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Effects of sex steroids on the Syrian hamster liver

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Summary. The primary objective of this research project was to study the role of sex steroids in the pathogenesis of cholelithiasis using the Syrian hamster as a model. In addition to the morphological examination of the gallbladder epithelium, we thought it imperative to observe the changes induced in the biliary tract in response to the sex steroid treatment. This report focuses on the morphological changes induced in the liver. The hamsters were randomly divided into 4 groups, control (C), estrogen-treated (E), estrogen and medroxyprogesterone-treated (E+MP), and medroxyprogesterone-treated (MP) groups. The E group hepatocytes demonstrated proliferation of the smooth endoplasmic reticulum, lipofuscin-like granules, aggregates of glycogen rosettes, and dense bodies. Lipid droplets in the hepatocyte cytoplasm as well as the nuclei were detected in this group. E+MP combined treatment induced an exacerbation of all the changes observed in the E group, furthermore, there appeared to be a disruption of the hepatic parenchymal architecture. The MP-treated group also exhibited the architectural changes observed in the E+MP group, but also showed sinusoidal dilation. In response to MP alone, the fatty changes in the liver appeared to be accentuated. A striking feature induced in response to MP treatment, was a focal area suggestive of adenomatous changes.

Key words: Liver, Biliary tract, Syrian hamster, Sex steroids, Ultrastructure

Introduction

Despite the fact that the development of oral contraceptive pills represents a major advancement in population control, the chronic administration of these drugs can interfere with various aspects of hepatic function. Contraceptive steroids are intrinsic hepatotoxins, which induce cholestasis (Thulin and Nermark, 1966; Dooner et al., 1971), and peliosis hepatis (Winkler and Poulsen, 1975; Balazs et al., 1981). Thrombosis of the hepatic veins or the inferior vena cava producing the Budd-Chiari syndrome, is a rare complication of contraceptive steroids administration (Ishak, 1981). In recent years, increasing incidence of hepatocellular neoplasms have been reported in patients on oral contraceptives, particularly hepatocellular adenomas with focal nodular hyperplasia and hepatocellular carcinomas (Klatskin, 1977; Moesner et al., 1977; Neuberger et al., 1980).

During pregnancy, liver function tests may frequently deviate from the normal range, but in general the liver adapts well to the increased metabolic demands of this physiological state. The incidence of liver injury is approximately 1 in 1500 pregnancies (Rustgi, 1989). Pregnancy is a state characterized by high levels of endogenous estrogen, progesterone, and metabolites, thus administration of female sex steroids to the hamsters for a prolonged period simulates extended gestation. Cholelithiasis is a common occurrence during or following pregnancy, therefore, in addition to the morphological examination of the female Syrian hamster gallbladder (Gilloteaux et al., 1992; Karkare et al., 1995; Karkare and Gilloteaux, 1995), it was thought that it would be enlightening to determine the changes occurring simultaneously in the liver and cystic duct in response to the sex steroid treatment.

A series of one, two, and three-month treatment studies were conducted (Karkare, 1993). However, in this report, the morphological changes observed in the female Syrian hamster liver in response to the one-month sex steroid treatment are presented and for the purpose of comparison and discussion references to the prolonged treatments (two and three month groups) are made.

Materials and methods

A. Induction of gallstones

A total of 22 nulliparous, F1B strain, female Syrian hamsters (85-90 days old) were purchased from

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BioBreeders (Watertown, MA.). All were housed two per cage and received standard rodent Purina formulab chow 5008 containing 6.5% of fat and 280.0 ppm of cholesterol and water ad libitum; they were kept at 20-21 ºC temperature and under 12 h/12 h dark/light cycles in the AAALAC approved Comparative medicine unit of the college. They were subdivided into the following groups: (1) One-month treatment: 22 hamsters were divided into the following 4 groups:

(a) Control (1C) group: 6 hamsters received one intraperitoneal injection (i.p.) of corn oil (Sigma Chem. Co. St. Louis, MO) every week for a month.

(b) Estrogen (1E) group: 6 hamsters received one weekly i.p. injection of estradiol benzoate (Sigma Chem. Co. St. Louis, MO) dissolved in corn oil (8-10 µg/100 gm body weight [b.w.]) for a one-month period.

(c) Estrogen and Medroxyprogesterone (E+MP) group: 6 hamsters received one weekly i.p. injection of estradiol benzoate dissolved in corn oil (8-10 µg/100 gm b.w.) on day 1 of each week and on day 5 of the same week they were injected intramuscularly (i.m.) with 8-10 mg/100 gm b.w. of medroxyprogesterone acetate (Depo-Provera from Upjohn Co. Kalamazoo, MI) for a one-month period.

(d) Medroxyprogesterone (MP) group: 4 hamsters were administered a weekly i.m. injection of medroxy-progesterone acetate (8-10 mg/100 gm b.w.) for a one-month period.

Two- and three-months treatments with female sex steroids were also studied but this publication will focus on the one-month treatment.

B. Blood samples

At the end of each of the treatments, the hamsters were euthanized with Nembutal (6-7 mg/100 gm b.w. i.p.). Approximately 3-4 ml of blood/hamster was collected by cardiac puncture from the hamsters of each treatment group.

C. Microscopy

Following collection of bile, the gallbladder with a portion of the cystic duct, liver and uterus were excised from each hamster per group and wet weight was recorded. Consequently, the gallbladders, cystic ducts, and pieces of liver were prepared for light and electron microscopical topographical, histo- and cytochemical examination. Gallbladder and liver samples for light microscopy were fixed by 10% neutral buffered formalin and embedded in paraffin. Five to six µm thick sections were prepared and stained using Hematoxylin-Eosin (H&E) and Alcian blue-Periodic acid Schiff (PAS) methods. For the purpose of ultrastructural study, the gallbladder and liver samples were fixed by 3.5% glutaraldehyde buffered with 0.1M sodium cacodylate (pH 7.35) for 15 minutes at room temperature and then for 2 hour at 4 ºC. The liver samples were cut into fine pieces prior to their fixation in glutaraldehyde. The gallbladder samples were split into two parts, and both the gallbladder and liver samples were washed in sucrose cacodylate buffer and fixed by 1% aqueous osmium tetroxide solution for 2 hours at 4 ºC. The washing step using sucrose cacodylate buffer was repeated and followed by dehydration by graded alcohols (30-100%). One half of each gallbladder was prepared for scanning electron microscope (SEM) examination. The other half of each gallbladder and the smaller pieces of liver were processed by propylene oxide and embedded in epoxy resin 812 (Polysciences, Warrington, PA.). After selection of an appropriate area on 1 µm-thick sections stained by Toluidine blue, grey-silver to grey ultrathin sections (65-95 nm in thickness) were cut and collected on 100 mesh copper grids and contrasted by uranyl and lead salts, and were examined at 80 kV in a Jeol JSM 100S transmission electron microscope (TEM). The gallbladder morphological changes have been reported elsewhere, and this report focuses on the morphological changes induced in the liver of the Syrian hamsters.

Results

Light Microscopy

Control (C) group (Fig. 1 row C)

The Syrian hamster liver is composed of four lobes, the right and left dorsocaudal, dorsal median, and the ventral median lobes. The dorsal median lobe is further divided into a larger right dorsal part and a smaller left dorsal part. An umbilical fissure subdivides the ventral median lobe into a right cranioventral part and lateral left cranioventral part (Nettleblad, 1954). The liver is covered by a thin connective tissue capsule and exhibits hepatic lobules, which like in the humans have sparse amount of perilobular connective tissue that does not form a continuous boundary between adjacent lobules and thus making it difficult to discern the limits of the various lobules. Each lobular unit is distinguished by the presence of a branch of the hepatic vein in its center (central vein) and peripherally located portal spaces of Kiernan (also named portal triads). The portal triads are present in the interlobular spaces and are each typically composed of at least an arteriole (hepatic artery branch), a venule (hepatic portal vein branch), and a bile duct with an occasional lymphatic embedded in a connective tissue sheath. The bile duct is lined by simple cuboidal epithelium.

In the Syrian hamster, hepatocytes are polyhedral in outline and are usually arranged in one-cell thick plates which run radially between the central veins and the portal triads. Located in the spaces between the cell plates we notice that the liver sinusoids are principally lined by discontinuous endothelial cells and subjacent Kupffer cells (darkly stained). The hepatocytes demonstrate spherical and centrally located nuclei with prominent nucleoli and scattered clumps of hetero-

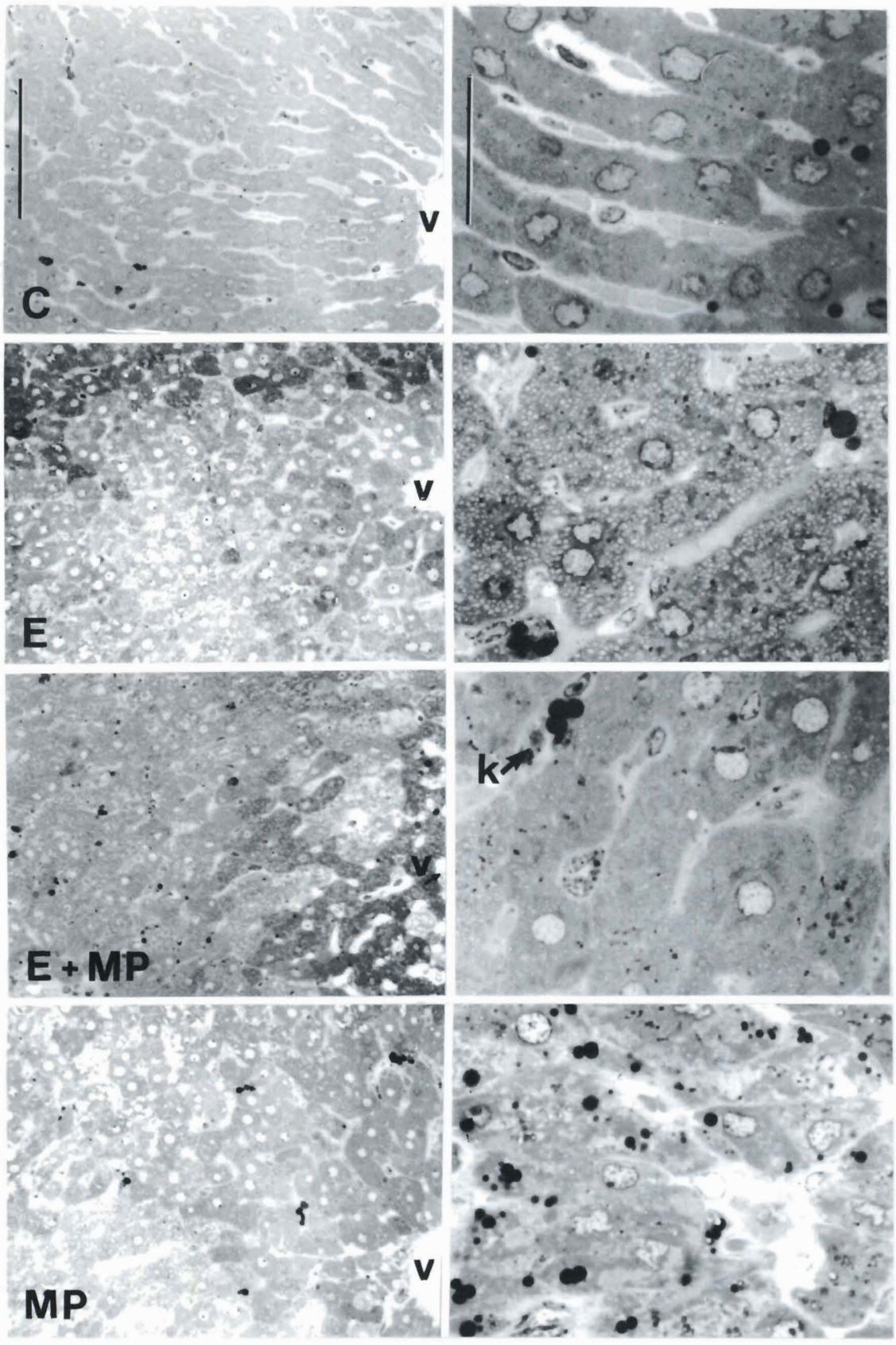


Fig. 1. Light microscopic montage of Toluidine-blue stained semi-thin section micrographs of one-month C (row C), E (row E), E+MP (row E+MP), and MP (row MP) treated livers. k: Küpffer cell; v: central vein. Scales are the same for each illustration of each column. Bar scale in right column= 100 µm; Bar scale in left column= 25 µm.

chromatin. It is not uncommon to find binucleated cells in the hamster liver. Following Toluidine blue staining, the cytoplasm of the parenchymal cells appears faintly granular, displaying densely stained granules as well as metachromatic inclusions which are probably of lysosomal origin. Dalcq (1963) reported that with Toluidine blue staining, lysosomes exhibited metachromasia, which was attributed to the presence of acid mucopolysaccharides in these organelles.

Estrogen-treated (E) group (Fig. 1 row E)

In response to estrogen treatment, the parenchymal architecture remained intact. The hepatocytes displayed enlarged euchromatic nuclei as compared to the C group. Nuclear indentations were commonly observed in this treatment group. An increased number of granules, and mitochondria were detected in the cytosol of the hepatocytes. The bile duct did not appear to demonstrate any specific morphological changes as compared to the C group.

Estrogen + Medroxyprogesterone-treated (E+MP) group (Fig. 1 row E+MP)

The changes observed in response to both sex steroids in combination were exacerbated as compared to those observed following treatment with estrogen alone. These included increase in cytoplasmic granulation/deposits, nuclear size and nuclear indentations. Administration of medroxyprogesterone in combination with estrogen appears to induce fatty changes, which were evident from the lipid droplets detected in the cytoplasm of the hepatocytes and some of which coalesced to form larger droplets. A predominantly striking feature of this treatment group was the disruption of the liver parenchymal architecture with a loss of the radial arrangement of cellular plates and dilatation of the liver sinusoids. Examination of the portal space revealed that some of the cuboidal cells lining the bile ducts demonstrated apical bulging in response to E+MP treatment. Prolonged duration of treatment appeared to accentuate all the above findings (three-month > two-month > one-month).

Medroxyprogesterone-treated (MP) group (Fig. 1 row MP)

The most striking feature of this treatment group was the occurrence of large, dense inclusions and abundant granules in the cytoplasm of the hepatocytes. Disruption of parenchymal architecture and sinusoidal dilatation observed following treatment with medroxyprogesterone alone was accentuated as compared to that noted in the E+MP group (Fig. 1 row E+MP and row MP). In addition to apical bulging, the epithelial cells lining the bile ducts exhibited cellular excrescences in the lumen.

Ultrastructural examination of liver

Control (C) group (Fig. 2)

Pale and dark hepatocytes were the predominant cell types in the hamster liver and formed cords or plates of cells which were one-cell thick (Fig. 2 A). The hepatocytes appeared polyhedral and multifaceted cells with diameters ranging between 18 to 20 µm. The plasma membrane of each hepatocyte revealed four distinct regions sinusoidal, lateral, junctional, and canalicular. Along the sinusoidal aspect, microvilli were present, whereas the lateral cell surface was devoid of them. A perisinusoidal space (space of Disse) was seen between the plasma membranes of the hepatocyte sinusoidal surface and the endothelial cells (Fig. 2 A). Bile canaliculi, the first and smallest passages of the bile duct system are gulf-like distensions of the intercellular space formed by the plasma membranes of contiguous hepatocytes to which the bile secreted by the hepatocytes is delivered.

The nucleus of the parenchymal cell, spherical to oval in shape and 8.0 to 8.5 µm in diameter was usually centrally located. Hepatocyte nuclei were euchromatic with scattered patches of heterochromatin, some of which were detected peripherally along the nuclear envelope. Organelles such as mitochondria, Golgi apparatus, rough and smooth endoplasmic reticulum, peroxisomes, and lysosomes in addition to microfilaments and intermediate filaments were clearly visible in the hepatocyte cytosol (Fig. 2 A-B). Glycogen and hemosiderin granules, and lipofuscin pigments were some of the commonly detected cytoplasmic inclusions in the C group hepatocytes.

Mitochondria (0.8-0.9 µm in diameter and 1-1.5 µm in length) were always the most abundant organelles in the liver cells and appeared round to oblong in shape. The mitochondrial matrix was moderately electron dense and the cristae appeared lamellar and widely spaced (Fig. 2 B). In the hepatocytes, the mitochondria were seen in close proximity to rough endoplasmic reticulum (RER) and peroxisomes. The hamster hepatocytes showed abundant RER as compared to the smooth surfaced endoplasmic reticulum (SER). The RER appeared to be dispersed in the cytoplasm, but was also observed to be arranged concentrically around the nucleus, and the cisternae typically appeared to be adjacent to the mitochondria, whereas the tubular SER networks were preferentially located in the peripheral areas of the hepatocyte and in the vicinity of glycogen granules. Each hepatocyte contained Golgi complexes which were abundant near the bile canaliculi and the perinuclear region. Peroxisomes, 0.4 to 0.5 µm in diameter appeared as round, single, membraned organelles with homogenous, granular, and electron dense matrices. Matrical rod-shaped crystalloid inclusions were characteristic of hamster peroxisomes (Fig. 2 B). Lysosomes of the hepatocytes were ovoid, electron dense structures

bound by single membranes, located in the perinuclear region and varied in size (0.5 to 1.0 µm), as seen in Figure 2 A. Lipofuscin granules represent a form of lysosomes which contain electron dense granules and lipid droplets, and were typically localized in the pericanalicular region. The cytoplasm of the hepatocytes showed dispersed α-glycogen aggregates, some of which appeared to be closely associated with elements of the SER.

Stellate cells (of von Küpffer, lipocytes, Ito cells, vitamin A-fat storing cells) were approximately 6.0 to 8.0 µm in diameter and were detected in the perisinusoidal spaces (Fig. 2 A). The nuclei of these perisinusoidal lipocytes varied in shape, however the cytoplasm showed a few, large lipid droplets and very few organelles were visible.

Kupffer cells are phagocytic cells (4 to 6 µm in width) present in the sinusoidal space (not illustrated

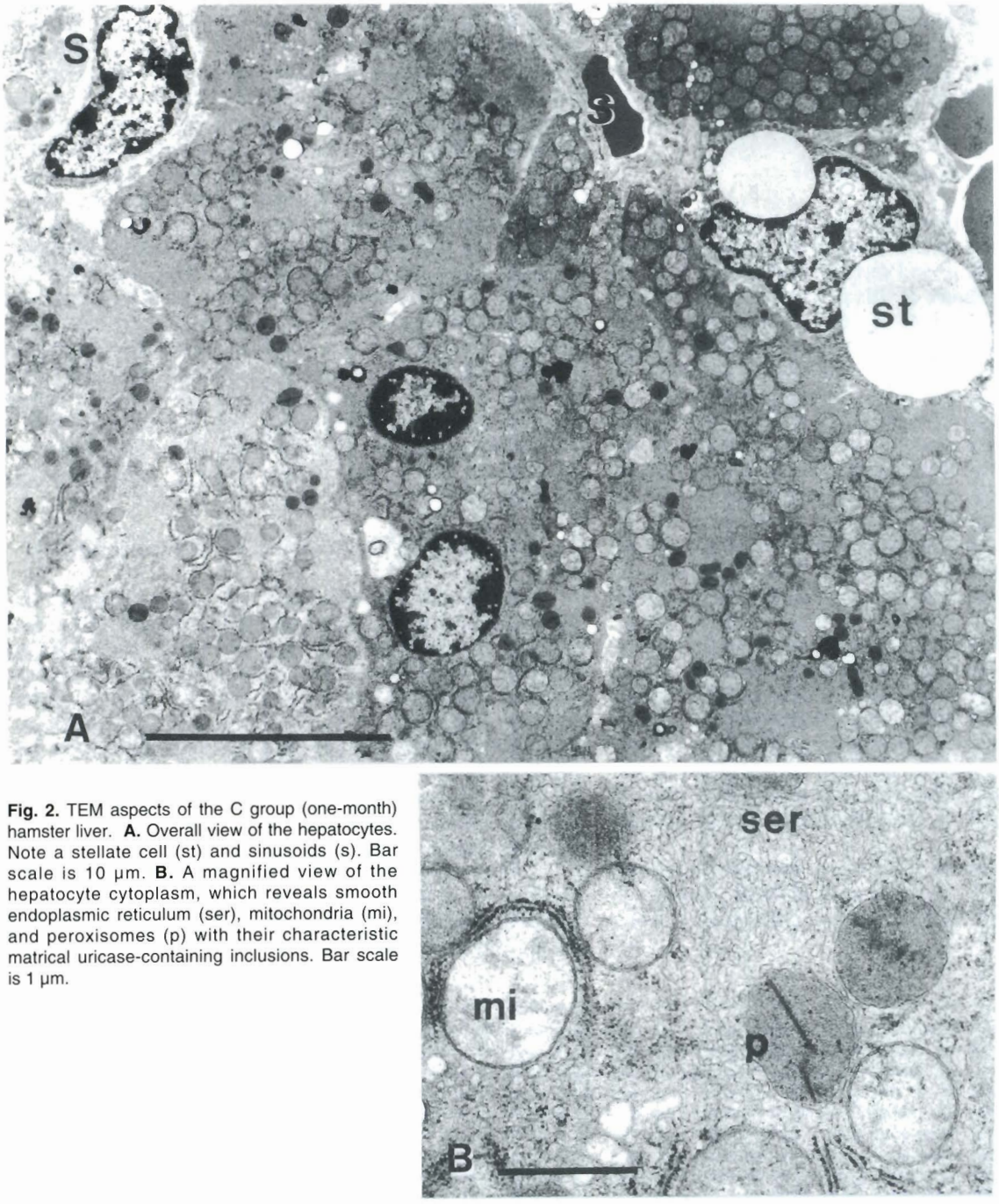


Fig. 2. TEM aspects of the C group (one-month) hamster liver. **A.** Overall view of the hepatocytes. Note a stellate cell (st) and sinusoids (s). Bar scale is 10 µm. **B.** A magnified view of the hepatocyte cytoplasm, which reveals smooth endoplasmic reticulum (ser), mitochondria (mi), and peroxisomes (p) with their characteristic matrical uricase-containing inclusions. Bar scale is 1 µm.

here, as the focus is on the hepatocyte). Cytoplasmic projections or pseudopodia contribute to the irregular shape of these hepatic macrophages and are consistent with their phagocytic functions. The cytoplasm of the Kupffer cells revealed many pleomorphic lysosomes and phagosomal inclusions.

The portal space showed a triad composed of branches of the hepatic artery, portal vein, and bile duct. Cuboidal epithelial cells with large nuclei, and scanty cytoplasmic organelles lined the portal bile ducts. Microvilli of varying heights (0.5 to 0.8 µm), with an occasional cilium were seen extending from the apical surface of the biliary epithelial cells. Cohesion of adjacent epithelial cells by lateral membranous interdigitations and desmosomal junctions were visible near the apical ends.

Estrogen-treated (E) group (Fig. 3)

In the one-month treatment group, the nuclei appeared indented and a few showed prominent nucleoli, nucleolonema, and nuclear pores (Fig. 3 A, B and G). A striking feature of this group was the presence of lipid droplets (Fig. 3 A, B, and G) and aggregates of fine granular particles in the hepato-cyte nuclei (Fig. 3 B and E). In this group the hepatocytes showed mitochondria of diverse sizes and shapes, some of them assuming giant forms (3.5-4.0 µm in length and 1.2-1.6 µm in diameter), as seen in Figure 3 A, F and G. These mitochondria displayed fewer cristae than the mitochondria of the C group hepatocytes. In response to E treatment an increase in the amount of SER, dense bodies, and lipofuscin granules was observed (Fig. 3 C, D and F). The aggregates of the smooth endoplasmic tubules appeared to be enclosed by pockets of mitochondria (Fig. 3 B and C). Cytoplasmic inclusions in the form of membrane-bound vacuoles enclosing concentric electron dense lamellar structures or empty spaces were detected (Fig. 3 C and D). Occasionally, these lamellar structures were seen without any membranous envelopes, but instead appeared to be closely associated with glycogen aggregates in the perinuclear region. Figure 3 E shows peroxisomes in close proximity of the nuclear envelope.

Prolonged estrogen treatment (two and three-months) induced accentuated vesiculization of SER, abundant dense inclusions and fatty changes as compared to one-month E group.

Estrogen + Medroxyprogesterone-treated (E+MP) group (Fig. 4 and 5)

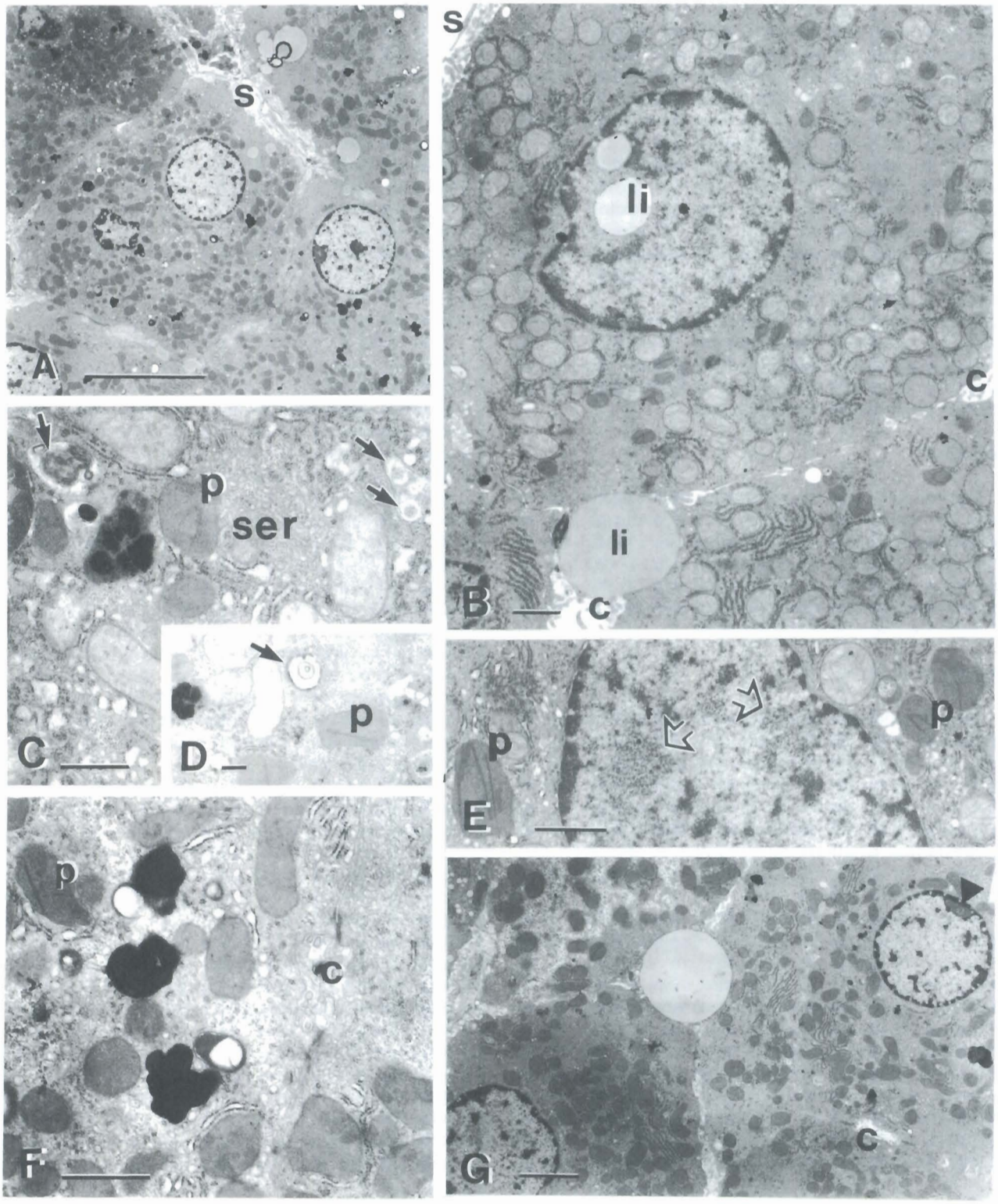
Similar to the observation in the E group, nuclear indentations were observed following one-month E+MP treatment (Fig. 4 B and C). The mitochondria of the liver parenchymal cells appeared pleomorphic and attained variable sizes (Fig. 4 C and D). Fewer cristae were visible in the mitochondrial matrices as compared to the C group. In response to estrogen and medroxyprogesterone combination treatment, marked vesiculization and dilatation of the SER was detected as compared to the E-treated group (Fig. 4 B, C, E, and F). Electron dense inclusions and lipid droplets were increased in number in the cytoplasm of the E+MP-treated hepatic cells as compared to the E-treated hepatocytes, as seen in Figures 4 B-D and 5. These inclusions and lipid droplets aggregated to form larger heterogenous bodies which were often visible in the canalicular lumen (Fig. 4 C). Along the pericanalicular region, in addition to dense inclusions, vacuoles with concentric lamellar inclusions, and glycogen rosettes were observed (Fig. 4 E, F, G, and I). Some of these lamellar inclusions are also detected in the space of Disse (Fig. 4 H and I). Due to the coalescing of the hepatocyte lipid droplets, and their superficial localization, it is difficult to delineate the hepatocyte boundaries as is exemplified in Figure 5. These aggregates of lipid deposits could also represent a segment of the fat storing (Ito) cells adjacent to a sinusoid space and apposed to a neighbouring hepatocyte.

Medroxyprogesterone-treated (MP) group (Fig. 6)

In response to medroxyprogesterone treatment the parenchymal architecture appeared disrupted. The nuclei of medroxyprogesterone-treated hepatocytes showed increased condensation of heterochromatin along the nuclear envelopes, which enhanced the dilated nuclear pores (Fig. 6 A). Administration of medroxy-progesterone induces the most striking increase in the SER of the liver cells among all the treatment groups (Fig. 6 A). In addition, the hepatic SER appeared hypertrophic and closely associated with glycogen granules and intermediate filaments (Fig. 6 B).

The mitochondria appeared pleomorphic and enlarged (2.0-2.5 µm in length and 1.0-1.2 µm in diameter) exhibiting highly electron dense matrices (Fig. 6 A). An increase in the number, size (0.9-1.0 µm in

Fig. 3. TEM micrographs of E-treated (one-month) hamster liver. **A.** An overall view of the hepatocytes is presented (s: sinusoid). Small to large lipid droplets can be observed in the peripheral areas of the hepatocyte cytoplasm. **B.** Lipid droplets (li) in the nucleus and an example of cytoplasmic lipid vacuole is visible. c: bile canaliculus; s: sinusoid. **C.** Example of segregated islets of proliferated smooth endoplasmic reticulum (ser), among mitochondria and peroxisomes (p). Arrows indicate the whorl-like inclusions observed in the cytoplasm in this treatment group. **D.** An example of a concetric lamellar cytoplasmic inclusion is shown (arrow) adjacent to a peroxisome (p). **E.** An example of an euchromatic nucleus demonstrates aggregates of fine granular material (opened arrows). Note the perinuclear peroxisomes (p). **F.** Lipofuscin-like (dense) bodies were noticed in the cytoplasm as well as in the pericanalicular region. c: bile canaliculus. **G.** Examples of hepatocytes exhibiting mitochondria with electron dense matrices (similar to those observed in A and F). Compare these mitochondrial profiles with those detected in B to E. Arrowhead highlights the nuclear pores contrasted with the peripheral ring of heterochromatin. Bar scale in A is 10 µm and in B to G is 1 µm.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diameter), and matrical density of the hepatic peroxisomes was observed following treatment with medroxyprogesterone. Most of these peroxisomes appeared to be distributed diffusely or in small clusters in the cytoplasm. Numerous electron dense inclusions and lipid droplets occurring individually or in aggregates were detected in the cytoplasm (Fig. 6 A, C, and E). Some of the lipid droplets revealed membranous whorl-like inclusions (Fig. 6 C), similar membranous inclusions were also visible in the lumen of the congested bile canaliculi (Fig. 6 D-F). It was interesting to note that tight junctions of the bile canaliculi appeared to have disrupted, thereby resulting in elongation of the canaliculi (Fig. 6 A). In Figure 6 G, two pale hepatocytes are observed. Figure 6 H is an enlarged view of the cytoplasm of these altered hepatocytes. An increase in glycogen rosettes and an apparent disarray of bundles of intermediate filaments were striking features of these unique hepatocytes (Fig. 6 H).

Discussion

The primary objective of this research project was to study the role of sex steroids in the pathogenesis of cholelithiasis using the Syrian hamster as a model. Many of the prior studies conducted in this area which have used other animal models, have focused on the role of the diet and the subsequent biochemical changes which influence the formation of gallstones.

Estrogen and Liver

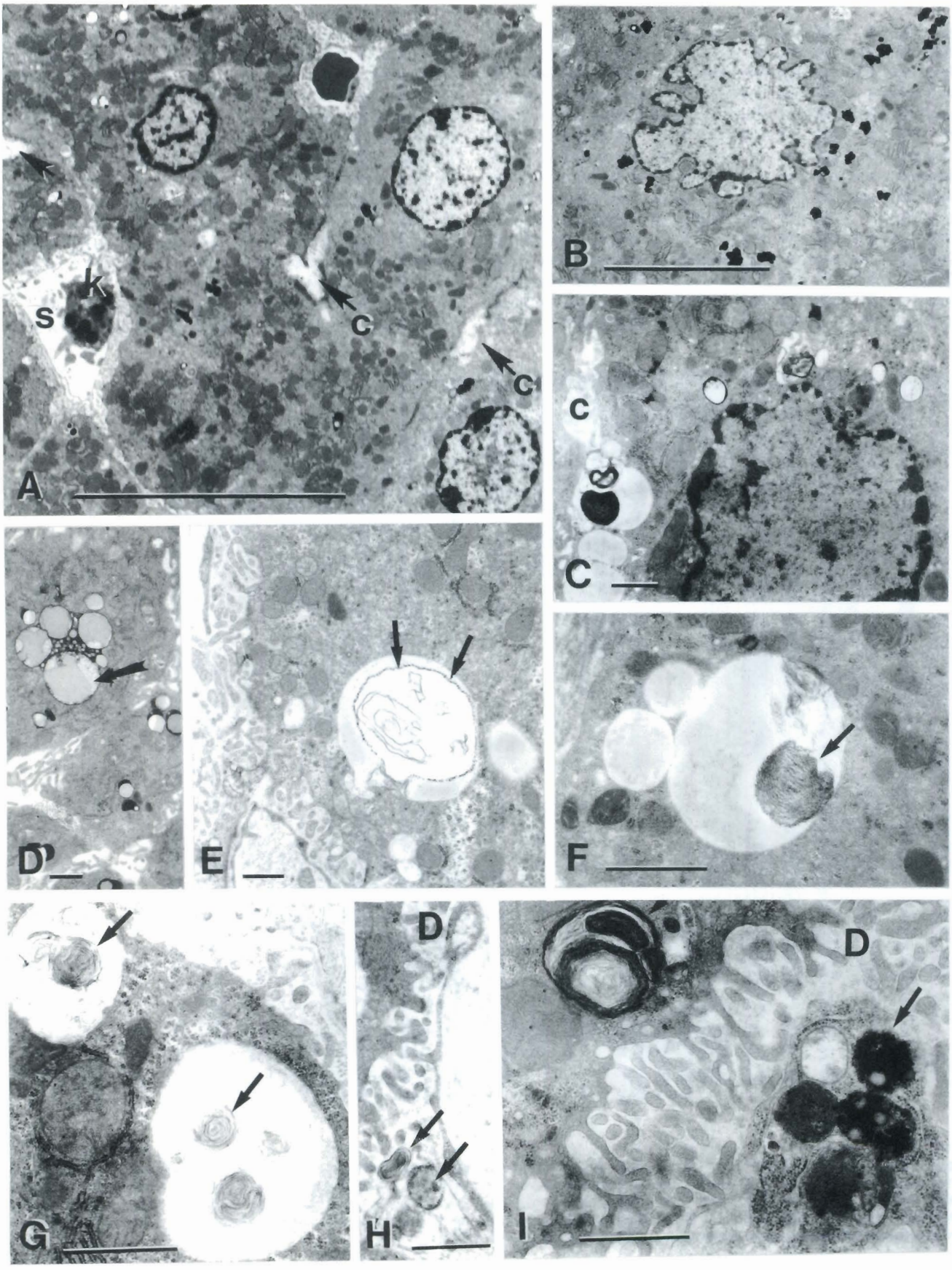
Specific estrogen receptors have been detected in the mammalian liver (Duffy and Duffy, 1976; Aten et al., 1978). The liver appears to be an unique target organ, in that the steroids administered are rapidly metabolized, processed, and excreted by this organ as sulpho- and glucoro-conjugates. Exogenous sex steroids interact with the substances routinely metabolized by the liver, and this interaction exacerbates the stress on the hepatocytes (von Schultz et al., 1989).

From the report of von Schultz et al. (1989), it is important to note that the effects of exogenous estrogens on liver metabolism depend on the route of administration and the type and dose of estrogen. Following oral administration of estrogen, at equilibrium the estrogen concentrations in the liver was five times greater than in the peripheral target organs. However, there was no difference in the estrogen concentrations in the liver and peripheral target organs following parenteral administration of estrogens (de Liguières and Basdevant, 1987). While oral administration of estrogens has been known to have effects on liver-derived plasma proteins, lipoproteins, and triglycerides, parenteral estrogen administration has practically little or no effect on these liver functions (Elkik et al., 1982; Fahreaus and Wallentin, 1983). Modern oral contraceptive preparations contain ethinyl estradiol in a dose of less than 50 µg. The ethinyl group of the ethinyl estradiol affects the drug metabolizing activity of the microsomal enzyme 17ß-dehydrogenase (O'Malley et al., 1972). Thus, the synthetic estrogens would be far more potent as compared to naturally occurring estradiol. The synthetic progestogens in the oral contraceptive preparations are structurally similar to testosterone and estrogen, as opposed to progesterones (Dickey and Stone, 1976), and in addition to their progestional activity, they also possess androgenic properties.

In a study carried out by Manautou-Martinez et al. (1970), it is reported that liver cells of women on oral contraceptive therapy exhibit modifications of the cell organelles, especially the smooth endoplasmic reticulum and mitochondria. These changes appeared to be more prominent, when the oral contraceptive contained estrogen. Dilatation and vesiculization of the SER and crystalloid inclusions in the mitochondrial matrices were the significant changes observed by Manautou-Martinez et al. (1970). Perez et al. (1969) reported increases in the amount of SER, and alterations in the shape, increases in the size of mitochondria, and presence of paracrystalline inclusions in the mitochondrial matrices of the hepatocytes. An increase in the number of peribiliary dense bodies and lipofuscin droplets in the hepatocytes were also observed by Perez et al. (1969). Several investigators have reported that the epithelial cell lysosomes which were depleted in the spayed animals. were restored with estrogen treatment (Nilsson 1962; Manning et al., 1967).

Following E treatment, the hamster liver cells demonstrated similar changes of the cell organelles as those reported by Perez et al. (1969) and Manautou-Martinez et al. (1970). Hypertrophy of the SER and alterations in the shape and size of the mitochondria were also observed in this study. The mitochondria appeared swollen and the cristae appeared dilated. Increased number of dense bodies and lipofuscin granules were detected in the hepatocyte cytosol. Lipid droplets were detected in the nuclei of the estrogen-treated hamster hepatocytes. Ghadially (1988) mentions that intranuclear lipid inclusions are possibly the commonest forms of intranuclear inclusions and that they tend to occur in conditions when there is an increase in the cytoplasmic lipid droplets. Prolonged E treatment (two and three- month) not only induced exacerbation of these changes, but also induced fatty changes in the liver. Similar to the observations in the

Fig. 4. TEM micrographs of the E+MP-treated (one month) hamster liver. **A-C.** Overall aspects of hepatocytes showing altered nucleus morphology, including chromatin and nucleoli patterns. Pleomorphic mitochondria and lipofuscin bodies can be detected. Examples of bile canaliculi (c), sinusoids (s), and Küpffer cell (k). **D-I.** Examples of different types of vacuolated structures detected in the periphery of the hepatocytes. Arrow in D illustrates a foamy (lysosomal) body. In E-G, arrows point to suggestive stages of formation of electron dense whorl-like inclusions. The space of Disse (D) shows whorls, which could suggest their apparent release from the hepatocytes in H-I. Bar scale in A is 10 µm; B is 5 µm; and C to I is 1 µm.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gallbladder epithelial cells (Karkare, 1993), the mitochondria in response to sex steroid treatments demonstrated vacuolated matrices with multilamellar inclusions. Larsson-Cohn (1965) has also reported similar mitochondrial alterations which were manifested by enlargement, parallel arrays of osmiophilic membranes and myelin figures in the mitochondrial matrices. Mitochondrial alterations and inclusions have been detected in a variety of hepatic and non-hepatic conditions (Svoboda and Manning, 1964; Sternleib, 1968; Bhagwat and Ross, 1971). Although these alterations have also been reported in normal liver cells (Willis, 1965), C groups did not exhibit these changes, only the sex steroid treated hamster livers demonstrated these alterations. Burns et al. (1972) indicated that this is suggestive that the mitochondrial deformities could possibly occur as a result of hepatocellular injury and reorganization of the mitochondrial membrane lipoproteins into these inclusions. Reynolds (1965) indicated that calcium or iron could be associated with these reorganized membrane lipoproteins, resulting in the electron dense nature of these inclusions. Preliminary X-ray microanalysis of E-treated livers detected relatively higher levels of iron as compared to the C, E+MP, and P-treated livers (not shown in this report). This finding appeared to complement the suggestion that iron could be associated with the formation of the electron dense mitochondrial inclusions.

The morphological changes observed in the SER confirmed that this organelle plays a significant role in

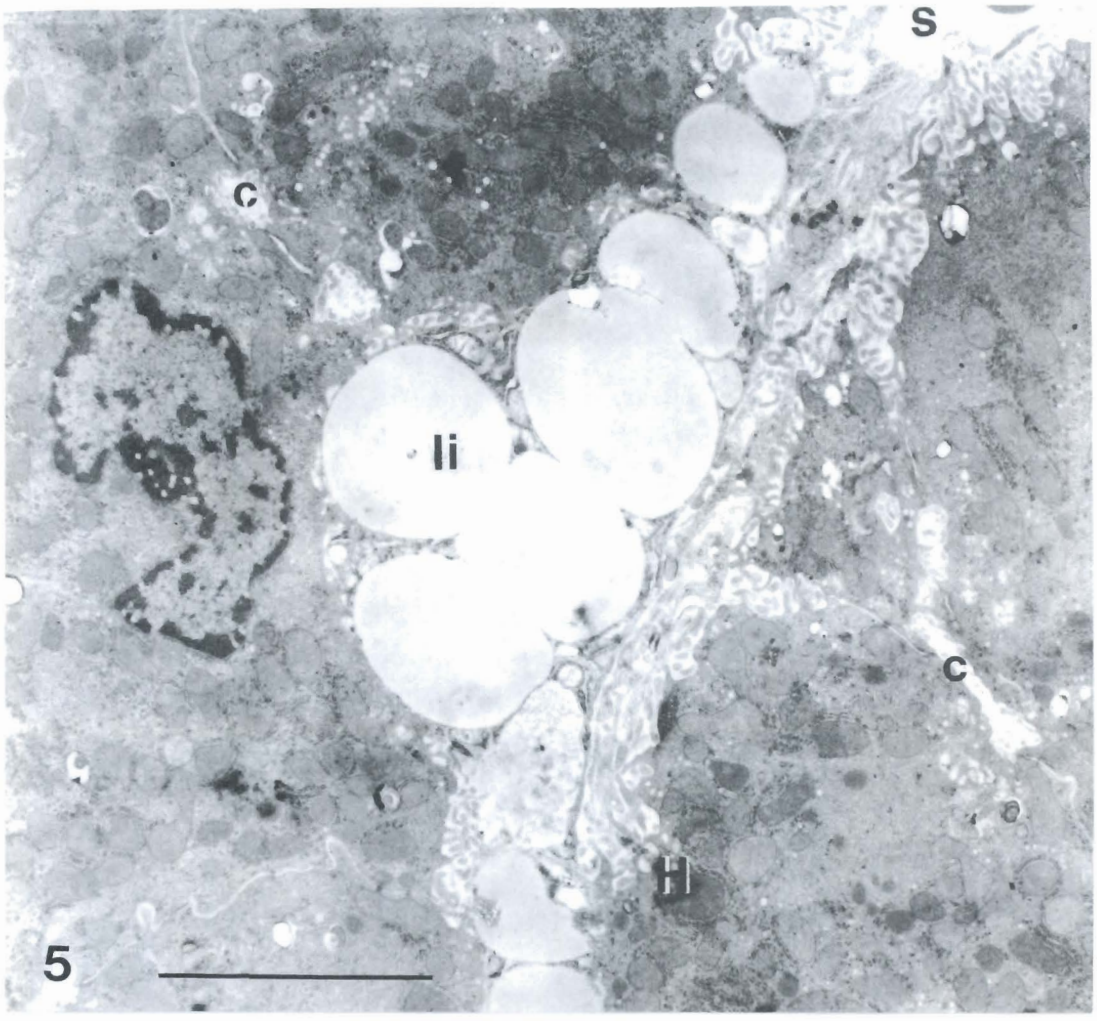
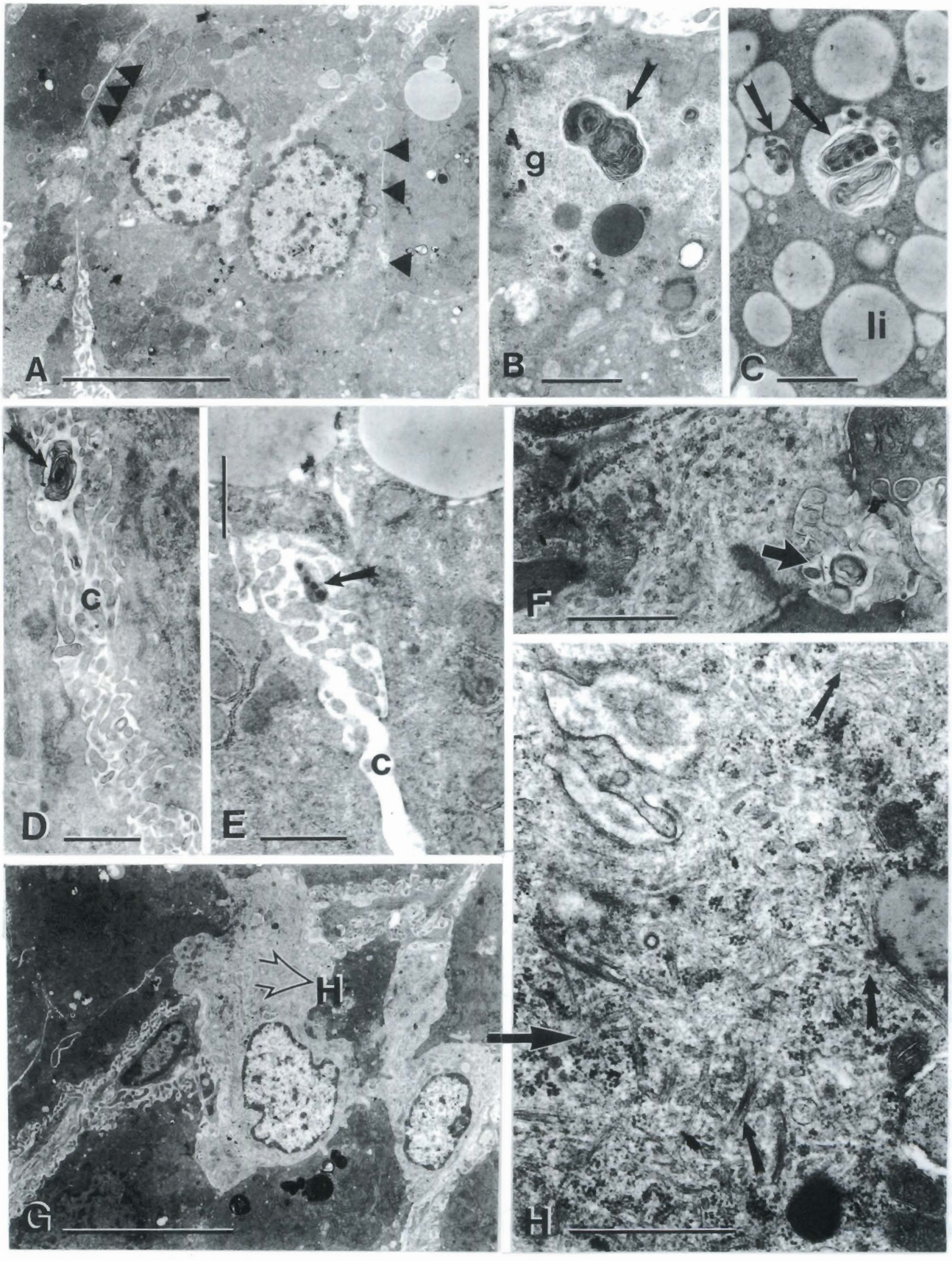


Fig. 5. TEM micrograph of E+MP-treated hamster hepatocytes. Peripheral induction, accumulation, and aggregation of lipid droplets (li); c: bile canaliculus; H: hepatocyte; s: sinusoid. Bar scale is 5 µm.

Fig. 6. TEM micrographs of MP-treated hamster liver. **A.** Overall view of hepatocytes illustrating fatty changes and elongation of the bile canaliculi (arrowheads) in response to MP treatment. **B-F.** Arrows demonstrate intracellular (B and C) and apparently released (D-F) membranous whorl-like structures and their associated densities. c: bile canaliculus; g: glycogen; li: lipid droplets. **G-H.** Micrograph G shows an example of a focal area exhibiting metaplastic hepatocytes. H is a magnified view of the hepatocytes in G showing cytoplasmic disarray of intermediate filaments (small arrows), ribosomes, and glycogen rosettes. Bar scale in A is 10 µm; in B-G is 1 µm.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the metabolism of drugs and that the hypertrophy of this organelle occurred in order to enable the liver to metabolize the exogenous lipid soluble substances efficiently (Jones and Fawcett, 1966). The report by O'Malley et al. (1972) suggests that estrogens influence the bile formation and composition by altering the hepatic membrane lipids and thus affecting the transport of organic anions. Ethinyl estradiol reduces both bile acid dependent and bile acid independent flows (DelPino et al., 1976). The action on the bile acid independent flow may be attributed to the reduced activity of the Na-K-ATPase enzyme, which is an ethinyl estradiol effect (Keefe et al., 1979).

Bonorris et al. (1977) have reported that the activity of the cholesterol 7ß-hydroxylase enzyme, which catalyzes the rate-limiting step in the synthesis of bile acid from cholesterol, is decreased by ethinyl estradiol. In addition, estrogen increases the activity of the cholesterol esterifying enzyme, cholesterol acyl CoA transferase (ACAT), and this further reduces the amount of cholesterol available for bile acid synthesis (Davis et al., 1978).

The estrogen + medroxyprogesterone effect on liver

The histopathological changes induced in the liver following the treatment with contraceptive steroids, are primarily due to the estrogenic components. Progestogens affect the liver to a smaller extent, in fact it has been reported by Nervi et al. (1983), that they oppose the estrogenic effect on the ACAT enzyme and favor bile acid synthesis. It is well known that progesterone, which is an inhibitor of smooth muscle contractility, reduces gallbladder and gastrointestinal motility (Ryan and Pellachia, 1982; Radberg et al., 1989).

In response to estrogen and medroxyprogesterone combination treatment (E+MP), the hamster livers exhibited changes similar to those detected in the E groups. However, these changes appeared accentuated and in addition to hypertrophic SER, mitochondrial alterations, and accumulation of dense bodies, predominant fatty changes were induced. Further, the multilamellar mitochondrial inclusions and dense lamellar bodies were detected in the bile canalicular lumens. The presence of these whorl-like structures and electron dense bodies in the canalicular lumens was indicative of their expulsion by the hepatocytes into the bile. These along with the contributions of the bile duct, cystic duct, and gallbladder epithelial cells could facilitate the pathogenesis of gallstones. Energy dispersive X-ray microanalysis of the E+MP-treated livers detected high levels of calcium, this evidence was supportive of the association of calcium with the mitochondrial inclusions. The largest number and size of gallstone-like deposits were observed in the E+MP-treated gallbladder lumens, and preliminary observations obtained with X-ray microanalysis of crystal-like deposits revealed that among all the elements analyzed (Cl, P, S, Fe, etc) approximately 70% was calcium. Thus, providing a possible confirmation of the assumption that these expelled mitochondrial inclusions and dense bodies could be involved in the formation of gallstone-like deposits. Sanchez-Bueno et al. (1991) observed an increase in the cytosolic calcium in the rat hepatocytes following injections of progesterone and estradiol. Calcium ions have been known to cause mitochondrial swelling (Chappel and Crofts, 1965). These reports could explain the swollen mitochondria observed in the hamster livers following sex steroid treatment. Increased accumulation of glycogen rosettes was detected in the hepatocytes following E+MP treatment.

The MP-treated livers demonstrated disruption of normal liver architecture, dilatation of sinusoids, and abundant lipid droplets. Similar to the E+MP-treated groups, dense bodies and whorl-like structures were expelled into the bile canaliculi. Focal areas of pale hepatocytes with clear cytoplasm and abundant glycogen and fatty changes, suggestive of hepatocellular metaplasia leading to adenoma formation were observed in the MP-treated livers. Hepatocellular adenomatous changes have been known to develop in women on long term oral contraceptives (Fechner, 1977; Klatskin, 1977). An interesting feature detected during the TEM examination of the MP-treated hamster livers was the disruption of the bile canaliculi tight junctions, resulting in elongated and dilated bile canaliculi. Increased permeability of the rat hepatocellular tight junctions in response to ethinyl estradiol has also been reported previously (Rahner and Landmann, 1992). These alterations could result in cholestasis and subsequent hepatocellular damage.

The morphological changes observed in the liver in response to the sex steroid treatments are suggestive of alterations in the hepatic functions and metabolism. Contraceptive steroids induce the formation of supersaturated bile, decrease bile acid synthesis from cholesterol, and cause the gallbladder to empty slowly. It is possible that the bile content is altered by the contributions at the level of the hepatocytes and bile duct cells induced by the treatments. As a result all these changes favor the formation of gallstones.

In previous reports we have presented the morphological and histochemical studies on the hamster gallbladder in response to sex steroids (Karkare et al., 1995; Karkare and Gilloteaux, 1995). Thus, in order to put all the factors involved in the cholelithiatic process in perspective, as well as understand the connection between contraceptive steroids and the pathogenesis of gallstones, it was imperative to study the ultrastructural changes in the Syrian hamster liver as well.

It has been noted previously that the Syrian hamster exhibits similar gallbladder surface epithelial morphology (no goblet cells in body and fundic regions), bile composition (Andersen et al., 1972), lipid metabolism (Kuroki et al., 1983; Andersen and Cook, 1986; Berr et al., 1992), and receptors for female sex steroids which are not only present in the musculature

(Fridhandler and Shaffer, 1983; Daignault et al., 1988) but also at the epithelial sites (Yamamoto et al., 1990) as in the humans. Interestingly, the morphologic data obtained from this study demonstrates that the microscopic anatomy of the Syrian hamster liver closely resembles that of the human liver. The morphological and biochemical similarities in the gallbladder and biliary tract of the Syrian hamster and the humans, further justifies the use of the the Syrian hamster as a model to understand the cholelithiatic process in the humans.

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