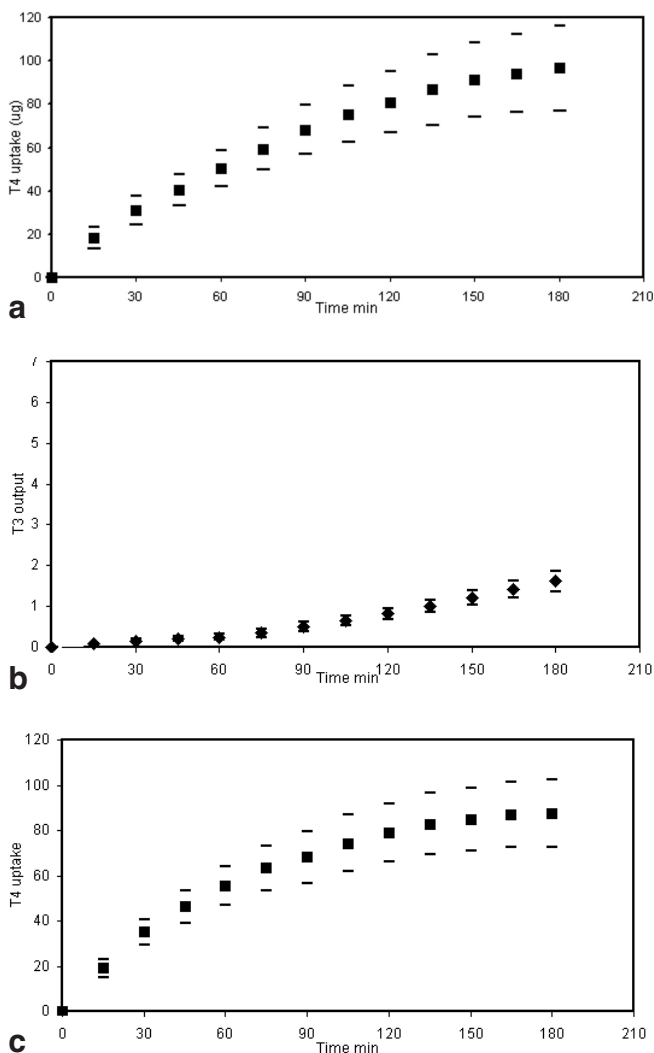
#### Thyroxine metabolism by isolated perfused livers

In livers perfused without MTX (Group 2), there was a significant difference between T4 concentration in the influent medium and effluent perfusate. The mean cumulative net T4 uptake ( $96.9 \pm 20.6 \mu\text{g}$ ) is shown in Table 2 and Fig. 6a. The mean cumulative T3 release in the perfusate was clearly detectable ( $1.61 \pm 0.84$ ), (Fig. 6b). T4 uptake and T3 release were clear evidence of maintained metabolic activity of the hepatocytes perfused in vitro for 3 hours (group 2). In livers perfused with MTX (Group 3), the mean cumulative net T4 uptake ( $87.6 \pm 6.5 \mu\text{g}$ ) (Table 3, Fig 6c) was not significantly different from the control untreated livers (Group 2). Interestingly, in this group T3 release was not detectable in the perfusate.

#### Discussion

This study describes, for the first time, the short-term effects of HD-MTX infusion on the cell surface, microvillar system and cell organelles and correlates structural changes with the peripheral thyroxine metabolism by the isolated rat liver. In vivo MTX-treated rats were employed as an additional control. The liver is the main site for drug metabolism and is therefore often associated with untoward reactions to drugs and their metabolites. The special pattern of the local hepatic vascular system and presence of the microvilli are of advantage for investigating a direct action of drugs administered by portal perfusion (Merker et al., 1983). The complementary use of SEM and TEM employing the isolated liver perfusion model have added significantly to our understanding of the early effects of HD-MTX on the hepatocytes.

Extensive studies have been reported on the effects of MTX in patients (Hendel et al., 1985; Bjorkman et al., 1988; Boffa and Chalmers 1996; Bathon et al., 2000; Fathi et al., 2002; Batchleor et al., 2003; Aithal 2004; Cosnes et al., 2005), in laboratory animals (Bremnes et al., 1991; Smeland et al., 1994; Bessler et al., 1996; Fuskevag 2000a,b; Tada et al., 2005) and in cell culture (Hamkalo et al., 1985). As mentioned in the Introduction, pre-existing conditions in patients may influence the course of the effects of the drug. The in vivo studies in laboratory animals are complicated by the fact that substrates in the blood or in the gastrointestinal tract may alter or modify the drug or its course. On the



**Fig. 6.** a. Mean cumulative net thyroxine (T4) uptake plotted against time, in 6 control isolated livers perfused without MTX (group 2) for 3hrs. b. Mean cumulative triiodothyronine (T3) output (release) plotted against time in 6 control isolated livers perfused without MTX (group 2) for 3hrs. c. Mean cumulative net thyroxine (T4) uptake plotted against time, in isolated livers perfused with MTX (group 3) for 3hrs. No T3 was detected in this group. All data are shown in Table 2 and Table 3.

other hand, hepatocyte cultures lack the normal intercellular cohesion and coordination and therefore are further removed from the physiological environment. The isolated rat liver perfusion model, therefore, offers the best compromise, provided the viability of hepatocytes is maintained, monitored and evaluated morphologically as well as functionally. This model allowed us to study the direct effects of HD-MTX on the hepatocytes independently of other factors, which may have been encountered in patients prior to the treatment. Furthermore, the hepatocytes were directly exposed to HD-MTX by perfusing the liver via the portal vein, i.e., bypassing the gastrointestinal tract and the circulatory blood. However, the possibility cannot be totally excluded that some fine-structural changes in the hepatocytes may be due to the *in vitro* setup rather than drug-induced reactions. Therefore, by running the control *in vivo* experiments along side the *in vitro* experiments and by lowering the perfusion temperature to 30°C, the possibility of such artefactual alterations has been minimized.

MTX acts as an inhibitor of the enzyme dihydrofolate reductase (DHFR), thereby inhibiting DNA synthesis (Beck and Foged, 1983; Maia et al., 1996; Calabresi and Chabner, 2001). The high dose (HD-MTX) schedule over a short period has been recommended to achieve an excess of intracellular MTX in concentrations that inactivate DHFR (Scheufler et al., 1981; Fuskevag et al., 2000a,b; Abrey et al., 2003). In patients, rescue schedules employing folonic acid, thymidine, leucovorin or other agents have been used to protect normal tissue from the effects of HD-MTX (Calabresi and Chabner, 2001; Buchen et al., 2005; Rigacci et al., 2005). In this study HD-MTX was administered without rescue tactics in order to evaluate its direct effects on normal hepatocytes.

Histopathological examination is still generally regarded as more reliable than the standard liver function tests for assessing hepatotoxicity in MTX-treated patients (Lawrence et al., 1983; Zimmerman and Ishak, 1995). In this investigation, although focal changes were seen in both groups (Group 1 and 3), these changes were not related to a specific zone within the liver lobule. The same liver lobule contained areas with normal appearing hepatocytes and other areas with altered cells. Interestingly, similar findings have been reported in human liver biopsies and in MTX-treated laboratory animals (Hendel et al., 1985; Kremer et al., 1995). It is unknown why some hepatocytes appeared to be more susceptible to the cytotoxicity of MTX than others. It has been reported that focal microcirculatory insufficiency could be the primary cause for focal hepatotoxicity (Fuskevag et al., 2000b). Cell death, however, was not a prominent feature in this study. A similar conclusion was drawn from MTX-treated rabbit limb bud (DeSesso, 1981), and MTX-treated kidney (Fuskevag et al., 2000b).

There is a limited number of different types of changes in the ultrastructural morphology of cell

organelles that occur following the administration of a variety of cytotoxins (Phillips et al., 1987; Ghadially, 1997; Hinton and Grasso, 1999; Kumar et al., 2005). In this study a combination of SEM and TEM was used to reveal the early indications of cytotoxicity.

Our results showed marked proliferation of mitochondria that displayed a diversity in shape and size, as well as changes in the matrix and cristae. A similar observation has been described in liver biopsies in patients and has been termed as onchocytic changes (Phillips et al., 1987). All these changes, including the presence of flocculent wooly focal inclusions, were strongly suggestive of toxic injury resulting probably from a biochemically defective mitochondrial membrane since similar changes have been found in hepatocytes and other cells after administration of a variety of cytotoxic chemicals (Bessler et al., 1996). The accumulation of many altered mitochondria and the increased oxygen consumption following HD-MTX infusion may be either a compensatory reaction occurring at the organelle level or the result of the lesion's high demands upon the cell's energy resources (Myagkaya et al., 1985; Ghadially, 1997).

The present study also showed the dispersion of polyribosomes, disruption of GER, vesiculation and proliferation of SER and changes in the distribution of the nuclear chromatin. Similar ultrastructural changes have also been described after administration of other drugs with diverse pharmacological properties and chemical structures indicating that such alterations are generally not drug-specific (Phillips et al., 1987; Ghadially, 1997; Hinton and Grasso, 1999).

The glycogen content following HD-MTX treatment varied, with glycogen granules either being depleted or accumulated in large pools within the cytoplasm, similar changes occurred in livers as a consequence of administration of several drugs and toxins (Phillips et al., 1987). The granules were largely of the beta type, fine and less electron dense than in control livers. Similar ultrastructural changes in glycogen stores were seen in isolated dog livers perfused with a variety of cytotoxins (Merker et al., 1983).

Cytoplasmic vacuolation and cell swelling occur in certain hepatopathies in humans and experimental animals (Crawford, 2005; Kumar et al., 2005). In this study, the hepatocytes in the affected zones became swollen, round, and vacuolated. Cytoplasmic vacuolation is known to develop when cellular semipermeability is increased but not totally destroyed (Dixon et al., 1994). Other investigators have raised the possibility of an early manifestation representing an acute protective adaptation associated with the tolerance of the liver cells to acute hepatotoxic injury (Nayak, 1981; Kumar et al., 2005).

HD-MTX induced striking changes in the cell membrane and microvilli of the hepatocytes. These changes included alteration in the microvilli, loosening of intercellular junctions, bleb formation and shedding of the plasma membrane. It has been stated that shedding

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and bleb formation of the plasma membrane represent early primary events in the action of drugs and toxins on the hepatocytes (Phillips et al., 1987). The intercellular junctional complexes play an important role in normal function of the liver and other organs (Guan and Ruch, 1996; Hayashi et al., 1997; Defamie et al., 2000; Sai et al., 2001; Hu et al., 2002; Velazquez et al., 2003). The breakdown of this system was evidenced by enlargement of intercellular spaces, hepatocyte swelling and detachment of the endothelial lining of the sinusoids as revealed by SEM and TEM. Such changes have also been reported in MTX-treated renal tubules (Fuskevag et al., 2000b) and in MTX-treated limb buds (DeSesso, 1981).

The results in the present study showed that the livers perfused in vitro for 3 hrs without MTX retained the ability for T4 uptake and T3 release, whereas in livers perfused with HD-MTX this activity was altered. T4 uptake and T3 release are energy dependent processes and require an intact cell membrane. The significant difference between T4 concentration in the influent and effluent perfusate in both the control and MTX treated livers indicates a sustained integrity of the metabolic function of the hepatocytes. In contrast to the control experiments, MTX treated liver showed no T3 release in the effluent perfusate. The non-detectability of T3 in the MTX perfused livers could be due to one or more of the following: (a) The presence of MTX may alter the chemical configuration of T3, which makes it immeasurable by RIA. (b) MTX may have a strong affinity towards T3 antibody, which leads to inhibition of its binding capacity. (c) MTX may block the release of T3 from hepatocytes. (d) The inhibition of T3 release from the hepatocytes might be due to partial injury of the hepatocyte cell membrane which was confirmed by SEM and TEM.

Infusion of HD-MTX induced early alterations in cell membrane, intercellular junctions, and cell organelles and in the hepatic thyroxine metabolism. These changes may reflect a depletion of energy stores that resulted in disturbance of the structural and functional integrity of the cell membrane and thus an early disturbance in the intracellular ionic and water homeostasis.

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