A BRIEF COMMUNICATION

Marginal Zinc Deficiency Increased the Susceptibility to Acute Lipopolysaccharide-Induced Liver Injury in Rats

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Lipopolysaccharide (LPS) triggers a global activation of inflammatory responses leading to liver injury in humans. Zinc pretreatment has been shown to prevent LPS-induced hepatic necrosis. In North America, suboptimal zinc status is more common than once realized. However, the effect of inadequate zinc nutrition on the host's susceptibility to LPS-induced liver injury is not known. The objective of this study was to determine whether marginal zinc deficiency would render rats more susceptible to LPS-induced liver injury. Weanling Sprague-Dawley rats were assigned to one of three dietary treatment groups: marginally low zinc *ad libitum* (Z3; 3 mg zinc/kg diet), adequate zinc *ad libitum* (Z30; 30 mg zinc/kg diet), or adequate zinc pair-fed (Z30P) group. After 6 weeks, each dietary treatment group was further divided into LPS-control (saline) groups (C-Z3, C-Z30P, C-Z30) and LPS-treatment (1 mg/kg body weight, intraperitoneal, 8 hrs) groups (LPS-Z3, LPS-Z30P, LPS-Z30). LPS reduced the serum zinc concentration and increased the liver zinc concentration regardless of dietary zinc intake. Serum alanine aminotransferase level was higher in the LPS-Z3 rats than in the LPS-Z30P and LPS-Z30 rats. LPS also induced hepatocyte necrosis and neutrophil infiltration into the liver sinusoids. This LPS-induced liver damage was more severe in the LPS-Z3 rats than in the LPS-Z30P and LPS-Z30 rats. Together these findings have demonstrated that marginal zinc deficiency increased the susceptibility to LPS-induced liver injury in rats. These results indicate that patients with sepsis who have suboptimal zinc nutrition status may be at higher risk of developing greater liver damage. Exp Biol Med 231:553–558, 2006

Key words: lipopolysaccharide; liver; necrosis; neutrophil; zinc

Lipopolysaccharide (LPS) is an endotoxin that triggers a global activation of inflammatory responses that can lead to tissue damage, including liver dysfunction, liver failure (1, 2), and even death (3, 4). In LPS-induced liver injury, the presence of LPS activates hepatic Kupffer cells to produce and release chemokines, which then signal the infiltration of neutrophils into the hepatic sinusoids (5). Neutrophil infiltration is associated with the development of hepatocyte necrosis in mice (6).

Zinc is important for immune responses, including the anti-inflammatory response (7). In mice, zinc pretreatment (two doses of 5 mg zinc ion/kg body weight, intraperitoneal [ip]) prevented LPS-induced increases in plasma alanine aminotransferase (ALT) activity, indicating that zinc can reduce LPS-induced liver injury (8). In contrast, zinc deficiency in rats enhances the cytotoxicity of LPS on bone marrow (9); however, it is not known if inadequate zinc nutrition could render the host more susceptible to LPS-induced liver damage.

In North America, severe zinc deficiency is rare; however, suboptimal zinc status is more common than is

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realized. For example, in Ontario, Canada, inadequate zinc intake ranged from 9% (31–50 years) to 43% (71–74 years) in men and from 15% (19–31 years) to 30% (71–74 years) in women, using the Estimated Average Requirement as the criteria (10). Similarly, the third National Health and Nutrition Examination Survey found that in the United States, only 50% of men aged 71 and over and of women aged 19 and over had adequate zinc intakes, using the Recommended Dietary Allowance as the criteria (11). Since zinc has been implicated in protecting the host from LPS-induced liver injury, the high prevalence of suboptimal zinc status reported in these two dietary surveys indicates a potentially serious public health concern. Therefore, this study was carried out to determine whether marginal zinc deficiency would render rats more susceptible to LPS-induced liver injury.

Materials and Methods

Weanling female Sprague-Dawley rats (43.0 ± 2.3 g; Animal Care Center, The University of British Columbia, Vancouver, Canada) were assigned to one of three dietary treatment groups: marginally low zinc *ad libitum* (Z3; 3 mg zinc/kg diet), adequate zinc *ad libitum* (Z30; 30 mg zinc/kg diet), or adequate zinc pair-fed (Z30P). Rats in the Z30P group were fed the adequate-zinc diet, but at the amount consumed by the individual Z3 rats during the previous 24 hrs. The diets were egg white–based AIN-93G diets for rodents (12), with modifications described previously (13). The adequate-zinc level was maintained at the same level as in the AIN-93G diet to produce adequate-zinc status (12). The low-zinc level was based on the established level designed to produce marginal zinc deficiency (13–15). The rats were fed the assigned diet for 6 weeks with free access to double-deionized water. To prevent potential zinc contamination, precautions were taken throughout the study. Precautions included the use of covered plastic containers for diet storage; glass feed jars, polypropylene water bottles, and stainless steel cages; the rats being housing in separate rooms according to dietary zinc intake; and thorough handwashing between handling of the different diets. The rats were housed in thermo-regulated rooms with 12:12-hr light:dark cycles and were cared for according to the guidelines of the Canadian Council on Animal Care.

At the end of the feeding trial, each dietary treatment group was further divided into LPS-control and LPS-treatment groups ($n = 6$), for a total of six zinc-LPS treatment groups: LPS-control Z3 (C-Z3), Z30P (C-Z30P), and Z30 (C-Z30) groups, and LPS-treated Z3 (LPS-Z3), Z30P (LPS-Z30P), and Z30 (LPS-Z30) groups. Rats in the LPS groups were injected with LPS (*Escherichia coli* serotype 026:B6; Sigma Chemical Co., St. Louis, MO) at a dose of 1 mg/kg body weight in saline ip, as reported previously (16). The control rats were injected with saline at the same volume. After 8 hrs of LPS exposure, blood samples and the livers were collected. The LPS exposure time was determined based on a preliminary study. One portion of the liver was immediately snap frozen in liquid nitrogen and stored at −76°C until analysis, while the other portion was fixed in 10% buffered formalin. Blood samples were collected by cardiac puncture and allowed to clot in capped polypropylene test tubes overnight (about 14 hrs) at 4°C. After centrifugation (850 *g*; 20 mins), serum was transferred into polypropylene microcentrifuge tubes, capped, and stored at 4°C for determining ALT activity within 48 hrs. A portion of the serum was stored at −20°C for determination of zinc concentration within 1 week. To prevent potential zinc contamination, only disposable plastic labware, including test tubes, microcentrifuge tubes, syringes, and transfer pipettes, were used in collecting and handling of blood and serum samples.

For histologic analyses, liver samples were fixed in formalin for 24 hrs and then embedded in paraffin wax for sectioning, followed by hematoxylin and eosin (H&E) staining. The liver sections (5 μm) were evaluated under a light microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany), and the images were captured with a digital camera (AxioCam MRm; Carl Zeiss). Necrotic cells were identified by the presence of brightly eosinophilic cytoplasm; small, condensed nuclei; and the loss of cellular detail. Counting of necrotic hepatocytes and neutrophils present in the sinusoids was carried out systematically under the guide of a 10 × 10-mm micrometer grid at a magnification of ×200. Necrotic hepatocytes and neutrophils present in the center four grids were counted, resulting in an average of 53 analyzed fields/liver section. For analysis of apoptosis, liver slides were stained using the TUNEL method according to the manufacturer's protocol (DeadEnd; Promega, Madison, WI) and were then counterstained in Harris hematoxylin (Fisher Scientific, Ottawa, Canada) for 1 min and rinsed for 5 mins under running tap water.

Serum ALT was determined using a diagnostic kit (Diagnostic Chemicals Limited, Charlottetown, Canada) according to the manufacturer's instructions. Serum and liver zinc concentrations were determined as described previously (15).

Data on body weight gain and total feed intake were analyzed by a one-way analysis of variance (ANOVA) for dietary zinc intake followed by Tukey's HSD test (JMP for Windows, Release 5.0; SAS Institute, Inc., Cary, NC) ($P < 0.05$). Data on serum and liver zinc concentrations, serum ALT level, necrotic hepatocytes, and neutrophil infiltration were analyzed by a two-way ANOVA for dietary zinc intake, LPS, and their interactions, where appropriate, followed by a *post hoc* test using Tukey's HSD for main separation ($P < 0.05$). Data sets were tested for normality using Normal Quantile Plot and equal variance using the O'Brien test. Data points that exceeded mean ± 3 standard deviations (SD) were considered outliers and were excluded from calculating the means and from statistical analyses.

Table 1. Effects of Dietary Zinc Intakes on Body Weight Gain and Total Feed Intake[a]

Diet	Body weight gain (g)	Total feed intake (g)
Z30	193 ± 7 [a]	699 ± 8 [a]
Z30P	143 ± 8 [b]	481 ± 20 [b]
Z3	150 ± 8 [b]	503 ± 23 [b]

[a] Values are means ± SEM ($n = 12$ rats). Female weanling rats were fed an egg-white–based, modified AIN-93G diet for 6 weeks. Z30 and Z3 rats were fed the adequate-zinc and marginally low-zinc diet *ad libitum*, respectively. The Z30P rats were pair-fed to the Z3 rats individually. Body weight gain was the difference between the initial body weight and the final body weight. Total feed intake was the total amount of feed consumed by the rats over the 6-week feeding period. Means within each column sharing a common lowercase letter were not significantly different ($P < 0.05$). Z30, adequate-zinc *ad libitum* control group; Z30P, adequate-zinc pair-fed control group; and Z3, marginally low-zinc group.

Results

Feeding the marginally low-zinc diet resulted in reduced body weight gain and total feed intake (Table 1) and lowered serum zinc concentration (Table 2) in the Z3 rats compared to the Z30 rats. Total feed intake and body weight gain were not significantly different between the Z3 and Z30P rats, but serum zinc concentration was lower in the Z3 rats compared to the Z30P rats. This lack of significant difference in feed intake between the Z3 and Z30P rats confirmed the success of the pair-feeding protocol. At the same level of feed intake, body weight gain was also the same between these two groups of rats; however, serum zinc concentration was lower in the Z3 rats than in the Z30P rats, confirming the development of marginal zinc deficiency status in the Z3 rats.

Serum zinc concentration was lower in the Z3 rats than in the Z30 and Z30P rats, regardless of LPS exposure (Table 2). Similarly, liver zinc concentration was also lower in the Z3 rats than in the Z30 and Z30P rats, regardless of LPS exposure (Table 2). LPS exposure lowered serum zinc concentration but increased liver zinc concentration, regardless of dietary zinc intake.

Serum ALT level in the LPS-control rats was not different regardless of dietary zinc intake. LPS exposure increased serum ALT level in the LPS-Z3 rats compared to their LPS-control rats (Table 2). Serum ALT level was not different among LPS-Z30 and LPS-Z30P rats and their corresponding LPS-control rats. Among the LPS-treated rats, serum ALT level was higher in the LPS-Z3 rats than in the LPS-Z30P and LPS-Z30 rats, but it was not different between the LPS-Z30P and LPS-Z30 rats.

H&E staining revealed very few necrotic hepatocytes in all LPS-control rats (Fig. 1A). LPS exposure induced necrosis in hepatocytes in all the dietary treatment groups, especially in the LPS-Z3 rats (Fig. 1A). The number of necrotic hepatocytes was 5-fold higher in the LPS-Z3 rats and about 2-fold higher in LPS-Z30P and LPS-Z30 rats than in their corresponding LPS-control rats (Fig. 1B). Among the LPS-treated rats, necrotic hepatocytes were about 3-fold higher in the LPS-Z3 rats than in the LPS-Z30P and LPS-Z30 rats, but these numbers were not different between LPS-Z30 and LPS-Z30P rats. TUNEL staining revealed a lack of apoptotic hepatocytes.

Neutrophils were present at a low frequency in the liver sinusoids of all LPS-control rats (Fig. 2A). LPS exposure increased the number of neutrophils present in the sinusoids by 1.9-fold in the LPS-Z3 rats, by 1.5-fold in the LPS-Z30P rats, and by 1.4-fold in the LPS-Z30 rats compared to their corresponding control rats (Fig. 2B). Among the LPS-treated rats, neutrophils present in the sinusoids were greater in the LPS-Z3 rats than in the LPS-Z30P (1.4-fold measure) and LPS-Z30 (1.3-fold measure) rats, but these numbers were not significantly different between the LPS-Z30 and LPS-Z30P rats.

Discussion

An elevated level of serum ALT is a well-established indicator of liver injury (17–21). Numerous studies have shown that the level of serum ALT is elevated in rats in

Table 2. Serum and Liver Zinc Concentrations and Serum Alanine Aminotransferase Activity[a]

	Serum zinc[b] (μmol/liter)		Liver zinc[b] (μg/g dry tissue)		ALT[c] (units/liter)	
Diet	Control	LPS	Control	LPS	Control	LPS
Z30	1.3 ± 0.05	0.4 ± 0.08	115 ± 2	126 ± 5	13 ± 0.7 [bc]	18 ± 1.5 [b]
Z30P	1.3 ± 0.09	0.6 ± 0.05	108 ± 8	126 ± 9	14 ± 0.8 [bc]	17 ± 2.0 [bc]
Z3	0.8 ± 0.14	0.2 ± 0.04	85 ± 4	108 ± 6	12 ± 0.7 [c]	24 ± 0.9 [a]

[a] Values are means ± SEM ($n = 6$ rats, with the exception of ALT Z3 control group and LPS group and the ALT Z30P LPS group. In these three groups, $n = 5$ rats). Female weanling rats were fed an egg-white–based, modified AIN-93G diet for 6 weeks. Z30 and Z3 rats were fed the adequate-zinc and marginally low-zinc diet *ad libitum*, respectively. The Z30P rats were pair-fed to the Z3 rats individually. LPS, lipopolysaccharide; Z30, adequate-zinc *ad libitum* control group; Z30P, adequate-zinc pair-fed control group; and Z3, marginally low-zinc group.
[b] There were significant main effects of dietary zinc intake and LPS for serum and liver zinc concentrations. Serum and liver zinc concentrations were lower in the Z3 rats than in the Z30 and Z30P rats, regardless of LPS exposure ($P < 0.05$). LPS exposure reduced serum zinc concentration and increased liver zinc concentration, regardless of dietary zinc intake ($P < 0.05$).
[c] There was a significant interaction between dietary zinc and LPS. Means sharing a common lowercase letter were not significantly different ($P < 0.05$).

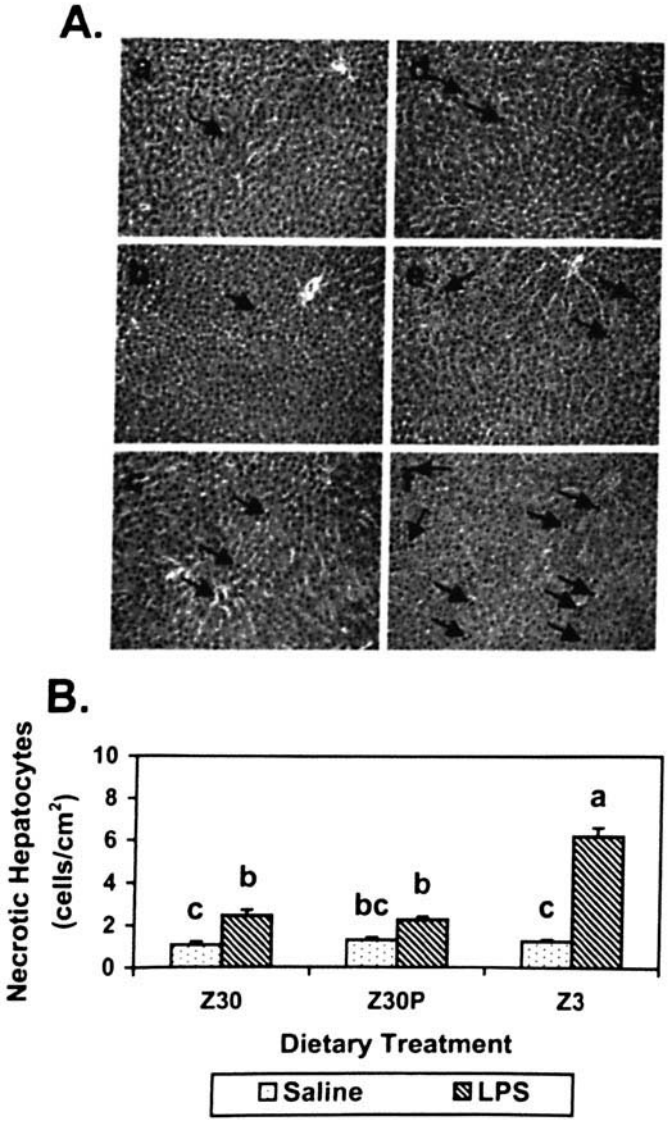

Figure 1. LPS-induced hepatocyte necrosis. Female weanling rats were fed an egg-white–based, modified AIN-93G diet for 6 weeks. Z30 and Z3 rats were fed the adequate-zinc and marginally low-zinc diet *ad libitum*, respectively. The Z30P rats were pair-fed to the Z3 rats individually. (A) Hematoxylin-eosin staining of the necrotic hepatocytes in the LPS-control Z30 (a), Z30P (b), and Z3 (c) rats and the LPS-Z30 (d), LPS-Z30P (e), and LPS-Z3 (f) rats. Magnification: ×200. (B) Frequency of the necrotic hepatocytes. There was a significant interaction between dietary zinc intake and LPS. Values represent means ± SEM ($n = 6$ rats, with the exception of Z30 saline group and Z3 LPS group. In these two groups, $n = 5$ rats). Means sharing a common letter were not significantly different ($P < 0.05$). LPS, lipopolysaccharide; Z30, adequate-zinc *ad libitum* control group; Z30P, adequate-zinc pair-fed control group; and Z3, marginally low-zinc group.

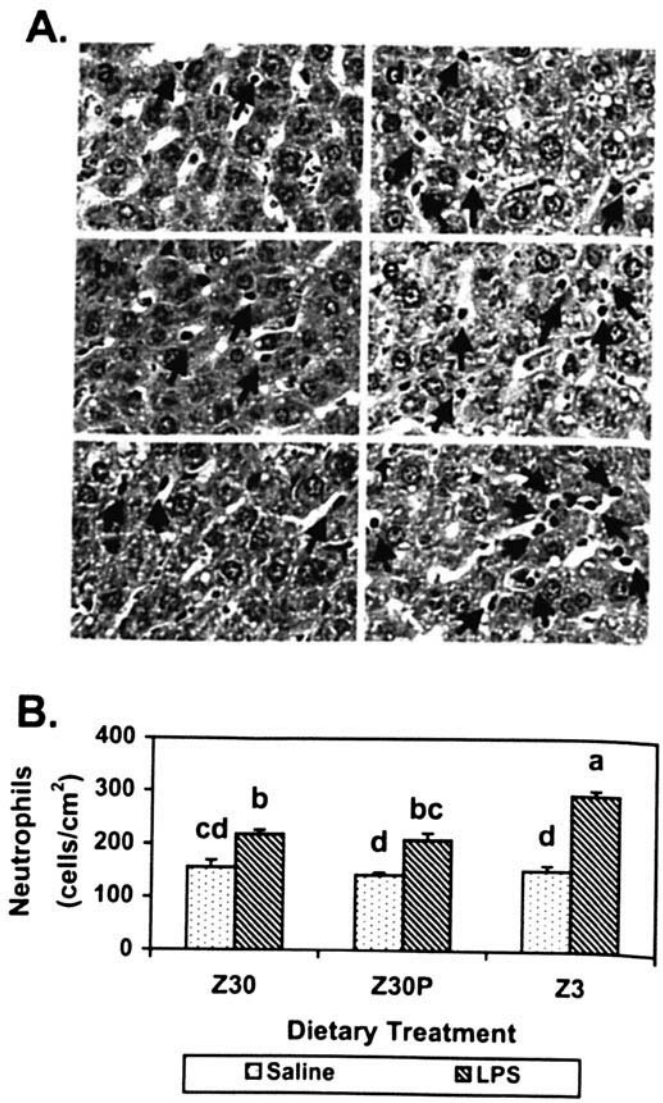

Figure 2. Neutrophil infiltration into liver sinusoids. Female weanling rats were fed an egg-white–based, modified AIN-93G diet for 6 weeks. Z30 and Z3 rats were fed the adequate-zinc and marginally low-zinc diet *ad libitum*, respectively. The Z30P rats were pair-fed to the Z3 rats individually. (A) Hematoxylin-eosin staining of neutrophils in liver sinusoids in the LPS-control Z30 (a), Z30P (b), and Z3 (c) rats and the LPS-Z30 (d), LPS-Z30P (e), and LPS-Z3 (f) rats. Magnification: ×400. (B) Frequency of the neutrophil infiltration. There was a significant interaction between dietary zinc intake and LPS. Values represent means ± SEM ($n = 6$ rats). Means sharing a common letter were not significantly different ($P < 0.05$). LPS, lipopolysaccharide; Z30, adequate-zinc *ad libitum* control group; Z30P, adequate-zinc pair-fed control group; and Z3, marginally low-zinc group.

response to LPS injection (22–24), making it a useful indicator for LPS-induced liver injury. In the present study, LPS exposure resulted in an elevated level of serum ALT in the LPS-Z3 rats compared to the C-Z3 rats. Furthermore, the level of serum ALT was also significantly higher in the LPS-Z3 rats than in the LPS-Z30P and LPS-Z30 rats, indicating that LPS-induced liver injury was more severe in the marginal zinc-deficient rats than in rats with adequate zinc status.

LPS exposure dramatically lowered the serum zinc concentration, but it increased the liver zinc concentration, a result that is consistent with the well-documented acute phase host response to stress, trauma, inflammation, and infection (25–27). Since there was a lack of interactions between dietary zinc intake and LPS on serum and liver zinc concentrations, this shift in tissue zinc concentration indicates that LPS induced zinc redistribution from the serum to the liver, regardless of dietary zinc intake. Treating

mice with a higher dose of LPS (5 mg/kg, ip) results in a greater decrease of the plasma zinc than treating mice with a lower dose of LPS (1 mg/kg, ip) (28). Thus, it appears that the zinc redistribution from serum to the liver is a function of LPS dosage.

LPS exposure increased the frequency of necrotic hepatocytes and induced an extensive infiltration of neutrophils into the sinusoids in the LPS-Z3 rats compared to the LPS-Z30P and LPS-Z30 rats. Neutrophil infiltration into the sinusoids in the liver is a common cytokine-induced inflammatory response (29) that has been shown to play a role in organ damage during sepsis (30, 31). Neutrophil infiltration is stimulated by LPS-induced tumor necrosis factor–α production and, ultimately, triggers hepatocyte necrosis (32, 33). As a key event in the pathologic pathway of LPS-induced liver injury, increased neutrophil infiltration in the LPS-Z3 rats compared to the LPS-Z30P and LPS-Z30 rats strongly indicates an increased severity of LPS-induced liver injury in the LPS-Z3 rats. Therefore, increased frequency of necrotic hepatocytes and neutrophil infiltration in LPS-Z3 rats demonstrated that marginal zinc deficiency renders rats more susceptible to LPS-induced liver damage.

Acute zinc loading appears to provide protection against LPS-induced tissue injury in a number of animal models. Zinc pretreatment prior to LPS exposure inhibits LPS-induced liver injury in rats (8) and grossly improved pulmonary function in pigs (34). In contrast, zinc treatment during LPS exposure resulted in a deleterious outcome (35), while zinc treatment shortly after LPS exposure offered no protection to pigs against LPS-induced lung injury (36). These apparently contradictory findings indicate that the timing of zinc loading is critical to realizing the potential protective effects of zinc. Since endotoxemia often occurs without warning, it would be quite a challenge to precisely determine the appropriate time for loading zinc in its clinical application. Therefore, body zinc status becomes an important component of the host's defense against potential LPS-induced tissue injury. To this end, observations reported herein for the first time provide direct evidence demonstrating that zinc deficiency renders the host more susceptible to LPS-induced liver injury. However, further study of the effect of zinc deficiency on LPS-induced damage in extrahepatic tissues (i.e., lung) and of the mechanisms involved are warranted.

In summary, our data have shown that LPS exposure resulted in an elevated serum ALT level, higher frequency of necrotic hepatocytes, and increased neutrophil infiltration into the hepatic sinusoids in the marginally zinc-deficient rats compared to rats with adequate zinc status. Collectively, these data showed that marginal zinc deficiency increased the susceptibility to LPS-induced liver injury in rats. Since LPS induces liver injury in humans, patients with sepsis who have suboptimal zinc status could be at risk of developing greater liver injury. Moreover, suboptimal zinc status in our society is more prevalent than was once realized. Therefore, these findings may have clinical implications for the nutritional intervention and treatment of patients with sepsis, especially those with inadequate zinc status.

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