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Effect of Ursodeoxycholic Acid on Experimental Hepatic Porphyria Induced by Griseofulvin

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Griseofulvin(GF) has become the drug of choice as an antifungal agent for patients who suffer from many kinds of fungal infection. In order to clarify hepatic injury by griseofulvin(GF) overload and the effect of UDCA on GF-induced hepatic injury, the authors carried out biochemical, histologic, and ultrastructural studies of liver following treatment with griseofulvin and ursodeoxycholic acid(UDCA) in mice. Urine porphobilinogen excretion in the group treated with GF alone was significantly increased and reached the highest level in the 4th week and declined thereafter. Biochemical studies of the liver function showed no remarkable changes of serum bilirubin levels throughout the experimental period in all groups, except for SGPT and alkaline phosphatase activities which were significantly elevated and reached the highest level in the second week. Then they slightly decreased in GF treated groups(GF alone and GF plus UDCA) in comparison with the control group. Pathologic findings in the group treated with GF alone include focal liver cell necrosis(esp, zone 3), Mallory bodies in hepatocytes(esp, zone 1), Kupffer cell activation, and brown protoporphyrin pigments in the hepatocytes, bile canaliculi and interlobular bile ducts with a marked inflammatory cell infiltration in the portal tracts. Under the polarizing light microscope, bile ductular and canalicular thrombi showed a "Maltese cross" birefringence in mice treated with GF alone. There is no definite finding of fatty change in hepatocyte. Under the microscope, the liver appeared normal with an intact lobular architecture in the GF plus UDCA treated group. Electron microscopically, GF-induced changes include swelling of mitochondria, globular protoporphyrin crystals in the hepatocyte cytoplasm, markedly dilated bile cannaliculi and bile ducts, and the formation of a Mallory hyaline bodies in the hepatocytes. There were no noticable structural changes in the GF plus UDCA-treated group. Therefore the results suggest that GF causes hepatic injury, namely porphyria and cholestasis, and the

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treatment of UDCA may have cytoprotective and choleretic effects on GF-induced hepatic injuries.

Key Words : *Griseofulvin, Hepatic porphyria, Ursodeoxycholic acid.*

INTRODUCTION

Most drugs are metabolized in the liver, causing hepatic damage in many cases. Some authors report that grisepfulvin(GF), an antifungal agent, induces serious hepatic damage (cholestasis, hepatic necrosis, liver cirrhosis) because of abnormal heme metabolism and hepatic porphyria caused by this drug(Adler et al., 1979; Franke et al., 1979).

It was suggested that GF disturbed the activation of hepatic ferrochelatase in mitochondria, which might lead to hepatic prophyria, hepatic damage, hepatomegaly, liver cirrhosis by biliary excretion abnormalities due to porphyrin accumulation in the hepatocytes(Barnes et al, 1968; Donaldson et al., 1971). Recently, it was suggested that ursodeoxycholic acid(UDCA), which activated bile acid independent bile excretion, might be available for prevention and treatment of cholesterol stone formation and primary biliary cirrhosis(Leuschner et al., 1987).

The aim of the present study was to investigate the hepatic damage induced by long-term administration of GF alon and the effect of UDCA on GF-induced hepatic injuries in view of hepatocytic morphology and functions.

MATERIALS AND METHODS

Subjects

In this experiment, 120 randomly-bred ICR mice 20-25gm each were fed a powdered diet. The antibiotic griseofulvin(Fulvicin, Hyun Dai Pharmacy Co., Korea) was administered in a 2.5% concentration and UDCA(Dae Woong Pharmacy Co., Korea) in a 1.0% concentration.

Materials

As follows, the experimental mice were divided into 3 groups, and each group contained 40 mice. Five mice were sacrificed every week in each group. The mice that expired during the experimental period were excluded, and total length of the period was 8 weeks.

Group 1 is the mice fed with control diet, Group 2 is the mice treated with GF alone. Group 3 is the mice treated with GF and UDCA.

Sampling

Five mice were sacrificed weekly. Before sacrifice, each mouse was weighed, and about 2 ml of blood was obtained through the abdominal aorta to measure serum bilirubin, SGPT, and alkaline phosphatase(ALP) values. During sacrifice, the common bile duct and macroscopic changes of the liver were observed and the liver weight was measured.

For quantitative analysis of urine porphobilinogen(PBG), a 24-hour urine collection from 10:00 a.m. to the same time the next day was performed. Acetic acid was mixed with the collected urine specimen in a 10:1 concentration, and after elimination of blunt tint, the same amount of Ehrich's solution was added to the specimen.

The PBG was quantitatively measured by optical density under 555.5μm colorimeter after 15 minutes(Davis et al., 1967).

Tissue preparation

After sacrifice, the obtained liver tissues were observed under light and electron microscopes.

1) Light microscope preparation. Specimens were fixed in a 10% formalin solution and processed in the usual manner. Paraffin sections were stained with hematoxylin and eosin and examined under light microscope.

2) Electron microsc pe preparation. Each liver specimen was sectioned in 1mm × 1mm size and put into 2% glutaraldehyde(phosphate buffer, pH 7.2), then fixed in 4% OsO4 (phosphate buffer, pH 7.4) for 2 hours at 4 ℃, dehydrated serially with ethanol, and embedded in epon 812. Each tissue section,

which was 0.5 to 1.0μm thick, was prepared by LKB microtome and stained by 1% toluidine blue, and we observed a central vein, portal tracts, and the distribution of bile ducts under light microscope. For electron microscopic examination, ultrathin sections, 600 nm thick, were made by ultratome and stained with acetate and lead itrate(Reynold et al., 1963), then examined under an electron microscope(JEOL-100B).

RESULTS

The relationship of liver to body weight

When sacrificed the liver weight was compared to the body weight of each mouse. In group 1, the ratio was 6.1±1.2% ; group 2, it increased from the end of the second week, maximally to 10.6±3.5%, and significantly increased to 10.5±2.5% at the 8th week($P<0.05$). In group 3, the ratio did not increase more than in group 2 but significantly increased more than in group 1($P<0.05$)(Table 1).

Results of quantitative analysis of urine porphobilinogen

In group 1, the urine PBG concentration was 1.5±0.4 mg/dl, and in group 2, it increased to 1.8±0.2 mg/dl at the second week and maximally to 3.4±1.5 mg/dl at the 4th week, then decreased to 2.1±1.4 mg/dl at the sixth week. In group 3, the urine PBG was not significantly different from in gro 1 (Table 1).

The changes in values of serum total bilirubin, SGPT, and ALP

In serum total bilirubin value, it was 0.8±0.1 mg/dl in group 1, and all group were the within normal limits. In serum SGPT value, it was 63±11 IU/dl in group 1, but in group 2, it maximally increased to 1316±211 IU/L at the second week and reached to 617±121 IU /L, 657±98 IU/L, and 784±12 IU/L at the 4th, 6th, and 8th week, resp tively, which significantly increased more than in group 1 ($P<0.001$, $P<0.01$).

In ALP value, it was 102±23 IU/L in group 1, but in group 2, it maximally increased to 1902±321 IU/L at the second week and decreased after that but singnificantly increased more than in group 1 at any time($P<0.001$).

In group 3, not more significantly increased than group 2, it maximally increased to 621 ±172 IU/L at the second week, which was also more significantly increased than in group 1($P<0.001$) (Table 2).

Light microscopic findings

In group 1, there were no significant macroscopic or microscopic changes in the hepatocytes, bile ducts, or portal areas(Fig. 1). In group 2, at the 3rd week, bile duct enlargement in the portal area was seen, and bil ducts were filled with protoporphyrin pigment thrombi of brown pigment(Fig. 2). At the 6th week, aggregated necrotic hepatocytes were observed in zone 3 in hepatic lobules(Fig. 3). At the 3rd week, oxyphilic degeneration leading to Councilman body and

Table 1. Changes of Liver Weight per Body Ratio and Urinary Porphobilinogen Excretion.

	Group	Week			
		2	4	6	8
Liver / body	1	6.1±1.2	6.3±2.0	6.2±1.7	6.4±2.5
Weight	2	8.5±2.9	9.4±4.1	10.6±3.5*	10.5±2.5*
Ratio(%)	3	7.4±3.6	8.3±3.9	10.2±1.8**	11.1±3.7**
Urine	1	1.5±0.4	1.3±0.4	0.9±0.2	1.0±0.2
Porpho−	2	1.8±0.2	3.4±1.5*	2.1±1.4	0.8±0.9
Bilinogen	3	1.0±0:1*	0.9±0.1	0.8±0.1	0.9±0.3

1 : control diet 2 : griseofulvin treated alone 3 : griseo ulvin plus UDCA treated
The data represents the menan±standard deviation.
* : $P<0.05$ ** : $P<0.01$ *** : $P<0.001$

Mallory hyaline bodies in cytoplasm were observed around necrotic hepatocytes in the hepatic lobules(zone. 1)(Fig. 4), in addition to inflammatory cell infiltration in the portal areas and brown-pigmented thrombi in the portal tracts. "Maltese cross" birefringens were seen in the bile ducts and ductules under polarizing light microscope, and smaller "Maltese cross" were observed in the hepatocytes(Fig. 5). In group 3, it was observed that the bile ducts were enlarged, but the hepatic lobules, central veins, and hepatocytes were normal(Fig. 6).

Electron microscopic findings

In group 2, characteristic changes were as follows : crowding and swelling of mitoc hondria, aggregation of globular crystalline materials in the bile ducts, ductules and hepa-tocyte cytoplasms, inflammatory cell infiltration in the portal areas, and destruction of bile duct epithelium. Ductular enlargement, loss of microvilli, and bleb formation of them, and lymphocytic infiltration between hepatocytes were seen(Fig. 7, 9). Protoporphyrin pigment infiltrated the cytoplasms of the hepatocytes in the form of globular aggregation or filamen-

Explanations of Figures

Fig. 1. Photomicrograph of control mouse liver which showed normal lobular architecture.
P:portal tract, C:central vein(H & E, 100).

Fig. 2. Photomicrograph of a mouse liver 3 after feeding griseofulvin. Ther is marked dilatation of the bile ducts of the portal tracts in which there is a striking deposition of brown protoporphyrin pigmentsin the bile ducts andductules and scattered in the parenchyme(H & E, 100).

Fig. 3. Photomicrograph of a mouse liver 6 weeks after feeding griseofulvin. A large zone of liver cells hae undergone necrosis(H & 100).

Fig. 4. Photomicrograph of a mouse liver 3 weeks after feeding griseofulvin. Acidophilic body(arrowhead) and Mallory hyalin body (arrow) are observed. There are inflammatory cell infiltrations in the sinusoidal space(H & E, 400).

Fig. 5. Photomicro raph of mouse liver 4 weeks after feeding griseofulvin. A birefringent "Maltese cross" and "starry sky" materials in the bile duct are observed by polarized light (H & E, polarized, 100).

Fig. 6. Photomicrograph of a mouse liver 4 weeks after feeding griseofulvin and UDCA. There is a marked dilatation of the bile duct in the portal tract, but normal lobular architecture is observed(H & E, 100).

Fig. 7. Electron micrograph of 3 hepatocytes of mouse liver 1 week after feeding griseofulvin. Bile canaliculus(BC) is markedly dilated and contains globular protoporphyrin (PP) crystal. The mitochondria are sw llen and crowdde. The lymphocytes(L) directly contacted with hepatocytes. S:sinusoid(6, 665).

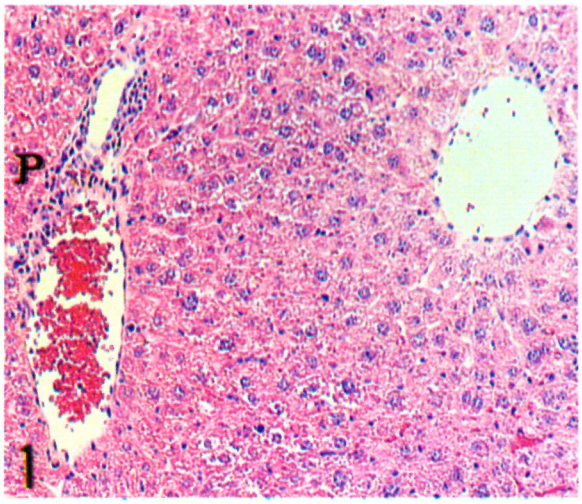
Fig. 8. Electron micrograph of portal tract of mouse liver 6 weeks after feeding griseofulvin. Macrophages phagocyte numerous protoporphyrin(PP) crystals of varying size and shape. Ploymorphonuclear leukocyte(L) and plasma cell(I) are observed(7.500).

Fig. 9. Electron micrograph of bile canaliculus 6 weeks after griseofulvin feeding in which a large protoporphjyrin crystal occupied neafrly all lumen. Hepatocytes contain intracytoplasmic protoporphyrin crystals, many of which show needle-like crystals (arrow) (7.500).

Fig. 10. Electron micrograph of a hepatocyte with Mallory hyalin 3 weeks after feeding griseofulvin. Several clusters of globular hyalin body in hepatocytic cytoplasm can be observed. N: nucleus(30,000).

Fig. 11. Higher magnification of Mallory body seen in Fig. 18 Mallory(hyalin) body appears as a conglomeration of fibrillar material, consisting of loosely arranged fine fibrils with random direction(75,000).

Fig. 12. Electron micrograph of interlobular bile duct of mouse liver 6 weeks after feeding griseofulvin and UDCA. Bile ductular epithelial cells show normal appearing cytoplasmic organelle(75,000).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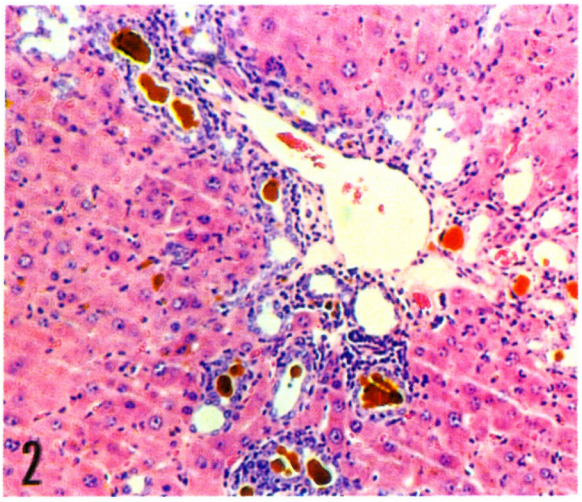
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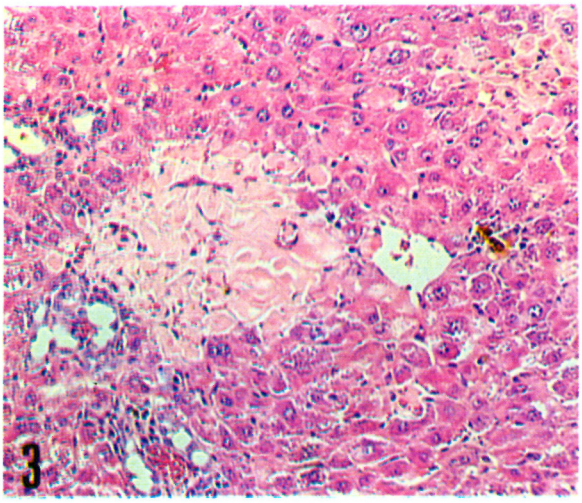
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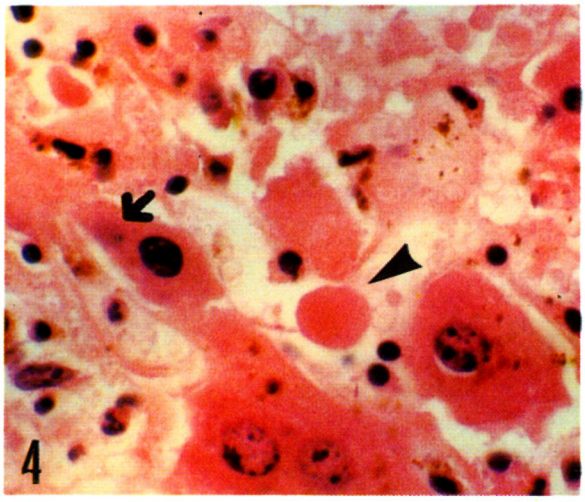
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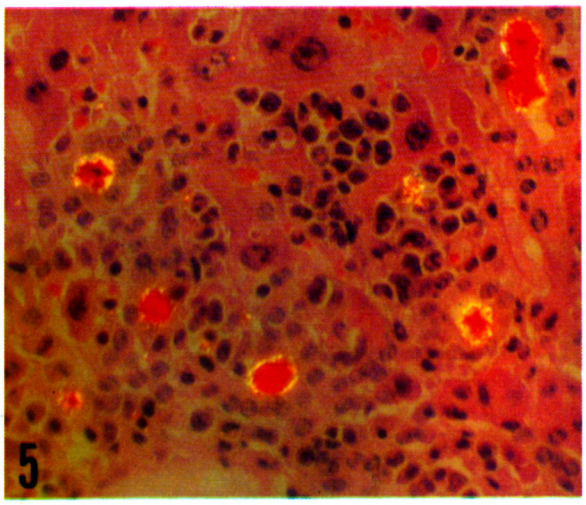
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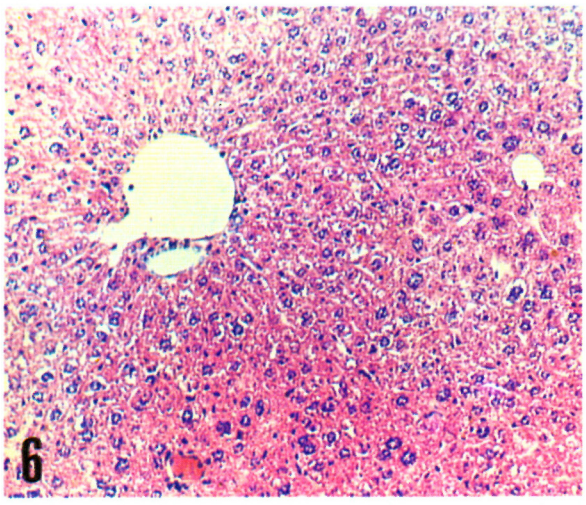
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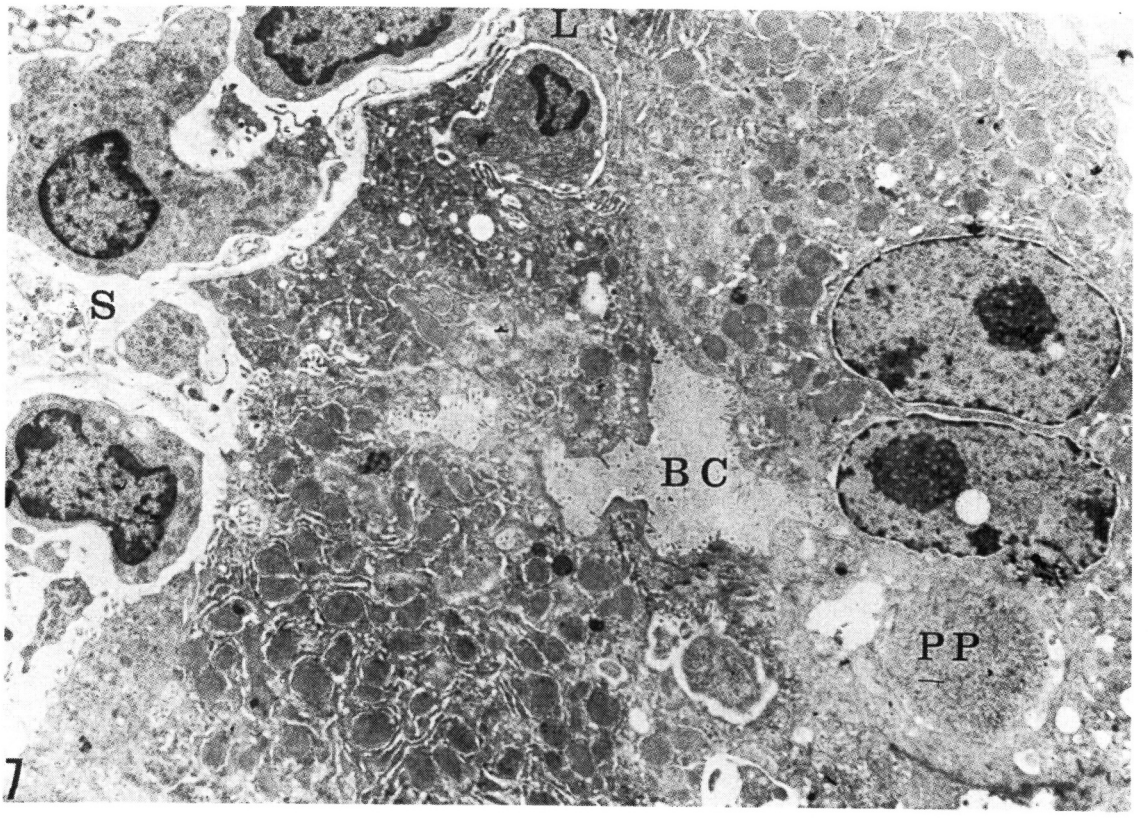
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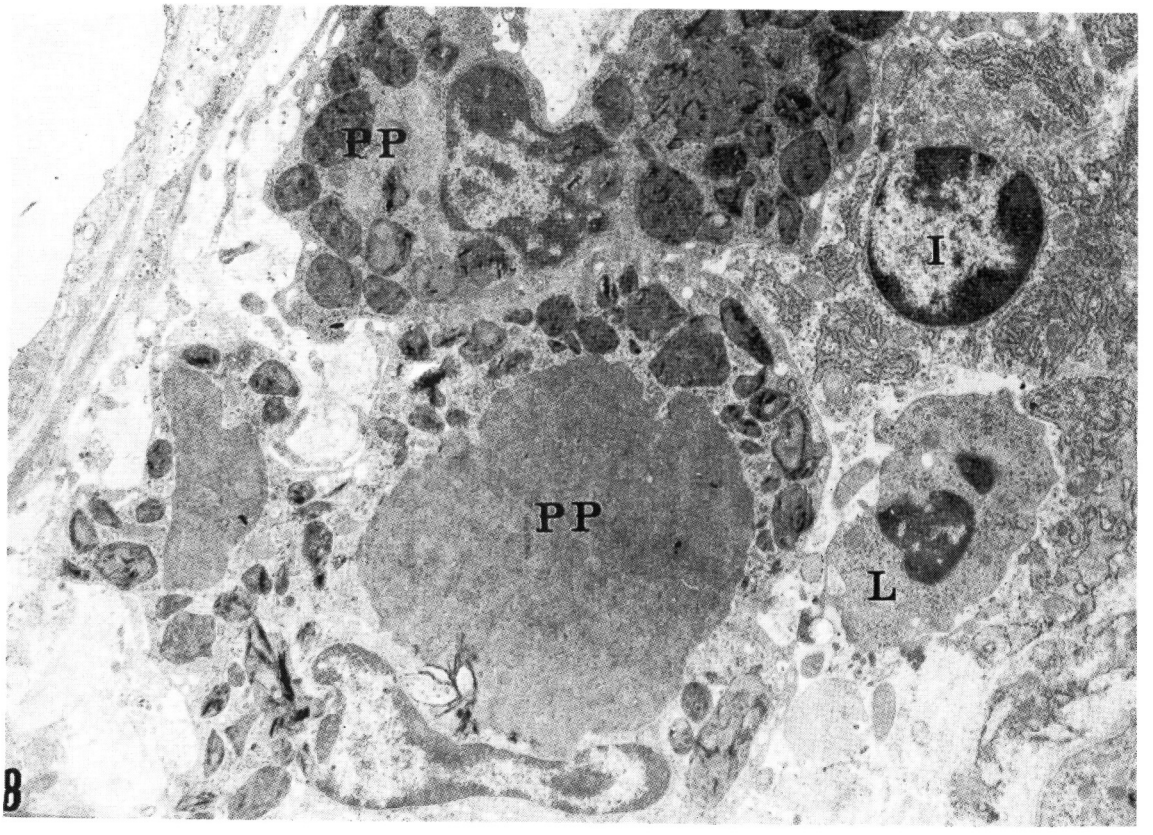
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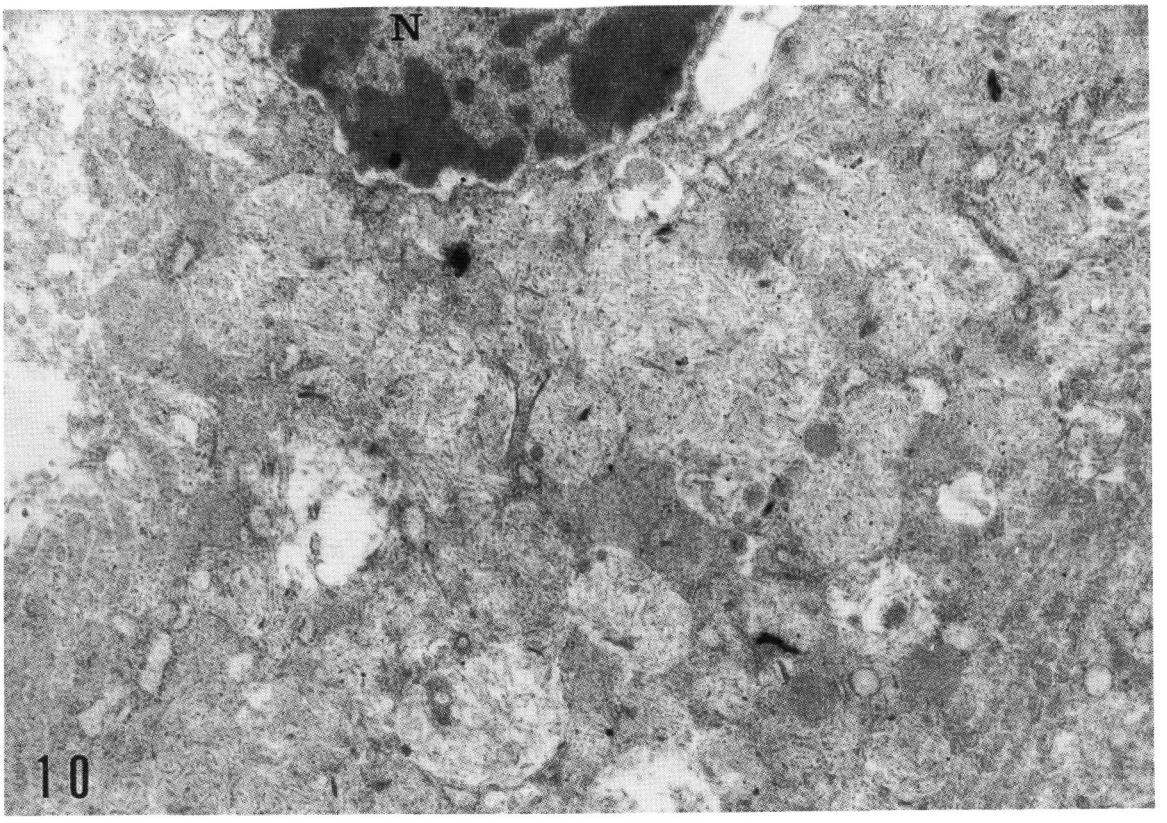

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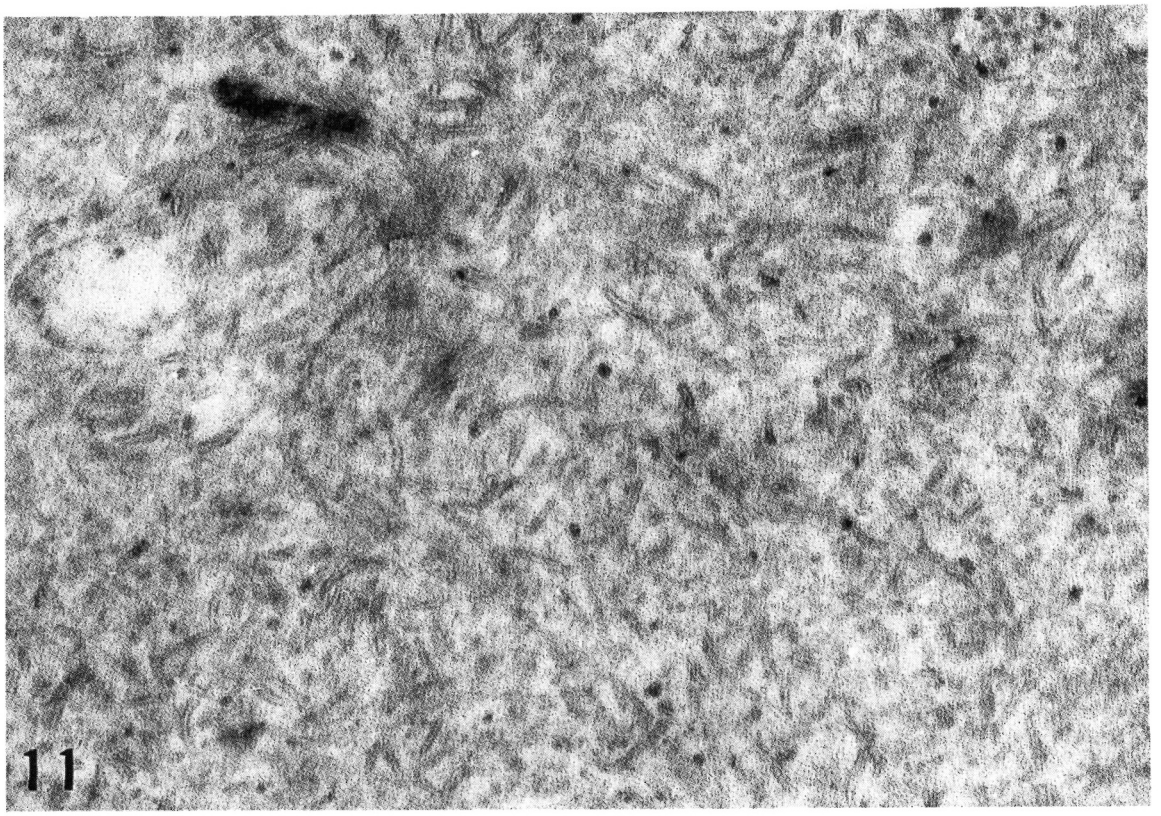
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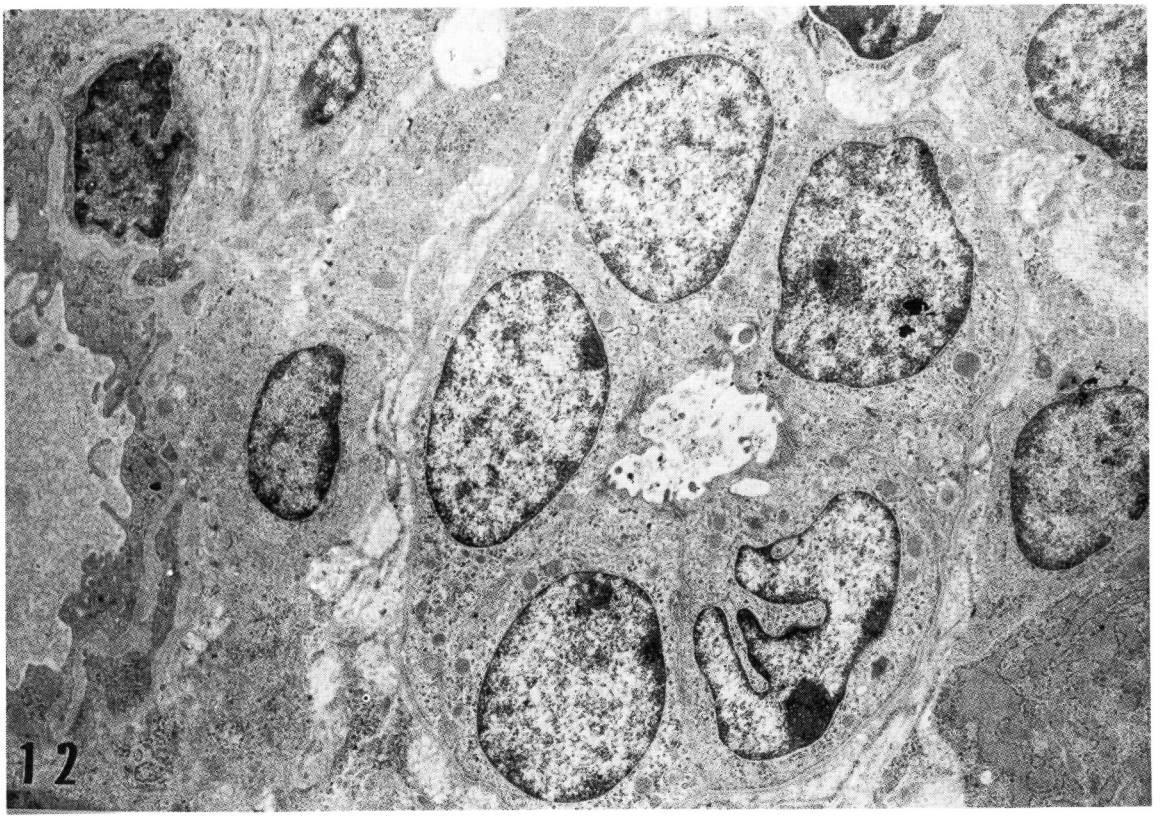
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Table 2. Biochemical Changes of Liver Function.

	Group	Week			
		2	4	6	8
Total	1	0.8±0.1	0.7±0.2	0.9±0.2	0.6±0.1
Nilirubin	2	0.7±0.1	0.8±0.2	0.9±0.1	1.1±0.1
(mg/dl)	3	0.6±0.2	0.9±0.1	1.0±0.2	0.8±0.1
SGPT	1	63±11	56±9	74±16	66±21
(IU/L)	2	1316±211***	617±121***	657±98***	784±123***
	3	356±79***	250±84***	201±78**	67±16
Alkaline	1	102±23	117±14	116±26	124±16
Pholsphatase	2	1902±321***	987±145***	870±98***	705±67***
(IU/L)	3	621±172***	600±115***	311±45***	190±27**

1 : control diet 2 : griseofulvin treated alone 3 : griseofulvin plus UDCA treated
The data represents the menan±standard deviation.
* : P<0.5 ** : P<0.01 *** : P<0.001

tous crystals(Fig. 7, 9). In the portal areas, many macrophages containing protoporphyrin pigment crystals were observed, which were different in electron density and which contained filamentous crystals(Fig. 8).

Mallory(hyaline) bodys came to appear 3 weeks after GF treatment, which was located principally in the juxtanuclear zone and round in shape(Fig. 10).

Cells containing Mallory body showed vacuolization and hydrophic changes, while organelles in the hepatocytes were severely changed and so not typically identical.

They had fibrillar shapes, no membranes around them, and were seen in a clustered, straight, or curved fashion. The major structural components of Mallory body were unbranched filament rods; each filament was 228.00±25.17(190-270) nm in length, 37.50±5.17(30-48) nm in width, and covered by a double fimbriated coat(Fig. 11).

In group 3, the hepatocytes were normal, and no marked changes of organelles were seen in the cytoplasms. Ductal epithelial cells in the portal areas, sinusoidal endothelial cells, and Disse's space were observed (Fig. 12).

DISCUSSION

GF, an antifungal agent, has been used widely, and the liver has an important role in the metabolism of this agent. Orally ingested GF is bound to serum proteins, metabolized by enzymes in the endoplasmic reticulum, and excreted through the bile duct and kidney.

In our study, long-term oral administration of large amounts of GF resulted in an increase of SGPT, ALP level, and no changes in serum total bilirubin level, suggesting that GF-induced hepatocyte damage and disturbances of biliary excretion.

Microscopic findings after GF treatment revealed severe hepatocyte damage, which was considered nonspecific, along with inflammatory cell infiltration, hepatocyte degeneration, necrosis, and a dark brownish pigment accumulation in the hepatocytes, bile ducts, and Kupffer cells, macrophages, birefringent pigment was observed under polarizing light microscope.

In the GF treated group, the urine PBG concentration was increased significantly than that of the control group, which suggested increased heme metabolism in liver tissue due to hepatic porphyria. Urine PBG level also maximally increased at the 4th week and decreased thereafter. In the GF and UDCA-treated group, the urine PBG level maximally increased at the second week. Therefore, rapid urinary excretion of PBG was noted after UDCA treatment.

In our study, protoporphyrin pigments appeared in the hepatocytes and intrahepatic ducts as dark brown color microscopically,

and pigment deposits were typically birefringent. Also, filamentous crystals, in electron microscopic findings, were seen in rosette formation, which was the same finding described by Gescheit et al(1975).

In our study, porphyrin pigments and bile were mixed and found in the bile ducts or ductules, and in the hepatocytes due to cholestasis by protoporphyrin pigments.

In 1983, Avner and his colleagues suggested the mechanism of protoporphyrin induced cholestasis : protoporphyrin pigments are excreted only into the bile ducts and decrease in the activities of liver surface Na^{+}-K^{+} ATPase, Mg^{+2} ATPase, 5'-nucleotidase and also succinyl-cytochrome C reductase due to toxic e ects on cytoplasmic membrane and mitochondria, resulting in cho-lestasis.

It was postulated by Matilla and Mollaud in 1974 that protoporphyrin pigments made in mitochondria were excreted from hepato-cyte like bilirubin and had an affinity to certain components of bile juice in view of the fact that protoporphyrin pigments were mixed with bile.

Another hepatic toxicity is the appearance of the Mallory body. Mallory(hyaline) body, first described by Mallory in 1911, can be seen in alcoholic hepatitis, jejunoileal bypass(Mallory, 1911), obesity(Adler et al., 1979), non-insulin dependent diabetes mellitus(Falchuk et al., 1980 ; Thaler et al., 1975), and hepatoma.

Our study showed that the Mallory body was composed of unbranched filament rods each being 228.00 ± 25.17 nm in length, 37.50 ± 5.17 nm in width, and covered by a double-dense fimbriated coat at, and aggregated by connection of their filaments. These findings were similar to alcoholic hyaline in humans(Franke et al., 1979).

In group 3, light microscopic findings were nearly normal, and there were no Mallory bodies, ductular dilatation, blunting of mi-crovilli, porphyrin pigmentation, or choles-tasis. UDCA was the major constituent in the bile of bear (Hammerstein et al., 1902), and cholesterol stones could be successfully dissolved by use of UDCA, which was 7-epimer of chenodeoxycholic acid(Nakano et al., 1973).

Recently, it was proposed that UDCA was not only effective in primary biliary cirrhosis but also in chronic cholestasis(Leuschner et al., 1987 : Yoon et al., 1986).

In GF-induced porphyria, the accumulation of toxic endogenous bile acids(taurocholic and taurodeoxycholic acid) in the hepatocytes resulted in hepatic damage(Hatoff et al., 1981 : Hertz et al., 1971; Schaffner et al., 1971), which was the same as our results.

It was reported that UDCA, hydrophilic bile acid, changed componets of endogenous bile acid pool, which was the underlying cause of cholestasis(Tint et al., 1986), and reduced bile acid pool by disturbance of the bile acid absorption of the small intestine(Stiel et al., 1990).

In this study, hepatic toxicity due to GF was partially prevented by adding UDCA, suggesting that UDCA partially prevented GF hepatotoxicity and facilitated porphyrin excretion. It was found that by ameliorating the abnormalities of cholestasis and hepatic injuries (porphyria), which in turn, were caused by toxic endogenous bile acid deposition in hepatocyte by GF, the UDCA treatment restored the hepatocyte function and morphologic changes through cytoprotective effects and by faciliati the excretion of bile acid.

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