Protection Against Microcystin-LR-Induced Hepatotoxicity by Silymarin: Biochemistry, Histopathology, and Lethality

Kay A. Mereish,[1,4] David L. Bunner,[1] Danny R. Ragland,[2] and Donald A. Creasia[3]

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Microcystin-LR, a cyclic heptapeptide synthesized by the blue-green algae, *Microcystis aeruginosa*, is a potent hepatotoxin. Pathological examination of livers from mice and rats that received microcystin-LR revealed severe, peracute, diffuse, centrilobular hepatocellular necrosis, and hemorrhage. These changes were correlated with increased serum activities of sorbitol dehydrogenase, alanine aminotransferase, and lactate dehydrogenase. Pretreatment of either rats or mice with a single dose of silymarin, a flavonolignane isolated from the wild artichoke (*Silybum marianum* L. Gaertn), completely abolished the lethal effects, pathological changes, and significantly decreased the levels of serum enzymes induced by microcystin-LR intoxication.

KEY WORDS: alanine aminotransferase; flavonoid; hepatic necrosis; hepatotoxin; lactate dehydrogenase; microcystin-LR; silymarin; sorbitol dehydrogenase.

INTRODUCTION

Microcystin-LR (MCLR) is a cyclic heptapeptide produced by several strains of the blue-green alga *Microcystis aeruginosa* (1). MCLR is a highly hepatotoxic peptide in cultured hepatocytes (2) and in mice (3) and rats (4). Administration of MCLR to mice and rats causes death within 1–3 hr (3,4), apparently as a result of hypovolemic shock secondary to massive hemorrhagic hepatocellular necrosis (3). It has been shown that liver damage induced by CCl_4 prior to MCLR administration protected mice from MCLR toxicity (5). In addition, mature mice are found to be more sensitive than young mice to acute toxicity of MCLR (5). These observations suggest that the liver is involved in activating the toxin. Silymarin is a flavonoid in which a phenylchromanone basic frame of taxifolin type is bound with one coniferyl alcohol molecule (6). Silymarin was shown to be protective against CCl_4 hepatotoxicity in rats (7) and against MCLR-induced toxicity in cultured rat hepatocytes (8). In this study, we extended our *in vitro* studies to *in vivo* and tested the use of silymarin against MCLR toxicity in mice and rats.

MATERIALS AND METHODS

Materials. Silymarin was purchased from Aldrich Chemical Co. (Milwaukee, WI). MCLR isolated from cultured *Microcystis aeruginosa* strain 7820 was obtained from Dr. W. Carmichael, Wright State University, Dayton, OH. MCLR purity was greater than 95% as verified by high-performance liquid chromatography.

Animals. Male Fischer-344 rats (250–300 g) and male CD-1 mice (25–30 g) were obtained from Charles River (Willimington, MA). All animals were maintained on standard laboratory diet (open formula 31, NIH, Bethesda, MD) and tap water ad libitum throughout the experiments. Five animals were maintained in each cage under a 12–12-hr light–dark cycle and room temperatures of 22–24°C. The animals were housed for 1 week prior to experimental use.

Surgery. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). After anesthesia, a PE-50 cannula was placed in the left carotid artery for collecting blood samples and right jugular vien for drug administration (9). All surgical procedures were performed under clean conditions. After surgery, rats were housed in individual cages and allowed to recover for 24 hr before the studies were begun.

Treatment Regimen. Rats received silymarin (15–50 mg/kg, i.v.) or its vehicle (0.2 ml of propylene glycol in saline, 75/25) 30 min prior to the administration of a sublethal, but hepatotoxic, dose of microcystin-LR (30 μg/kg, i.v.). Blood samples of 0.2 ml were collected 10 min prior to silymarin administration, and 0.5, 1, 2, 4, 6, 8, and 24 hr post-MCLR treatment. Blood samples were centrifuged at 5000 rpm for 10 min in an Eppendorf centrifuge, Model 5414, and the sera were collected and stored at −20°C for enzyme analysis (maximum storage time was 3 days). After 24 hr, livers were removed, weighed, and processed as described under histopathology.

Lethality. Mice received a single dose of silymarin (0.15–0.5 g/kg, i.p. or orally) or its vehicle (0.2 ml of propylene glycol in saline, 75/25) as specified in each case, prior to the administration of a lethal dose of MCLR (80 μg/kg, i.p.). Mice were checked for mortality every 20–30 min for 9 hr, then at 20 hr, and then every 20–30 min for an additional 4 hr. Livers were removed, weighed, and then processed as described under histopathology.

Serum Enzyme Activities. Serum samples were analyzed for enzyme levels of sorbitol dehydrogenase (SDH), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) with reagent kits obtained from Sigma (St. Louis, MO).

Histopathology. Livers were fixed in 10% buffered formalin solution and embedded in paraffin. Sections were cut at 4 to 6-μm thickness and stained with Mayer's hematoxylin and eosin. Liver sections were examined under light microscope and graded as normal, minimal, mild, or severe for hepatocellular changes.

Statistical Analysis. Results are presented as mean ± SE. Significance was determined by using analysis of vari-

[1] Medical Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702–5011.
[2] Pathology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702–5011.
[3] Pathophysiology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702–5011.
[4] To whom correspondence should be addressed.

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ance (ANOVA) followed by Duncan's new multiple-range test or *t* test where applicable.

RESULTS

Effects of Silymarin Pretreatment on Hepatotoxicity of MCLR in Mice. The effects of silymarin pretreatment on MCLR lethality, time to death, percentage liver to body weight, and histopathology of the liver were studied. Intoxication of mice with 80 μg/kg, i.p., of MCLR proved to be a lethal dose (LD_{99}), with death occurring at an average of 103 min, and with hepatomegaly due to intrahepatic hemorrhage (Table I). Percentage liver-to-body weight of the toxin treated mice increased from normal levels of 6.1 to 9.9% (Table I). The administration of silymarin (0.15–0.5 g/kg, i.p.) 1 hr prior to a lethal dose of MCLR was effective in preventing MCLR lethality and prevented hepatomegaly when given in doses greater than 0.35 g/kg (Table I).

Pretreatment of mice with silymarin in a single oral dose ranging from 0.1 to 0.5 g/kg, 1 hr prior to MCLR administration, offered no protection (data not shown). Even changing the silymarin pretreatment time from 1 to 16 hr, to allow for absorption and distribution, at a dose of 0.5 g/kg orally, did not provide protection against MCLR hepatotoxicity or lethality (Table II).

In contrast, changing the pretreatment time of silymarin (0.5 g/kg, i.p.) to 0.3, 0.5, 1, or 24 hr prior to MCLR intoxication provided full protection at all time points (Table III) against MCLR toxicity. Administration of silymarin (0.5 g/kg, i.p.) 10 min prior to toxin or simultaneously with a lethal dose of toxin resulted in partial protection in which 70 and 60% of the mice died within 24 hr, respectively (Table III). The administration of silymarin with the toxin delayed the time of death from 1–2 hr to about 11 hr (Table III). The dead mice showed typical liver enlargement, while those surviving >24 hr revealed a macroscopically normal liver with a normal ratio of liver to body weight (Table III).

Table I. Effect of Silymarin on the Toxicity of Microcystin-LR in Mice[a]

Silymarin dose (g/kg, i.p.)	24-hr mortality (dead/total)	Time to death (min)	Liver/body wt ratio (%)
Control[b]	0/3	—[d]	6.1 ± 1.2
Control[c]	0/3	—[d]	6.3 ± 0.8
0	5/5	103.0 ± 17.7	9.9 ± 0.8
0.15	4/5	309.0 ± 201	9.9[e] ± 0.5
0.25	1/5	285	8.9[e]
0.35	2/10	98.0 ± 18.3	9.2[e] ± 0.1
0.50	0/5	—[d]	6.5 ± 0.8

[a] Silymarin dose was in 75% propylene glycol in saline. All animals, except control groups, received a lethal dose of MCLR (80 μg/kg, i.p.). Silymarin treatment was 1 hr prior to toxin administration.
[b] Control mice received nothing.
[c] Control mice received only a silymarin dose of 0.35 g/kg, i.p.
[d] Experiment was terminated after 24 hr.
[e] Data represent the mean of dead animals only. The liver/body weight ratios for surviving animals were in the normal range (6.0–6.5%).

Table II. Effect of Pretreatment Time of Silymarin[a] Administered Orally on the Toxicity of Microcystin-LR in Mice[b]

Pretretment time (hr)	24-hr mortality (dead/total)	Time to death (min)	Liver/body wt ratio (%)
Control[c]	0/5	—[d]	5.1 ± 0.4
Toxin only	5/5	62.2 ± 16.5	10.4 ± 1.1
−1	5/5	64.6 ± 15.1	10.4 ± 0.9
−2	5/5	69.5 ± 21.1	10.1 ± 0.2
−3	5/5	62.6 ± 11.9	11.8 ± 2.9
−4	5/5	58.7 ± 7.3	9.7 ± 1.1
−16	5/5	97.8 ± 10.8	11.6 ± 0.7

[a] All animals except control received a lethal dose of MCLR i.p.
[b] Silymarin-pretreated animals received an oral dose of 0.5 g/kg.
[c] Control mice received vehicle (propylene glycol/saline, 75/25).
[d] Experiment was terminated after 24 hr.

Effects of Silymarin Pretreatment on Hepatotoxicity of MCLR in Rats. The effects of silymarin pretreatment on the MCLR-induced increase in plasma LDH (data not shown), SDH (data not shown), and ALT (Fig. 1) levels as a measure of liver damage were evaluated. Rats receiving a sublethal, but hepatotoxic, dose of MCLR (30 μg/kg, i.v.) showed significant increases in plasma enzyme levels 3 hr after MCLR administration, and the levels continued to increase for 24 hr (Fig. 1). In animals pretreated with silymarin (50 mg/kg, i.v.) 30 min prior to MCLR, plasma enzyme activities were reduced compared to those in animals receiving only MCLR (Fig. 1) but were the same as those found in groups of rats that received only silymarin, vehicle, or sham-surgical preparation. Histopathological changes in livers of rats correlated well with the enzymatic data. Serum enzyme levels for groups of rats pretreated with 25 and 15 mg/kg silymarin, i.v., 30 min before MCLR were similar to those presented in Fig. 1. Representative time points at 6 hr after MCLR treatment were selected for the presentation of dose effect. All silymarin doses (15–50 mg/kg) tested in rats were effective in preventing the release of LDH, ALT, and SDH (Table IV).

Table III. Effect of Pretreatment Time of Silymarin[a] Administered Intraperitoneally on the Toxicity of Microcystin-LR in Mice[b]

Pretreatment time (hr)	24-hr mortality (dead/total)	Time to death (min)	Liver/body wt ratio (%)
Control[c]	0/10	—[d]	5.8 ± 0.7
Toxin only	10/10	167.0 ± 64.9	8.8 ± 1.0
+0.17	7/10	119.3 ± 40.3	7.9[e] ± 0.8
0	2/5	11 hr	6.5[e] ± 0.9
−0.33	0/5	—[d]	5.6 ± 0.2
−0.5	0/5	—[d]	6.7 ± 0.5
−1	0/5	—[d]	5.7 ± 0.3
−24, −1	0/5	—[d]	5.5 ± 0.6

[a] All groups except control received a lethal dose of MCLR i.p.
[b] Silymarin (0.5 g/kg, i.p.)-pretreated animals.
[c] Control mice received vehicle (propylene glycol/saline, 75/25).
[d] Experiment was terminated after 24 hr.
[e] Data represent the mean of dead animals only. Liver/body weight ratios for surviving animals were in the normal range (5.5–6.7%).

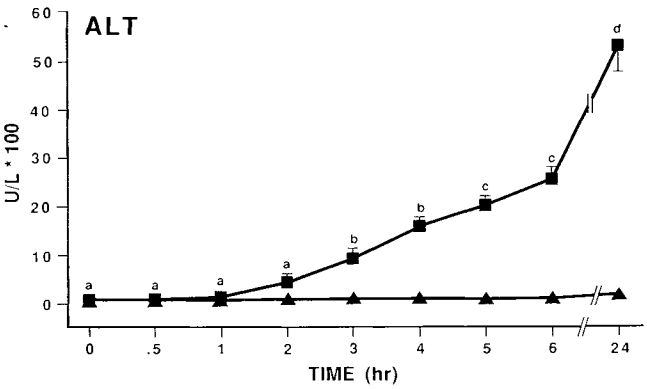

Fig. 1. Effect of silymarin on microcystin-LR-induced changes in serum ALT in rats. Rats received silymarin (50 mg/kg, i.v., ▲—▲) or propylene glycol in saline (■—■), 30 min before a sublethal dose of MCLR (30 μg/kg, i.v.). Thereafter, at the indicated times, blood samples were collected and serum enzyme activities were measured. Each point represents the mean ± SE; $n = 5$. Data points having no letters (a–d) in common were significantly different; $P < 0.05$.

Histopathology of Livers Treated with MCLR and Silymarin. Microscopic changes in mouse and rat livers treated with only MCLR exhibited diffuse, severe, centrilobular disruption of hepatic plates and dissociaton of individual hepatocytes. The hepatocytes were rounded and fragmented, with loss of nuclei, as well as occasional pyknosis and karyorrhexis. The cytoplasm was hyaline and hyperchromatic in appearance, with loss of detail. Sinusoids were markedly dilated, resulting in extensive pooling of blood. Central veins contained variable numbers of sloughed hepatocytes; some central veins were obliterated by the cellular debris. These changes often extended to the midzonal and infrequently into periportal areas (Figs. 2A, B). In silymarin-pretreated mice (data not shown) and rats (Fig. 2C), the livers were not different histologically from experimental controls (data not shown). Livers from rats and mice given silymarin alone were normal compared to controls (data not shown). The morphological data are summarized (normal, minimal, mild, severe), combined, and tabulated in Table V.

Table IV. Effect of Different Doses of Silymarin on Microcystin-LR-Induced Changes in Serum[a] ALT, SDH, and LDH in Rats

Dose (mg/kg)	ALT (U/L)	SDH (U/L)	LDH (U/L)
Control[b]	60.5 ± 14.6	6.5 ± 5.2	96.9 ± 30.3
Toxin[c]	2516.6 ± 239	561.8 ± 39	8659.2 ± 105
15[d]	134.2 ± 54.4	31.2 ± 18.2	126.7 ± 32.5
25[d]	166.8 ± 23.9	45.9 ± 20.2	167.5 ± 67.2
50[d]	81.4 ± 12.2	12.4 ± 5.9	152.8 ± 46.1

[a] Serum enzyme activities were measured 6 hr post toxin administration. Data represent the mean ± SD of $n = 5$.

[b] Control rats received vehicles only.

[c] Rats received silymarin vehicle 30 min before a sublethal dose of microcystin-LR (30 μg/kg, i.v.). Levels of serum enzymes were significantly different from control, $P < 0.05$.

[d] Rats received an i.v. dose of silymarin 30 min before a sublethal dose of microcystin-LR (30 μg/kg, i.v.).

DISCUSSION

This study shows that, in mice and in rats, silymarin pretreatment inhibited MCLR-induced hepatic damage and toxicity. Liver damage was evaluated by histopathology of liver tissues and by measurement of serum levels of LDH, SDH, and ALT. Silymarin pretreatment resulted in a significant decrease in serum enzyme levels.

The effectiveness of silymarin through the i.p. but not the oral route may indicate poor bioavailability. In addition, the silymarin protective dose in mice injected i.p. was about 10-fold higher than the protective dose when injected i.v. in rats. The bioavailability of silymarin in mice and rats will be determined in a future study.

The mechanism responsible for the protection by silymarin against MCLR lethal effects and liver damage is not presently known. Silymarin has been shown to increase the levels of glutathione and reduce the production of malondialdehyde, as a measure of lipid peroxidation in livers of rats acutely intoxicated with ethanol (10). In addition, silymarin has been shown to inhibit lipoxygenase specifically and, therefore, leukotriene synthesis (11); scavenge and neutralize free radicals generated during oxidative stress (12,13); and increase hepatocyte rRNA, ribosomal, and protein synthesis *in vivo* and *in vitro* (14).

Some of the effects associated with MCLR-induced hepatotoxicity are cell blebbing (2,8), increased intracellular calcium flux (15), increased phosphorylase-a activity (16), glutathione depletion (16), and arachidonic acid release (17). These observations are similar to the events, observed with oxidative stress, that result from other toxins such as the lipopolysaccharide endotoxins (18).

Biochemical and ultrastructural studies indicate that MCLR affects the plasma membrane of hepatocytes (19) and interacts with cytoskeletal microfilaments (2,4), cytokeratins, and actin (4). These early ultrastructural changes were detectable in cultured hepatocytes (2) and *in vivo* (18,20); however, how these initial changes are related to the hemorrhagic necrosis of the liver is not understood.

Experiments with isolated hepatocytes showed that silymarin and dithioerythritol prevent the protrusions observed at the surface of cells exposed to MCLR (8). Recently, we found that changes of protein-thiol induced by MCLR in isolated hepatocytes were prevented by silymarin (unpublished data). Plasma membrane protrusions in hepatocytes of menadione-induced toxicity are associated with the oxidation of thiol groups (21). Moreover, the addition of dithioerythritol to menadion (21)- or MCLR-treated hepatocytes (8) protected the cells from the appearance of surface blebs. The antagonistic effect of silymarin could be similar to that of dithioerythritol by stabilizing protein-thiol, which may be important to the structure of liver cells. In addition, silymarin might inhibit the binding of MCLR to the cell surface or inhibit its transport as seen with rifampicin (19). Further work is required to prove these hypotheses.

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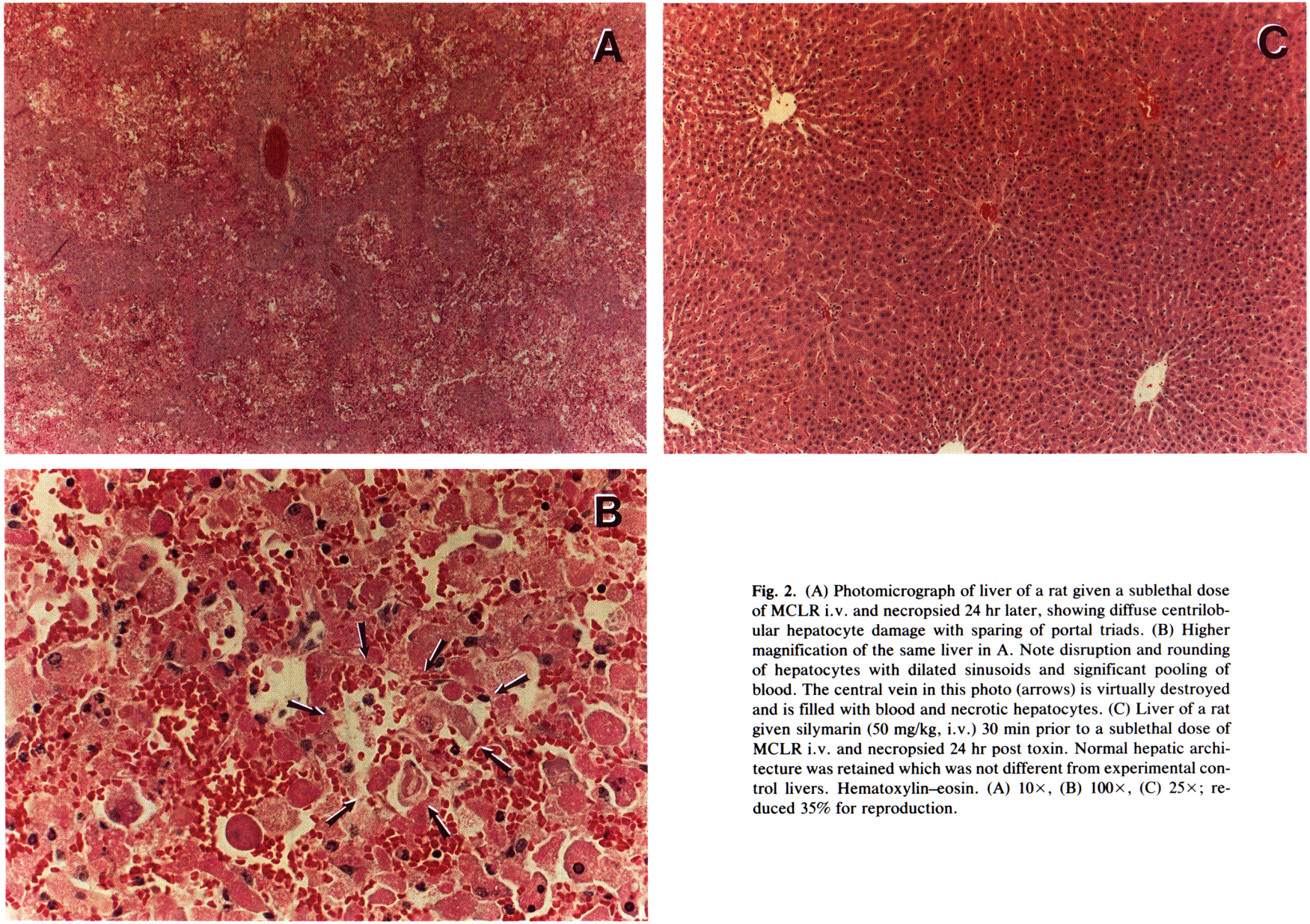

Fig. 2. (A) Photomicrograph of liver of a rat given a sublethal dose of MCLR i.v. and necropsied 24 hr later, showing diffuse centrilobular hepatocyte damage with sparing of portal triads. (B) Higher magnification of the same liver in A. Note disruption and rounding of hepatocytes with dilated sinusoids and significant pooling of blood. The central vein in this photo (arrows) is virtually destroyed and is filled with blood and necrotic hepatocytes. (C) Liver of a rat given silymarin (50 mg/kg, i.v.) 30 min prior to a sublethal dose of MCLR i.v. and necropsied 24 hr post toxin. Normal hepatic architecture was retained which was not different from experimental control livers. Hematoxylin–eosin. (A) 10×, (B) 100×, (C) 25×; reduced 35% for reproduction.

Table V. Effect of Silymarin on Microcystin-LR-Induced Hepatic Necrosis[a]

Treatment	Extent of hepatic necrosis (number of mice)			
	Normal	Minimal	Mild	Severe
Vehicle (3)	2	1	0	0
Silymarin (3)	3	0	0	0
MCLR (3)	0	0	1	2
MCLR/silymarin (3)	2	1	0	0

[a] Mice received silymarin (0.5 g/kg, i.p.) or its vehicle (75% propylene glycol in saline) 1 hr before a lethal dose of MCLR (80 μg/kg, i.p.). Surviving animals were killed 24 hr after treatment and the extent of liver necrosis was graded.

Army or the Department of Defense. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, NAS/NRC. The work described here was presented at the 8th European Symposium on Animal, Plant and Microbial Toxins, Porec, Yugoslavia, September 24–27, 1989.

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