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Acute Hepatotoxic Potential of Imazalil Fungicide in Rats

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Imazalil (IMZ; 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole) is a systemic imidazole fungicide used to control a wide range of fungi on fruit, vegetables and ornamentals (Food and Agriculture Organization of United Nations 1977). IMZ is also used as a seed dressing and for the postharvest treatment of citrus, banana, and other fruit crops to control storage decay. IMZ has been investigated *in vivo* and *in vitro* to assess various toxicological properties. Because the oral LD_{50} of IMZ in the rat is approximately 340 mg/kg (Thienpont *et al.* 1981), IMZ is classified as a moderately toxic compound. Serious effects of IMZ such as carcinogenicity, mutagenicity, and teratogenicity have not been found in rats (Thienpont *et al.* 1981). Despite this, the exposure of isolated rat hepatocytes to IMZ is more toxic than other fungicides (e.g. *ortho* -phenylphenol and thiabendazole) (Nakagawa and Moore 1995). IMZ causes a number of interrelated cellular effects which together result in the induction of malondialdehyde and the loss of cellular ATP and glutathione which precede cell death in the hepatocyte. Since there is little information on the acute organ effects of IMZ, the object of this study was to investigate the hepato-toxic potential of a single dose of IMZ and the effect of L-buthionine-*S,R*-sulfoximine, an inhibitor of glutathione synthesis, on rat liver following IMZ dosage.

MATERIALS AND METHODS

IMZ was obtained from Wako Pure Chemical Ltd. (Osaka, Japan) and L-buthionine-*S,R*-sulfoximine (BSO) from Sigma Chemical Co. (St Louis, MO). All other chemicals were of the highest purity available.

Male F344/DuCrj rats (Charles River Japan Inc., Atsugi), weighing 220-240 g, were divided into groups of four for each experiment, housed in stainless steel cages in rooms designed to maintain adequate environmental condition (22-24°C, 60% humidity and 12-hr light/dark cycle) and provided both commercial rat food (CE-2, Japan CLEA Co., Tokyo) and water *ad libitum.* The animals were starved

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overnight before use. IMZ (170 mg/kg, l/2-LD_{50}; 255 mg/kg, 3/4-LD_{50}; 340 mg/kg, LD_{50}) was dissolved in corn oil and given to each rat by stomach tube. To evaluate the effects of BSO on IMZ-induced toxicity, rats received BSO (900 mg/kg) in isotonic phosphate-buffered saline (pH 7.4, M.A. Bioproducts, Walkersville, MD) by intraperitoneal injection 1 hr prior to IMZ dosing. The corresponding control animals received either an equivalent volume of corn oil or BSO in isotonic phosphate-buffered saline. Two hours after administration of IMZ, all rats were allowed both food and water again.

Twenty-four hours after administration of IMZ, the rats were killed by decapitation while under general anesthesia with CO_2. The blood was allowed to clot for 30 min at room temperature and then was centrifuged. Serum was used to measure activities of alanine aminotransferase and aspartate aminotransferase and concentrations of urea nitrogen, cholesterol, phospholipids and triglycerides. The liver and kidney were removed and weighed. A piece of liver was homogenized with 1.15% KCl containing 10 mM phosphate buffer (pH 7.4) in a Potter-Elvehjem homogenizer and was used for determination of malondialdehyde.

The activities of serum aminotransferases and the concentration of serum urea nitrogen, cholesterol, phospholipids and triglycerides were determined by a Hitachi 7150 automatic analyzer and diagnosis kits (Autosera®) from the Daiichi Chemicals Co. (Tokyo). The concentration of hepatic malondialdehyde was assayed by the thiobarbituric acid reaction, according to the procedure of Ohkawa *et al.* (1979).

Liver and kidney sections (thickness, 5 μm) were fixed in buffered formalin solution, and paraffin sections were prepared and stained with hematoxylin and eosin. In addition, frozen sections (thickness, 5 μm) of liver were stained with oil red O or with sudan black B.

The mean values for each treatment group were compared to the appropriate groups using the Mann-Whitney ***U*** test, and data from organ weights were analyzed by the multiple *t* statistic of Dunnett.

RESULTS AND DISCUSSION

A single administration of IMZ or IMZ with BSO did not significantly affect body, liver or kidney weight in male rats after 24 hr (Table 1). Administration of IMZ alone caused a dose-dependent decrease in serum activity of aspartate aminotransferase, but not alanine aminotransferase (Table 2). Both serum parameters indicated that IMZ at doses used did not cause serious hepatic damage, which was supported by histological observation (Fig. 1). The highest dose of IMZ alone decreased concentration of serum lipids, cholesterol, triglycerides and

Table 1. Effects of IMZ and/or BSO on liver and kidney weights of F344 rats. Male rats pretreated with BSO (900 mg/kg; i.p. injection) were killed 24 hr after oral administration of IMZ. Each value represents the mean ± S.D. of four individual animals.

Treatments (mg/kg)		(g/100 g BW)	
BSO	IMZ	Liver	Kidney
0	0	4.00±0.19	0.69±0.02
900	0	3.98±0.23	0.71±0.02
0	170	3.90±0.19	0.72±0.03
0	255	3.85±0.16	0.73±0.02
0	340	3.43±0.53	0.73±0.05
900	170	3.81±0.24	0.73±0.07
900	255	3.77±0.11	10.72±0.03
900	340	3.67±0.16	0.78±0.07

There was no significant difference from control group.

phospholipids and these effects were enhanced by BSO except for triglycerides. In rats with high levels of serum triglycerides, liver histopathology showed the presence of small fat droplets, which were stained by oil red O (Fig. 1) or sudan black B (data not shown), dispersed mainly throughout the cytoplasm of midzonal and/or peripheral hepatocytes. Both dyes used here have a high affinity for intracellular neutral lipid. However, necrosis was not observed in regions of hepatic steatosis. In contrast, no histological changes were observed in rat livers treated with IMZ alone or BSO alone. A slight elevation of hepatic malondialdehyde level, an index of lipid peroxidation, was found in the highest-dose group of IMZ-treated rats (389±26 nmol/g tissue; 115% of control, $p < 0.05$) as well as in BSO plus the highest-dose of IMZ-treated rats (441±23 nmol/g tissue; 130% of control, $p < 0.05$). This result suggests that IMZ-induced hepatic lipid peroxidation is potentiated by BSO pretreatment. Kidneys in either IMZ alone- or BSO plus IMZ-treated rats had no apparent changes in either histological observation or serum concentration of urea nitrogen (26.5± 1.1 mg/dL; control rats) as an index of renal damage.

Hepatic or renal damage caused by some chemicals, which are converted to reactive intermediate(s) by microsomal monooxygenase system, is aggravated by the pretreatment of BSO in rats or mice (Miners *et al.* 1984; Nakagawa and Tayama 1988; Brittebo *et al.* 1993). However, the combination of BSO and IMZ produces a typical microvesicular steatosis without necrosis in rat livers (Fig. lB). It is well known that a number of chemicals produce liver injury accompanied by

Table 2. Effects of IMZ and/or BSO on serum biochemical parameters in F344 male rats. Male rats pretreated with BSO (900 mg/kg; i.p. injection) were killed 24 hr after oral administration of IMZ. Each value represents the mean ± S.D. of four individual rats.

Treatments (mg/kg)		Serum parameters				
BSO[a]	IMZ	AST (U/L)	ALT (U/L)	CHO (mg/dL)	TG (mg/dL)	PL (mg/dL)
0	0	136±19	64± 6	54± 3	141±18	118± 4
900	0	108±24	55± 8	48± 5	169±28	128±10
0	170	129±12	52± 2	52± 4	124±19	127±13
0	255	82±12*	51±10	41± 5*	149±34	95±13*
0	340	51± 4*	48± 3	39± 8*	104± 8*	79± 7*
900	170	64±19*†	39± 7*†	49± 1	127±13	128± 8
900	255	58± 2*†	43± 5*	42± 5*	250±30*†	99± 7*
900	340	50±10*	39± 4*	25± 5*†	241±41*†	59± 7*†

* Significant difference from control group ($p < 0.05$)

† Significant differencc between IMZ alone group and BSO plus IMZ group ($p < 0.05$)

[a] Abbreviations used: BSO, L-buthionine-S,*R*-sulfoximine; IMZ, imazalil; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CHO, cholesterol; TG, triglycerides; PL, phospholipids.

accumulation of abnormal amounts of lipid, predominantly triglycerides, and that the lipid accumulation is a result of an imbalance between the rate of synthesis and the rate of release of triglycerides by the hepatocytes (Zimmerman 1982). Although the mechanism by which the elevation of serum triglycerides concentration as well as induction of hepatic steatosis is induced by the combination of BSO and IMZ is unclear in the present study, it seems that the elevation of serum triglycerides levels depends on both stimulation of hepatic triglycerides synthesis and triglycerides release into the blood circulation.

With an administration of BSO (900 mg/kg) to male rat, hepatic levels of glutathione decreased to approximately 10% or 65% of untreated rat liver after 6 hr or 24 hr, respectively (Nakagawa and Tayama 1988). It is well established that glutathione plays a crucial protective role against cellular injury induced by oxidative stress or alkylation derived from a number of toxic compounds and their intermediates (Reed 1990; O'Brien 1991). Glutathione depletion to 20-30% of total glutathione levels can impair the cellular defenses against toxic compounds (Moldéus and Quanguan 1987). Therefore, low levels of hepatic glutathione over long periods in the presence of toxic compounds may be related to the hepatocellular alteration associated with lipid metabolism.

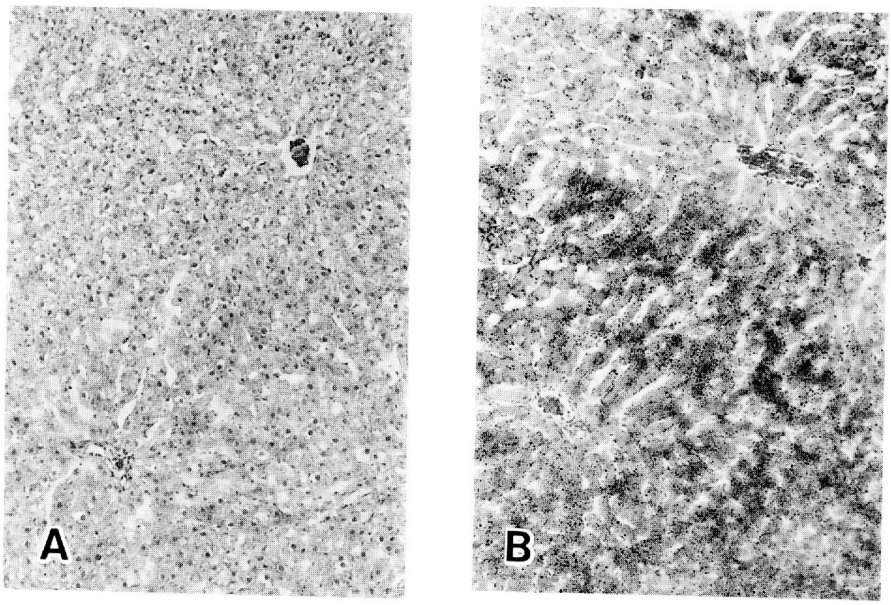

Figure 1. Liver morphology 24 hr after administration of (A) BSO (900 mg/kg) alone or (B) BSO (900 mg/kg) plus IMZ (255 mg/kg) in male rats. Small fat droplets, which were stained by oil red O, dispersed mainly throughout the cytoplasm of midzonal and/or peripheral hepatocytes in BSO plus IMZ-treated group. (x200)

The induction of steatosis does not necessarily lead to cell death of hepatocytes: ethionine, puromycin and cycloheximide result in lipid accumulation without producing necrosis (Hoyumpa *et al.* 1975; Zimmerman 1982). Thienpont *et al.* (1981) have reported that a chronic administration of IMZ at a dose of 80 mg/kg (approximately $l/4LD_{50}$) elicited slight swelling of hepatocytes without obvious degenerative changes. In a previous study using freshly isolated hepatocytes (Nakagawa and Moore 1995), IMZ caused acute cell killing accompanied by abrupt depletion of intracellular ATP and glutathione, with induction of malondialdehyde. Changes in these parameters may be directly or indirectly associated with the onset of cell death caused by IMZ. Lipid peroxidation observed in a later stage of IMZ-induced cytotoxicity has been proposed as a major molecular mechanism involved in cell or tissue injury induced by some chemicals (Comporti *et al.* 1965; Eklöw-Låtbom *et al.* 1986; Fawthrop *et al.* 1991; Nakagawa *et al.* 1991). Since the administration at the middle-dose of IMZ alone or at the same dose of IMZ plus BSO did not induce malondialdehyde formation even when the hepatic steatosis was found in rats, the lipid peroxidation may not be directly linked with the induction of steatosis.

A single administration of IMZ (170-340 mg/kg, p.o.) to male rats did not cause

an acute hepatic or renal damage. In BSO (900 mg/kg, i.p.)- pretreated rats, the administration of IMZ led to the elevation of serum triglyceride levels and the induction of hepatic steatosis without necrosis.

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