Effects of mild zinc deficiency, plus or minus an acute-phase response, on galactosamine-induced hepatitis in rats

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Zn deficiency is hypothesized to produce poor resistance to injury involving oxidative stress. This could occur by impairing Zn antioxidant function(s) or by indirectly limiting adaptive protective mechanisms such as a rise in acute-phase proteins. The present study examined rats fed diets adequate or moderately low in Zn (4 or 25 μg/g diet) for 9 d. The lower intake produced a mild Zn deficiency based on body weight, plasma Zn and plasma alkaline phosphatase (*EC* 3.1.3.1) activity. Galactosamine injection, an oxidative stress, produced much more liver injury in the mildly Zn-deficient rats. However, injury was strongly inhibited in rats from each dietary group by an acute-phase response due to turpentine-induced leg inflammation. Mild Zn deficiency did not prevent a rise in levels of the acute-phase protein caeruloplasmin (*EC* 1.16.3.1), but did limit the usual inflammation-induced rise in hepatic levels of metallothionein, a Zn protein with possible antioxidant function. In conclusion, high degrees of galactosamine-induced hepatitis were associated with mild Zn deficiency, but the liver injury was blocked by prior stimulation of an acute-phase response, regardless of Zn status.

Zinc: Galactosamine-induced hepatitis: Metallothionein

A recent review article (Bray & Bettger, 1990) summarizes the evidence for and against the hypothesis that Zn performs antioxidant function(s). On the negative side, Zn-deficiency symptoms do not resemble those of the antioxidant nutrients Se or vitamin E, nor do cell membranes from Zn-deficient rodents show compositions expected after oxidative damage. On the other hand, Zn inhibits oxidative reactions *in vitro*, depresses radical production by activated isolated phagocytes, and stabilizes membranes against stress *in vitro*. Also, metallothionein, a protein which contains Zn, can scavenge radicals *in vitro*. In addition, high-dose Zn injections in rats block injury due to CCl_4 and radiation. One limitation of these studies supporting an antioxidant function for Zn is that the Zn levels and/or binding ligands produced are not necessarily typical of most physiological conditions.

Possibly, the main effects of impaired Zn antioxidant function(s) occur only during oxidative stress. A few studies have related dietary Zn deficiency with the potential for oxidative injury. For instance, lung microsomes from Zn-deficient rats yield high quantities of carbon-centred radicals upon stimulation *in vitro* (Kubow *et al.* 1986). Also, liver microsomes from such rats show high lipid peroxidation induction *in vitro* (Sullivan *et al.* 1980). However, the relationship of these effects to oxidative injury *in vivo* is unclear.

Little research has actually examined the effects of varying dietary Zn on stress-induced injury *in vivo*. In one exception, Taylor *et al.* (1990) found that Zn-deficient rats show excessive hyperoxia-induced lung damage. However, at least some of the effects may not

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have involved impairment of specific Zn antioxidant functions. Instead, the high degree of injury may have resulted from an indirect effect on antioxidant enzyme levels due to a general impairment of protein synthesis. Hyperoxia injury develops over several days and can be influenced by the extent to which adaptive responses occur (Crapo *et al.* 1980). Taylor & Bray (1991) found that Zn-deficient rats did not show the rise in antioxidant enzyme levels (i.e. superoxide dismutase, *EC* 1.15.1.1) normally seen during hyperoxia.

The present study tested the hypothesis that Zn deficiency could affect resistance to an oxidative stress through mechanisms other than general restriction of protein synthesis. One way in which this was accomplished was by using galactosamine injection as the oxidative stress. This treatment injures the liver quickly while blocking hepatic protein synthesis (Decker & Keppler, 1974). Thus, impaired liver synthesis of serum acute-phase proteins or antioxidant enzymes within the liver should not have been dependent on Zn status. In effect, all galactosamine-treated rats, regardless of dietary treatment, would have an impairment. Furthermore, the study used a mild Zn deficiency which should not have a major impact on protein synthesis. This assumption about mild Zn deficiency was tested by examining its effects on the protection against galactosamine normally afforded by first elevating the synthesis of acute-phase proteins (Alcorn *et al.* 1992). In the present study this elevation was accomplished by pretreatment with leg inflammation induced by intramuscular turpentine injection.

Galactosamine is considered to be an oxidative stress resembling that of viral hepatitis (Shedlofsky & McClain, 1991). In both cases, liver injury is thought to occur because these agents increase the sensitivity of this organ to the low levels of endotoxin generally present in mammals (Chojkier & Fierer, 1985; Shedlofsky & McClain, 1991). Evidence for O radical involvement includes observations that galactosamine-induced injury is limited by injection with antioxidants such as superoxide dismutase, allopurinol or catalase (*EC* 1.11.1.6; Shedlofsky & McClain, 1991).

MATERIALS AND METHODS

Animal diets and treatments

The animal protocol was approved by The Ohio State University Institutional Laboratory Animal Care Committee. Male Sprague–Dawley rats, initially weighing about 150 g, were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA) and housed singly in stainless steel cages. Rats were given deionized water and a semipurified diet obtained from ICN Biochemicals (Cleveland, OH, USA) shown in Table 1. The low- and adequate-Zn diets contained 4 and 25 mg Zn/kg respectively, measured by atomic absorption spectrometry. For the first 5 d after arrival all rats were given the adequate-Zn diet *ad lib.* Rats were then either continued on the same dietary regimen, switched to the low-Zn diet or given the adequate-Zn diet pair-fed to the average intake for rats consuming the low-Zn diet. Rats used for injury evaluations were killed 9 d later. Rats used to measure caeruloplasmin and metallothionein were killed a day earlier. All rats were killed by decapitation after a brief exposure to CO_2. When an acute-phase response was produced, inflammation was initiated with turpentine (0·1 ml/rat, given intra-muscularly in the leg) 2 d before the rats were killed. Leg swelling was visible the day after turpentine injection. Galactosamine (Sigma Chemical Co., St Louis, MO, USA), when injected, was given intraperitoneally at 1 g/kg body weight in saline (9 g NaCl/1) 1 d before the rats were killed. Most serum measurements used to assess galactosamine-induced liver injury reach peak or near peak values in young rats about 1 d after injection (Platt *et al.* 1978; Tsuda *et al.* 1990; Farghali *et al.* 1991).

Table 1. *Composition of the experimental diet (g/kg)*

Egg white	180·0
Maize oil	100·0
Maize starch	443·0
Sucrose	200·0
Alphacel hydrolyzed	30·0
Choline bitartrate	2·0
Biotin	0·02
AIN-76C vitamin mixture	10·0
AIN-76 mineral mixture*	35·0

For the low-Zn diet, $ZnCO_3$ was omitted.

Analytical methods

Serum Zn was measured by atomic absorption spectrometry. Serum bile acids and activities of alanine aminotransferase (ALT; *EC* 2.6.1.2), β-glucuronidase (*EC* 3.2.1.31) and alkaline phosphatase (*EC* 3.1.3.1) were measured spectrophotometrically using kits from Sigma Chemical Co. ALT was measured by first catalysing the conversion of alanine plus 2-oxoglutarate to glutamate plus pyruvate. The latter acts as a substrate for lactate dehydrogenase (*EC* 1.1.1.27) which converts NADH to NAD, a process followed by a decrease in absorbance at 340 nm. β-glucuronidase was assessed by liberation of the dye phenolphthalein from phenophthalein mono-β-glucuronic acid. Alkaline phosphatase was detected by cleavage of phosphate from *p*-nitrophenyl phosphate at alkaline pH, forming the yellow compound *p*-nitrophenol. The bile assay used 3α-hydroxysteroid dehydrogenase (*EC* 1.1.1.50) to convert the bile acids to 3-oxo bile acids with the concurrent generation of NADH. The latter, through the action of diaphorase (*EC* 1.8.1.4), reacts with the dye nitro blue tetrazolium. Serum sialic acid levels were assayed with a kit from Boehringer Mannheim (Indianapolis, IN, USA) which uses a series of enzyme steps to cleave sialic acid from proteins and ultimately produces a red chromophore. Caeruloplasmin (*EC* 1.16.3.1) activities were evaluated by oxidation of *p*-phenylenediamine by the method of Rice (1962). Metallothionein levels were assessed by the ^{109}Cd–haemoglobin method (Eaton & Toal, 1982). Serum was stored for less than 2 d at 4° for caeruloplasmin, Zn and ALT determinations and at −80° for other analyses. Livers were stored at −20° before metallothionein assay. All results were analysed by ANOVA plus least significant differences (LSD; Steel & Torrie, 1980) with $P < 0.05$ considered significant.

RESULTS

Consumption of the moderately-low-Zn diet affected body-weight, serum Zn and serum alkaline phosphatase activity (Table 2). The degree of deficiency produced could be termed mild based on the values of Table 2 compared with those found for other studies of low Zn intake by rats (i.e. Luecke *et al.* 1968; Kubow *et al.* 1986; Taylor *et al.* 1990). For instance, the Zn-adequate rats in the study of Kubow *et al.* (1986) showed a mean plasma Zn value similar to that for adequate Zn in Table 2. In contrast, the mean value for the low-Zn group in the study of Kubow *et al.* (1986) was only about 40% of that in Table 2 for the rats given low Zn. The classification of mild Zn deficiency for the present study is also supported by the observation that rats given moderately low Zn showed normal liver metallothionein levels (Table 2). Levels are below normal in more severely Zn-deficient rats (Blalock *et al.* 1988). Pair-fed rats of Table 2 showed below normal body-weight gain, but not as low as seen during pair-feeding to more severely Zn-deficient rats (i.e. Luecke *et al.* 1968; Kubow

Table 2. *Effects of dietary treatment on Zn status assessors in rats: body weight (g), serum Zn (μmol/l), alkaline phosphatase (AP, EC 3.1.3.1; units/l) and liver metallothionein (nmol/g)**

(Mean values with their standard errors for five to seven rats)

	Body wt		Serum Zn		AP†		Metallothionein	
Diet group	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Zn-adequate	242[c]	13	2·6[b]	0·2	340[b]	20	1·9[a]	0·3
Pair-fed	217[b]	7	2·6[b]	0·2	280[b]	40	1·7[a]	0·5
Low-Zn	187[a]	5	1·8[a]	0·1	160[a]	30	1·7[a]	0·3

[a, b, c] Mean values within a column bearing different superscript letters were significantly different ($P < 0·01$) by one-way ANOVA and least square differences.

* For details of diets and procedures, see Table 1 and pp. 612–613.

† AP activity is given in Sigma units (1 unit is sufficient to liberate 1 μmol *p*-nitrophenol/h from *p*-nitrophenyl phosphate at alkaline pH).

et al. 1986; Taylor *et al.* 1990). Pair-feeding did not affect serum Zn nor alkaline phosphatase values (Table 2).

In all dietary groups, galactosamine injection produced a significant increase in serum ALT activities (Fig. 1), a measure of cell membrane damage (Korsrud *et al.* 1972). However, the mean increase was higher with mild Zn deficiency than for either group given adequate Zn. Both adequate-Zn groups showed the same amount of galactosamine-induced injury. For this reason, as well as the small difference in feed intake and body weight between the pair-fed and *ad libitum* adequate-Zn groups, a pair-fed group was omitted from the remainder of the study.

A new set of rats was fed on either moderately-low or adequate-Zn diets as done for Table 2 and Fig. 1, only without the pair-fed group. One purpose was to confirm the strong, though variable, effects of low Zn intake on injury due to galactosamine. In addition, this experiment evaluated whether Zn status would affect the protection against galactosamine normally produced by an acute-phase response (Alcorn *et al.* 1992). As found previously (Table 2), rats fed low-Zn diets showed mild Zn deficiency based on moderately low serum Zn (data not shown) and normal liver metallothionein values (Table 3). Galactosamine again produced a high degree of injury in the mildly Zn-deficient rats (Table 4). However, the protective effect of an acute-phase response, brought on by leg inflammation, overcame this tendency toward a high degree of injury. Elevated serum levels of caeruloplasmin, an acute-phase protein (Cousins, 1985), demonstrated that an increase in acute-phase protein levels could occur in the mildly Zn-deficient rats (Table 3). Caeruloplasmin was measured by activity, not protein, but the two variables are generally increased to the same extent by inflammation in rats (DiSilvestro *et al.* 1988). Serum sialic acid, a crude measure of acute-phase protein levels (Blatteis, 1985), was also increased with inflammation in both dietary Zn groups (data not shown). In contrast, the deficient rats, unlike those fed on the Zn-adequate diet, did not show increased hepatic levels of the Zn-containing protein metallothionein (Table 3).

Table 5 shows that two assessors of liver injury other than ALT activities showed the same patterns seen for ALT in Table 4. High β-glucuronidase values reflect hepatocellular necrosis (Ohta *et al.* 1992). High serum bile concentrations indicate liver functional impairment where bile leaks into the blood faster than it can be passed down the biliary system (Tsuda *et al.* 1990). Poor uptake of bile acids from portal blood may also occur.

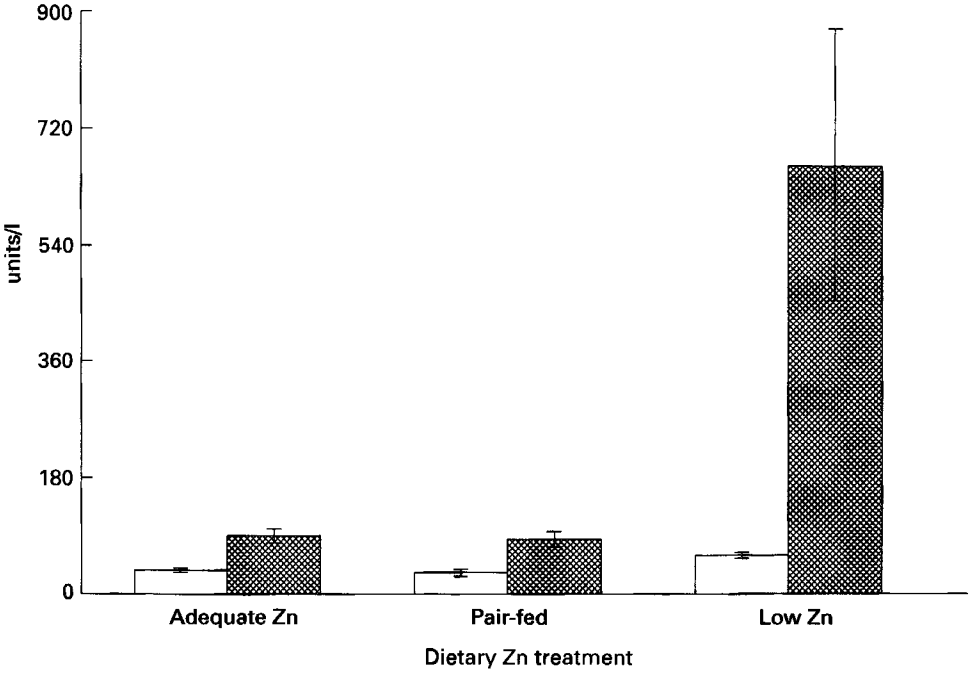

Fig. 1. Effects of Zn status and galactosamine injection on serum alanine aminotransferase (*EC* 2.6.1.2) activities in the rat. Values are means with their standard errors represented by vertical bars for five rats per control group (□) and six to nine rats for galactosamine-treated groups (⊠). One unit of activity was that required to produce 1 μmol NAD/min from NADH under standard conditions. Values for all galactosamine-treated groups were significantly different from untreated controls, and the treated, low-Zn group was significantly different from the other treated groups ($P < 0.01$, two-way ANOVA and least significant differences).

Table 3. *Effects of Zn intake plus or minus acute-phase response on liver metallothionein (nmol/g) and serum caeruloplasmin* (EC *1.16.3.1; units/l*) *in rats**

(Mean values with their standard errors for five rats)

	Metallothionein		Caeruloplasmin†	
Treatment	Mean	SE	Mean	SE
Adequate Zn	1·5[a]	0·1	800[a]	90
+inflammation‡	5·6[b]	0·8	2080[c]	100
Low Zn	1·3[a]	0·2	1280[b]	120
+inflammation‡	1·3[a]	0·3	3520[d]	650

[a,b,c,d] Mean values within a column bearing different superscript letters were significantly different ($P < 0.01$) by one-way ANOVA and least significant differences.

* For details of diets and procedures, see Table 1 and pp. 612–613.

† Caeruloplasmin units were arbitrarily defined as the change in absorbance at 540 nm over a 15-min period.

‡ An acute-phase response was produced by turpentine-induced inflammation of the leg. For details see p. 612.

A fairly high degree of variation occurred for the assessors of injury, but this is typical for studies of galactosamine-induced injury (Platt *et al.* 1978). The assays themselves show low variation as evidenced by between-day coefficient of variation values of 3% (ALT), 2% (β-glucuronidase) and 3·5% (bile acids).

Table 4. *Effects of Zn intake plus or minus an acute-phase response on serum alanine aminotransferase* (EC *2.6.1.2*)* *activities (units/l) in the rat*†

(Mean values with their standard errors for six to nine rats)

	Dietary group			
	Adequate Zn		Low Zn	
	Mean	SE	Mean	SE
Control	43^{ab}	5	65^{c}	6
Inflammation‡	32^{a}	5	35^{a}	6
Galactosamine	98^{d}	23	491^{e}	103
Inflammation‡ + galactosamine	40^{a}	5	55^{bc}	7

^{a,b,c,d,e} Mean values bearing different superscripts were significantly different ($P < 0.01$) by two-way ANOVA and least significant differences.

* One unit of activity is that required to produce 1 μmol NAD/min from NADH under standard conditions.

† For details of diets and procedures, see Table 1 and pp. 612–613.

‡ An acute-phase response was produced by turpentine-induced inflammation of the leg. For details see p. 612.

Table 5. *Effects of Zn intake plus and minus an acute-phase response on serum β-glucuronidase* (EC *3.2.1.31*) *activities (units × 10^{-3}/l) and bile acid concentrations (μmol/l) in the rat**

(Mean values with their standard errors for six to nine rats)

	Bile acids		Glucuronidase†	
Treatment	Mean	SE	Mean	SE
Adequate Zn	30^{a}	6	102^{a}	4
+ Inflammation‡	22^{a}	7	90^{a}	4
+ Galactosamine	58^{b}	6	195^{c}	14
+ Inflammation‡, galactosamine	40^{a}	3	135^{b}	11
Low Zn	12^{a}	5	93^{a}	10
+ Inflammation‡	9^{a}	5	82^{a}	6
+ Galactosamine	115^{c}	25	279^{d}	41
+ Inflammation‡, galactosamine	21^{a}	6	138^{b}	10

^{a,b,c,d} Mean values within a column bearing different superscript letters were significantly different ($P < 0.05$) by two-way ANOVA and least significant differences.

* For details of diets and procedures, see Table 1 and pp. 612–613.

† β-glucuronidase activity was measured in modified Sigma units (1 unit is sufficient for the formation of 1 μg phenolphalein/h from phenolphthalein mono-β-glucuronic acid under standard conditions).

‡ An acute-phase response was produced by turpentine-induced inflammation of the leg. For details see p. 612.

DISCUSSION

This study demonstrated that a mild Zn deficiency can produce poor resistance to injury from a type of oxidative stress. It is not yet known whether this effect resulted from impairment in some direct antioxidant function of Zn, or from some other alteration in the pathological process. However, the effect was not dependent on impaired ability to accumulate acute-phase proteins. The galactosamine treatment already restricts such accumulation (Decker & Keppler, 1974; Alcorn *et al.* 1992). Also, the capability to increase levels of an acute-phase protein, caeruloplasmin, was present in the mildly deficient rats

(Table 3). An increase in caeruloplasmin due to hyperoxia is inhibited in more severely Zn-deficient rats (Taylor & Bray, 1991).

The Zn-binding protein metallothionein has been proposed to protect against O radical-mediated injury (Sato & Bremner, 1993). However, variations in liver metallothionein levels did not contribute to the injury outcomes measured in this study. The high degree of galactosamine-induced liver injury in the mildly Zn-deficient rats occurred despite normal metallothionein values (Table 2, Fig. 1). Furthermore, inflammation pretreatment protected against liver injury in the Zn-deficient rats despite low Zn intake abolishing the rise in liver metallothionien concentrations (Tables 3 and 4). These results do not support an antioxidant role for metallothionein. However, this role is supported by work from our laboratory which examines another hepatoxin, CCl_4 (DiSilvestro & Carlson, 1993). In that case, the protection normally afforded by leg inflammation was partially lost when elevations in metallothionein levels were blocked by mild Zn deficiency.

In conclusion, the present study demonstrates that mild Zn deficiency can affect susceptibility to an injury thought to involve the actions of O free radicals. The mechanisms responsible for the Zn effects seen in this study await clarification. Possible mechanisms include membrane destabilization and exaggerated free radical production, both proposed consequences of impaired Zn function (Bray & Bettger, 1990).

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