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Dietary Zinc Supplementation Ameliorates LPS-Induced Teratogenicity in Mice

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ABSTRACT: Maternal infection during the first trimester of pregnancy has been associated with preterm birth, spontaneous abortion, growth retardation, and congenital anomalies. Previously, our group has shown that subcutaneous injection of zinc prevents endotoxin [lipopolysaccharide (LPS)]-induced teratogenicity. The purpose of this study was to investigate whether increasing or decreasing dietary zinc alters the teratogenic effects of LPS. Female C57BL6 mice were mated and fed diets containing 5, 35, or 100 mg/kg zinc. On gestational day (GD) 8, pregnant dams were injected with either LPS (0.5 mg/kg s.c.) or saline and killed on GD18. LPS-treated fetuses from dams fed 5 and 35 mg/kg zinc diet had a significantly higher number of abnormalities per litter (2- and 1- fold saline controls, respectively) compared with those from LPS + zinc supplemented dams, which were not significantly different from the saline control groups. The beneficial effect and importance of zinc was also reflected in the larger size of fetuses (weight and crown-rump length) from the LPS + zinc–supplemented treatment group. We have demonstrated that low dietary zinc during exposure to infection (*i.e.* LPS) in pregnancy augments the negative impact of LPS alone, and that dietary zinc supplementation throughout pregnancy ameliorates LPS-induced teratogenicity. **(*Pediatr Res* 59: 355–358, 2006)**

Gestational infections, such as maternal fever, and urinary tract infections, such as BV, have been associated with an increased risk for preterm birth, spontaneous abortion, growth retardation, and birth defects in humans and animals (1–8). However, the link between maternal fever, BV, and fetal morbidity has not been elucidated, with the exception of both being associated with the release of proinflammatory cytokines such as IL-1, IL-6, and TNF-α (9,10). Endotoxin or bacterial LPS, a component of the Gram-negative bacterial wall, is a potent inflammogen and is associated with the release of the above cytokines (10). Endotoxin was also found to occur in higher concentrations in women with BV (11). Previous studies in rodents have shown that administration of high intravenous doses (>10 mg/100 kg body weight) of LPS on GD8 caused 100% resorption of implantation sites (12), whereas lower dosing regimes led to development of fetal malformations involving the eye, brain (13), neural crest defects, cleft palate, and limb anomalies (12,14). In contrast, when LPS was given to mice subcutaneously later in gestation (GD13), fetuses that were not resorbed were phenotypically normal (15). This is perhaps not surprising inasmuch as the time of exposure was outside the critical window of organogenesis (GD8–12 in mice). Recently, work from our laboratory (14) has found a mechanistic explanation for LPS teratogenicity whereby MT is the key mediator.

MT is an intracellular, low-molecular-weight, cysteine-rich binding protein with a high affinity for Zn (16–18). Previous studies employing various teratogens(including urethane, α-hederin, 6-mercaptopurine, TNF-α, and valproic acid) suggested that MT and its associated changes in maternal-fetal Zn distribution were the key mediators of teratogenicity (19–24). We and others have shown that alcohol (25) and LPS (26,27) are all potent inducers of MT in the liver resulting in sequestration of plasma Zn in the liver, with a consequent decrease in plasma Zn concentration.

Zn is an important trace element that plays a critical role in growth and development, as it is a cofactor for more than 300 enzymes involved in metabolism and gene regulation. Because only 0.1% of total body Zn is in the plasma, a sudden increase in Zn uptake by the liver can greatly depress plasma Zn concentration (26,28). Such a scenario in early pregnancy is highly unfavorable because the maternal plasma compartment acts as the conduit for fetal Zn supply. Therefore, this change in plasma Zn pool can be likened to a transient fetal Zn deficiency with deleterious effects on the fetus. The involvement of MT in this process can be seen in studies where MT –/– (MT-1 and -2 knockout) mice exposed to alcohol or LPS on GD8 had fetuses that were morphologically unaffected by these teratogens (14,28). Our laboratory (14) has demonstrated that a single intraperitoneal injection of LPS increased liver MT by 30-fold basal from 6 to 24 h, which coincided with an 80% decrease in plasma Zn over the same time period. Moreover, in other studies where we induced MT with alcohol, not only was there a reduced transfer of Zn to the fetus but whole-fetus Zn concentration was significantly less (25).

Previous studies have demonstrated that there are striking similarities in fetal outcome resulting from LPS or alcohol exposure and maternal Zn deficiency in pregnancy. Pregnant mice treated with alcohol and LPS (14,28) presented with

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Abbreviations: BV, bacterial vaginosis; **GD,** gestational day; **LPS,** lipopolysaccharide or endotoxin; **MT,** metallothionein; **TNF-α,** tumor necrosis factor-alpha; **UTI,** urinary track infection; **Zn,** zinc

abnormalities similar to those observed in rodents fed Zn-deficient diet (≤1 mg Zn/kg diet) throughout pregnancy (29–31). The observed abnormalities include craniofacial, neural tube, and limb bud defects (14,28–31). Previously, Keppen and co-workers (32) demonstrated that Zn deficiency acts as a co-teratogen with alcohol and this was prevented in the chronic alcohol model by giving high Zn in the form of a liquid diet throughout pregnancy. Moreover, we showed that alcohol and LPS-induced teratogenicity can be prevented by subcutaneous Zn treatment (14,33). However, whether 1) a diet low in Zn is co-teratogenic with LPS and 2) dietary Zn supplementation throughout pregnancy can prevent the birth abnormalities caused by LPS are the foci of the present study. Here, we demonstrate that dietary Zn supplementation throughout pregnancy ameliorates teratogenicity associated with infection.

MATERIALS AND METHODS

Animals and mating procedure. C57BL6 mice were purchased from the Institute of Medical and Veterinary Science (IMVS) Animal Care Facility (Adelaide, SA, Australia). Mice were maintained in an animal house at 22°C, subject to a 14-h light/10-h dark cycle and had access to water *ad libitum*. All mice were fed a commercial nonpurified diet (Milling Industries, Adelaide, Australia) before and during the mating process.

Mating was carried out by pairing females (20–25 g) with males and examining the females every morning for the presence of a vaginal plug (GD1). Females were then removed from the males and housed individually throughout the duration of experimentation. On GD1, mice were randomly allocated to different treatment groups and fed specially formulated diets containing low Zn (5 mg/kg), normal Zn (35 mg/kg), or supplemented Zn (100 mg/kg) throughout their pregnancy.

All animal-related protocol followed the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes and was approved by the Animal Care and Ethics Committees of the Institute of Medical and Veterinary Science (IMVS), Adelaide, and the University of Adelaide.

Diet and composition. All animals were fed a purified diet containing spray-dried egg white (ICN Biomedicals, Aurora, OH) as a protein source. The basic diet composed of AIN-93G mineral mix with no added Zn and AIN-93VX vitamin mix (ICN Biomedicals, Aurora, OH). The basic mixture without Zn had a Zn content of approximately 1 mg/kg. Appropriate amount of aqueous $ZnSO_4$ was added to the mixture to produce diets deficient, normal, and supplemented with Zn. The low-Zn diet contained 5 mg/kg Zn, representing a moderate state of Zn deprivation, which at the same time can support pregnancy (34). The control diet contained 35 mg/kg Zn, whereas the supplemented diet contained 100 mg/kg Zn, which enabled maximal growth and reproduction (34,35). Zn concentrations in all diets were confirmed using flame atomic absorption spectrophotometry and food intake of all animals was measured every alternate day.

Dietary Zn and GD8 LPS treatment. Mice were randomly allocated into six different groups where they were either treated with LPS or saline (S), and fed diets containing low (LPS+Zn5 / S+Zn5), normal (LPS+Zn35 / S+Zn35), or supplemented Zn (LPS+Zn100 / S+Zn100) from GD1 throughout pregnancy. On GD8, mice in the LPS group were injected subcutaneously into the nape of the neck with 0.5 μg/g body weight LPS in 0.85% saline. *Escherichia coli* LPS (serotype O111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). Control mice were treated with 0.85% saline in a similar fashion. Food was removed for 4 h after treatment to control for any nutritionally related effects. This dose and route of administration has been demonstrated in studies from this laboratory to initiate highly reproducible induction of hepatic MT and associated hypozincemia. Dams did not exhibit any behavioral abnormalities or obvious signs of toxicity such as diarrhea after treatment.

LPS, Zn, and GD18 fetal morphology. Mice were killed on GD18 using halothane anesthesia and bled by cardiac puncture followed by cervical luxation. Uteri were immediately excised, weighed, and examined for number of resorption sites. Individual fetuses were separated from the placentas, weighed, and crown-rump length was measured. Fetuses were examined under low power magnification to determine the extent of physical abnormalities such as microphthalmia, anophthalmia, cleft lip, micrognathia, microcephaly, exencephaly, and other obvious malformations.

Statistical analysis. All data were compared with repeated measures ANOVA using general linear model. Where appropriate, the litter was taken as the unit of comparison. Due to nonhomogenous variances, data pertaining to fetal weight and crown-rump length were log transformed before using ANOVA. For presentation purposes, means were back-transformed into the usual units, which were reported in the tables. Nonparametric data such as resorption sites and percentage abnormalities were compared using the Mann-Whitney *U* test. Significance was determined using Tukey's post hoc test and the *t* test.

All statistical analysis was performed using Minitab statistical software (Minitab Inc., State College, PA). Results are presented as mean ± SEM and differences considered significant at $p < 0.05$, unless otherwise stated in the text.

RESULTS

Pregnancy success. LPS-treated mice completed pregnancy 13 out of 27 times a plug was detected (48% success), whereas saline-treated mice had 20 successful pregnancies out of 29 times a plug was observed (69% success). There were no differences in the percentage of successful pregnancies between LPS-treated mice fed 5, 35, or 100 mg/kg Zn diet. However, saline-treated mice fed the low-Zn(5 mg/kg) diet had the lowest pregnancy success (55%) compared with peers fed the normal (35 mg/kg) or supplemented (100 mg/kg) Zn diet (88% and 64%, respectively). The normal success rate as assessed in our laboratory over the years is between 80 and 90%. Only the saline-treated mice on normal Zn diet fell within this category.

LPS, Zn, and teratogenicity. LPS combined with Zn deficiency had a more severe effect on the fetus than LPS or saline combined with normal or supplemented Zn. There were more resorption sites in LPS-treated dams than saline control dams regardless of dietary Zn consumption. LPS dams had 26–57% resorptions per litter compared with 7–27% in saline controls. Although LPS dams on the low and supplemented Zn diets had slightly more resorptions than those fed the normal Zn diet, this observation was not significantly different. However, LPS dams on the normal Zn diet had significantly more resorptions than the saline dams on the same diet (22% *versus* 7%, respectively) (Table 1).

Fetal weights were lower in the LPS + Zn 5 and LPS + Zn 35 groups compared with LPS + Zn 100 group and saline + 35 Zn fetuses ($p < 0.001$), respectively (Table 1). However, there was no difference in fetal weights between saline control fetuses regardless of dietary Zn consumption. LPS-Zn supplemented dams had larger fetuses (in terms of crown-rump length) compared with those from LPS + Zn 35 group. There was no difference in size between LPS + Zn 5 and LPS + Zn 35 fetuses, although fetuses from the latter group were significantly smaller when compared with saline control fetuses exposed to the same diet ($p < 0.001$) (Table 1).

External malformations were most profound in fetuses from LPS + low Zn group (96%) compared with all other treatment/dietary groups (Table 1). The most common abnormalities present were anophthalmia (80%), exencephaly (60%), and microencephaly (40%). These abnormalities were consistent with those observed in a previous LPS study by our laboratory (14). Although these abnormalities were also present in the LPS + Zn 35 group, they occurred at a significantly less frequency. Fetuses on the Zn-supplemented diet were least affected by LPS, mainly exhibiting eye abnor-

Table 1. *GD18 fetal parameters following GD8 LPS treatment with 5, 35, and 100 mg/kg Zn diet throughout pregnancy*

	Treatment/dietary Zn (mg/kg)					
	LPS + 5Zn	LPS + 35Zn	LPS + 100Zn	Saline + 5Zn	Saline + 35Zn	Saline + 100Zn
Maternal data						
Coital plugs (*n*)	9	9	9	9	9	11
Successful pregnancy per litters (*n*)	4	5	4	5	8	7
Live fetuses (*n*)	21	34	21	22	55	44
Litter size	5.3 ± 1.0	6.8 ± 1.0	5.3 ± 1.3	5.5 ± 0.6	6.9 ± 0.8	6.3 ± 1.3
Resorptions (*n*)	12	9	11	6	5	6
Resorption sites per litter	3.0 ± 1.1	1.8 ± 0.5†	2.8 ± 1.6	1.5 ± 0.3	0.6 ± 0.2	0.9 ± 0.5
Fetal data						
Abnormal fetuses (*n*)	20	23	5	6	11	5
Litters with abnormal fetuses	4	5	4	3	4	1
Abnormal fetuses per litter	5.0 ± 0.9¶	4.4 ± 1.0‡	1.3 ± 0.3*	1.5 ± 0.6	1.4 ± 0.9	0.7 ± 0.9
% Abnormalities	96 ± 3.6¶	65 ± 8.7‡	30 ± 9.1*	30 ± 16.4	25 ± 11.8	14 ± 18.9
Weight (g)	0.58 ± 0.06§	0.61 ± 0.02†	0.68 ± 0.05	0.75 ± 0.08	0.74 ± 0.02	0.64 ± 0.05
Crown-rump length (mm)	17.16 ± 0.6	17.02 ± 0.2†§	18.28 ± 0.5	18.84 ± 0.9	18.96 ± 0.3	17.8 ± 0.7

Values represent mean ± SEM where applicable. Data were analyzed as three separate groups: 1) LPS *vs* saline, 2) LPS + 5 *vs* LPS + 35 *vs* LPS + 100 mg/kg Zn, and 3) saline + 5 *vs* saline + 35 *vs* saline + 100 ppm Zn. All data were tested via repeated measures ANOVA. Significance at $p < 0.05$ was determined using Tukey's post hoc test.

* Significantly different between dietary groups within treatment.

† Significantly different between treatments within dietary groups.

¶ and ‡ Significantly different from each other, and different between treatments (LPS or saline) and dietary groups.

§ Significantly different within dietary group within treatment.

malities commonly found in the strain of mice used. Other abnormalities observed included cleft lip, microphthalmia, micrognathia, agnathia, and spinal hemorrhage.

DISCUSSION

Previous studies in our laboratory have shown that a single subcutaneous Zn injection at the time of LPS exposure prevented the development of birth defects (14). In the present study, we demonstrate that feeding mice a low-Zn diet, which was sufficient to support pregnancy, nonetheless augmented the frequency of birth abnormalities when combined with exposure to LPS compared with that of LPS alone. We further demonstrated these abnormalities could be prevented through dietary Zn supplementation throughout pregnancy. These findings lend support to the fact that adequate dietary Zn, especially during pregnancy, plays an important role in fetal growth and development. Moreover, our findings are consistent with previous studies from our laboratory with LPS or alcohol where Zn injection was given at the same time as the teratogens (14,33), thus supporting the proposed mechanism involving MT-mediated Zn redistribution leading to impaired fetal Zn supply, hence jeopardizing the fetus.

Gestational infection, such as BV, urinary tract infections (UTI,) and maternal fever, has been associated with spontaneous abortion, still birth, growth retardation, and birth defects. The occurrence of such infections during the first trimester of pregnancy, the critical window of organogenesis, is particularly detrimental to the offspring, resulting in physiologic and neurologic defects. Both BV and UTI are commonly associated with the release of LPS or endotoxin, a component of the Gram-negative bacterial cell wall (11,13) and a potent inducer of inflammatory cytokines such as IL-1, IL-6, TNF-α, and acute-phase proteins such as MT (9,10,36). It is well documented that TNF-α plays a central role in mediating the pathophysiologic changes associated with LPS exposure by triggering the acute phase response leading to fetotoxicity and teratogenicity. Previously, Taubeneck and co-workers (23) have shown that TNF-α is a strong inducer of liver MT when given as an intraperitoneal injection in mice. Moreover, it was demonstrated that maternal serum TNF-α levels increased rapidly for 1–1.5 h after exposure to LPS (0.05 mg/kg), and, when administered on GD9, approximately 50% embryo lethality was observed within 24 h (10). Furthermore, work from our laboratory (28) has shown that administration of 0.05 mg/kg LPS caused a rapid increase in liver MT levels within the first 5 h, peaking at 30-fold basal after 24 h. This strongly supports the role of TNF-α as a central mediator of the acute-phase response triggered by LPS. However, as TNF-α was not found to directly induce MT in cultured hepatocytes, it most likely mediates its effect through the induction of other cytokines, in particular IL-6, which is a strong inducer of MT both *in vivo* and *in vitro* (37).

MT is a key Zn-binding protein that plays a major role in Zn homeostasis. Findings from our laboratory have shown that an increase in MT due to either LPS or alcohol not only caused a marked depression in plasma Zn that lasts for 12–24 h (14,28) but also decreased Zn transfer to the fetus (25). This impaired Zn supply to the fetus presents as a transient Zn deficiency, which has deleterious impacts on the fetus. Turks *et al.* (38) were the first to observe the teratogenic effects of Zn deficiency in chicks hatched from hens fed Zn-deficient diet. Furthermore, rodents fed Zn-deficient diet ($\leq$1 mg Zn/kg diet) throughout pregnancy had offspring with craniofacial, neural tube, and limb bud defects (29,30). However, whether or not a low-Zn diet combined with exposure to infection or LPS during pregnancy is co-teratogenic has not been studied.

Previously, Keppen *et al.* (32) showed that alcohol and Zn deficiency is co-teratogenic in a chronic alcohol model, which was prevented by high Zn throughout pregnancy.

In the present study, we found that mice fed a low-Zn diet (5 mg/kg) exposed to LPS on GD8 had fetuses with the worst outcomes compared with those from dams fed normal Zn diet (35 ppm) and saline controls on the same diets. Fetuses from the LPS + low Zn group presented with gross neural tube defects and craniofacial anomalies, and exhibited delayed growth and development as evidenced by the lower weights and crown-rump length. More importantly, we demonstrated that dietary Zn supplementation with exposure to LPS prevented the teratogenic effects to basal control levels, most of which consisted of spontaneous (*i.e.* nonteratogenic) malformations, in particular those relating to the eyes. Moreover, fetuses from the Zn-supplemented group regardless of treatment were heavier and had longer crown-rump length at birth compared with fetuses from other groups.

The abortigenic nature of LPS and importance of Zn were also demonstrated in this study. LPS-treated mice were less successful in completing their pregnancy compared with saline controls after detection of a vaginal plug (48 and 69%, respectively; normal, 80–90%). Mice that were fed a low-Zn diet were also unable to complete their pregnancy 55% of the time compared with mice on the normal and supplemented diet. This is indicative of complete resorption of fetal tissue. Furthermore, mice given LPS and fed the low-Zn diet that were successfully pregnant had the most resorption sites and fetuses that were grossly abnormal. Therefore, although LPS exposure may cause intrauterine fetal death and abnormal development of the fetus *via* a direct effect of TNF-α, the major effect appears to be indirect through the MT-mediated changes in maternal Zn homeostasis. This is supported by our previous studies where MT –/– (knockout) fetuses exposed to alcohol or LPS had less fetal resorptions and fewer birth abnormalities compared with their MT +/+ counterparts (14,28).

In conclusion, we have demonstrated that low dietary Zn combined with exposure to LPS in pregnancy augments the negative impact of LPS alone, and that Zn supplementation throughout pregnancy ameliorates LPS-induced teratogenicity and delayed development. This supports our hypothesis that LPS teratogenicity works through the MT-mediated Zn redistribution resulting in a transient fetal Zn deficiency, leading to development of birth defects. Our findings further emphasise the importance of Zn during pregnancy in terms of fetal growth and development. Therefore, in the clinical setting, dietary Zn supplementation during pregnancy appears to be beneficial for ensuring proper growth and development of the fetus and in preventing the development of birth defects.

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