

Zinc Deficiency Increases Plasma Lipids and Atherosclerotic Markers in LDL-Receptor-Deficient Mice¹

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ABSTRACT Low zinc concentration can be associated with an increased risk of cardiovascular diseases. In the current study, we hypothesize that zinc deficiency can increase and zinc supplementation can decrease proatherosclerotic events in LDL receptor knock-out (LDL-R^{-/-}) mice fed a moderate-fat diet. Mice were fed either a zinc-deficient (0 $\mu\text{mol Zn/g}$), a control (0.45 $\mu\text{mol Zn/g}$), or a zinc-supplemented (1.529 $\mu\text{mol Zn/g}$) diet for 4 wk. Mice fed the zinc-deficient diet had significantly increased concentrations of cholesterol and triacylglycerides in the VLDL and HDL fractions. Zinc supplementation decreased these lipid variables compared with control mice. We detected significantly higher concentrations of glutathione reductase mRNA in the thoracic aortae of zinc-deficient mice. Furthermore, inflammatory markers, such as nuclear factor- κB and vascular cell adhesion molecule-1, were significantly increased in zinc-deficient mice compared with mice of the control or supplemented groups. In addition, zinc deficiency significantly reduced the DNA binding activity of peroxisome proliferator activate receptors (PPARs) in liver extracts. Interestingly, mRNA expression levels of PPAR γ were significantly increased in thoracic aortae of zinc-deficient mice, indicating an adaptation process to decreased PPAR signaling. These data provide in vivo evidence of zinc deficiency inducing proinflammatory events in an atherogenic mouse model. These data also suggest that adequate zinc may be a critical component in protective PPAR signaling during atherosclerosis. *J. Nutr.* 135: 2114–2118, 2005.

KEY WORDS: • zinc deficiency • atherosclerosis • inflammation • PPAR • lipoproteins

The development of atherosclerosis is usually due to multiple causes. Current patterns of energy intake frequently show the portion of the daily energy intake that is derived from fat to be well over 30% (1). Such nutritional habits were shown to result in hyperlipidemia and dyslipidemia. Much less attention has been paid to micronutrients, and particularly to minerals. However, epidemiologic studies suggest that in some population groups, low serum concentrations of zinc are associated with coronary artery disease (2). Furthermore, zinc concentrations were significantly lower in atherosclerotic plaques of abdominal aortae of deceased patients with ischemic heart disease and acute myocardial infarction (3). Mechanisms of the protective functions of zinc in the pathogenesis of atherosclerosis,

including vascular cell dysfunction and the inflammatory response, are not clear.

We and others showed that zinc has antioxidant and anti-inflammatory properties (4–7). Zinc is a structural component of proteins that stabilizes thiol groups, thus rendering proteins less prone to oxidation (8). Oxidative stress can induce nuclear factor (NF) κB , a proinflammatory transcription factor involved in mediating endothelial cell activation [reviewed in (9)].

In addition to its antioxidant properties, we reported recently that zinc appears to be essential for the protective properties of peroxisome proliferator activated receptors (PPARs) α and γ in vascular endothelial cells (10). PPARs are nuclear receptors that are involved in many metabolic pathways, including lipid and glucose metabolism (11). PPARs appear to have anti-inflammatory properties that can protect against endothelial cell activation (12,13). All of these features make PPARs attractive targets for pharmaceutical intervention in cardiovascular diseases. Indeed, clinical and exper-

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⁴Abbreviations used: LDL-R^{-/-}, LDL-receptor-deficient; NF, nuclear factor; PPAR, peroxisome proliferator activated receptor; PPARE, PPAR response element; VCAM-1, vascular cell adhesion molecule-1.

imental evidence suggests that PPAR activation decreases the incidence of cardiovascular diseases (11).

Zinc deficiency in atherosclerosis has not been studied in an animal model suitable for atherosclerosis research. LDL-receptor-deficient (LDL-R^{-/-}) mice are a good model because, similar to humans, most cholesterol is carried in LDL (14). Here, we hypothesized that zinc deficiency can induce proatherogenic events such as oxidative stress and induction of inflammatory markers, and that zinc supplementation can reduce these oxidative stress and inflammatory markers. Furthermore, we aimed to determine whether the effect of zinc on inflammation was accompanied by modified PPAR activity.

MATERIALS AND METHODS

Animals and diets. The LDL-R^{-/-} deficient mice used in this study were obtained from The Jackson Laboratory (Stock Number: 002207). Mice were housed and killed at the University of Missouri, and all procedures were in compliance with the IACUC guidelines of the University of Missouri. All mice were 5 wk old at the start of the feeding study. Two batches of diets were prepared and analyzed for zinc concentration using duplicate measurements for both batches (Table 1). Mice were divided into 3 groups ($n = 11$ /treatment): 0 $\mu\text{mol Zn/g}$ diet (deficient), 0.45 $\mu\text{mol Zn/g}$ diet (control), and 1.529 $\mu\text{mol Zn/g}$ diet (supplemented). All diets were prepared based on AIN-93 diets (15) and modified only in their zinc content and protein source (16). Body weights of all mice were determined before, after, and throughout the study. After completion of the study (4 wk), the mice were killed by i.p. pentobarbital injections.

Zinc quantification. Rear limbs of mice were removed at the time of killing. Skin was removed and limbs were stored at -20°C pending analysis. Livers were flash frozen after excision from the mouse and stored at -20°C until analysis. Zinc concentration in femur and liver samples was determined as described previously (17).

Plasma cholesterol and lipoprotein profiles. Blood was drawn from exposed hearts using heparinized syringes. Plasma was obtained by centrifugation of whole blood at $1500 \times g$, 4°C , for 20 min. Plasma cholesterol concentration was determined enzymatically using a commercially available kit (Wako Chemicals).

The lipoprotein profile was determined by HPLC (Chemstation

LC/MSD 1100, Agilent Technologies) (18). The chromatographic profile was monitored by a Diode Array Detector at 280 nm and a Mass Selective Detector (Agilent Technologies). Electrospray and chemical ionization were used to detect triacylglycerides and phospholipids, respectively (19). Proteins were detected by measuring absorbance at 280 nm.

Immunohistochemistry for vascular cell adhesion molecule-1 (VCAM-1) expression in aortic roots. Hearts were separated from thoracic aortae, cut horizontally, immersed in optimal cutting temperature embedding medium, and frozen at -20°C . Sections (10 μm) were cut on a cryostat (Microm HM505N, Carl Zeiss). Immunocytochemistry was performed as described previously (20). Briefly, endogenous peroxidase was inactivated using hydrogen peroxide (3%) in methanol. Samples were blocked in the serum of the secondary antibody host. Primary antibodies for VCAM-1 (PharMingen) were detected using biotinylated secondary antibodies and peroxidase ABC kits (Vectastain, Cat. # PK-4000). Aminoethylcarbazole was used as a chromogen, and sections were counterstained with hematoxylin.

Gene expression analysis. Thoracic aortae were excised from mice and stored in RNeasy (Qiagen) until analysis. Total RNA was isolated from aortae using RNeasy (Qiagen) after removing surrounding adipose and connective tissues. cDNA was generated using the Reverse Transcription System (Promega). Gene expression was determined by real-time PCR using the ABI Prism 7000 and Taq Man Universal PCR Master Mix (Applied Biosystems). Primers and probes were designed to VCAM-1 (gi: 31981429, forward 5' CGT CGC GAG GTT GTT TAG AGT 3', reverse 5' CAA CAG TCA GTC CAA GCA ACA CT 3', probe 5' 6-FAM CAG CAC TCC ATA TGC AGG TCA GAA CGT AAT C TAMRA 3'), PPAR γ (gi: 6755137, forward 5' CAC AAT GCC ATC AGG TTT GG 3', reverse 5' GCT GGT CGA TAT CAC TGG AGA TC 3', probe 5' 6-FAM ACA GGT CGA GAA GGA GAA GCT GTT GG TAMRA 3'), and glutathione reductase (gi: 34785373, forward 5' CGA CCA TGA TTC CAG ATG TTG A 3', reverse 5' TCA GAT TCA GGC CCT TAG AAT TTG 3', probe 5' 6-FAM TGC CTG CTC TGG GCC ATT CGA C/36-TAMRA 3') using the Primer Express 2.0 software (Applied Biosystems) and synthesized by IDT DNA. Detection of 18S RNA utilized predeveloped assay reagents (Applied Biosystems). Relative quantifications employed standard curves for individual genes using serial dilutions of RNA samples expected to show induced gene expression, i.e., a random sample from the zinc-deficient group for VCAM-1 and a random sample from the zinc-supplemented group for glutathione reductase and PPAR γ gene expression studies.

Transcription factor (NF- κ B and PPAR γ) activation studies: electrophoretic mobility shift assay. Left liver lobes were flash frozen and stored at -80°C until analysis. Nuclear extracts containing active proteins were prepared according to the method of Dignam et al. (21). Nuclear extracts were incubated for 25 min with ^{32}P -end-labeled oligonucleotide probes containing the enhancer DNA element for NF- κ B (Promega) or the PPAR response element (PPRE) (Santa Cruz). Incubation at room temperature was performed in the presence of nonspecific competitor DNA. After binding, the complexed and uncomplexed DNA in the mixture was resolved by electrophoresis in a 6.5% (wt:v) nondenaturing polyacrylamide gel and visualized by autoradiography. Control reactions using a supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes. The antibodies used were obtained from Santa Cruz.

Quantifications and statistical analyses. Samples of all mice ($n = 11$) were subjected to analyses of zinc concentrations, plasma lipoproteins, mRNA expression, and transcription factor binding activities. VCAM-1 staining in aortic roots took place in 5 mice/treatment group. Numeric data were analyzed using SYSTAT 7.0. Comparisons between treatments were made by 1-way ANOVA with post-hoc comparisons of the means made by Tukey's least significance difference procedure. Differences with $P < 0.05$ were considered significant. Photomicrographs of VCAM-1 staining in aortic roots were analyzed using a double-blind design. Uncropped pictures of the same magnification were assigned random labels. Staining intensity of photomicrographs was then evaluated by 5 individuals by ranking the specimen according to staining intensity.

TABLE 1

Experimental diets

Ingredient	Zn-deficient	Zn control	Zn-supplemented
	g/kg		
Protein			
Egg white	200	200	200
DL-Methionine	3	3	3
Carbohydrate			
Cornstarch	397.5	397.5	397.5
Sucrose	100	100	100
Cellulose	35	35	35
Dextrose	131	131	131
Fat			
Corn oil	50	50	50
Safflower oil	20	20	20
Choline bitartrate	2.5	2.5	2.5
Minerals			
Mineral mix AIN 93 ¹	50	50	50
Zinc, ² mg/kg	0	30	100
Vitamin mix 93	10	10	10
Biotin	1	1	1

¹ The mineral mix used did not contain zinc (15,16).

² Actual zinc concentrations of the diets as determined by atomic absorption were 0.4 ± 0.1 , 33.1 ± 0.3 , and 109.9 ± 1.5 mg/kg, respectively.

TABLE 2

Lipid concentrations in plasma lipoproteins of mice fed control, Zn-deficient, or Zn-supplemented diets for 4 wk^{1,2}

Component	Zn-deficient	Zn control	Zn-supplemented
Free cholesterol			
VLDL	18978 ± 1688 ^a	14811 ± 1138 ^{ab}	12035 ± 939 ^b
LDL	4811 ± 723	5245 ± 352	5366 ± 434
HDL	636 ± 52 ^{ab}	689 ± 46 ^a	489 ± 25 ^b
Cholesterol esters			
VLDL	2863 ± 228 ^a	1093 ± 205 ^b	696 ± 86 ^b
LDL	2121 ± 103 ^a	1022 ± 180 ^b	767 ± 111 ^b
Phospholipids			
VLDL	59582 ± 5779 ^a	48338 ± 3602 ^{ab}	37398 ± 3247 ^b
LDL	62119 ± 5767	67803 ± 2894	64873 ± 3240
HDL	11150 ± 2163 ^a	9770 ± 1213 ^{ab}	4949 ± 496 ^b
Triglycerides			
VLDL	17818 ± 975 ^a	9394 ± 1897 ^b	2077 ± 1186 ^c
LDL	13162 ± 675 ^a	8190 ± 1512 ^a	1451 ± 1201 ^b

¹ Data are presented as means ± SEM, *n* = 11. Means in a row without a common letter differ, *P* < 0.05.² Values shown are arbitrary units derived from detection peak height.

RESULTS

Body weight and tissue zinc concentrations. The combined body weight of all mice at the beginning of the study was 19.0 ± 0.3 g. After the 4-wk study period, the weight of mice fed the zinc-deficient diet decreased to 18.4 ± 0.7 g, whereas the weights of the control or zinc supplemented mice were 21.0 ± 0.4 g and 20.8 ± 0.4 g, respectively. Femur zinc concentration changed significantly according to the dietary zinc mice received. Zinc concentrations were significantly lower in zinc-deficient mice and significantly higher in zinc-supplemented mice compared with control mice (data not shown). However, there was no change in total zinc concentration in liver tissues (data not shown).

Plasma total cholesterol and lipoprotein concentrations of triacylglycerides, cholesterol, and phospholipids. Although the LDL fraction was not affected, free cholesterol was significantly reduced by zinc supplementation in the VLDL and HDL fractions compared with the zinc-deficient mice (Table 2). Cholesterol esters were significantly higher in VLDLs and LDLs of zinc-deficient mice compared with the 2 other groups (Table 2). Triacylglycerides differed significantly in VLDLs among all groups, with the lowest concentrations in zinc-supplemented mice. In addition, total cholesterol, as determined enzymatically, was modified by zinc. Zinc-supplemented

mice had significantly lower (4.24 ± 0.25 mmol/L) total plasma cholesterol concentrations than zinc-deficient mice (5.22 ± 0.33 mmol/L). Control mice had cholesterol values (4.59 ± 0.26 mmol/L) that did not differ from those of the other groups. Triacylglycerides in the LDL fraction also were significantly different in zinc-supplemented mice compared with the other 2 groups (Table 2). Phospholipids did not differ among treatment groups in the LDL fraction but were significantly different in the VLDL and HDL fractions of zinc-supplemented compared with zinc-deficient mice, with the highest concentrations in zinc-deficient mice (Table 2).

mRNA expression of glutathione reductase. Mice deficient in zinc (0 μmol/g) had a significantly higher mRNA expression of the antioxidant enzyme glutathione reductase as determined by RT-PCR of RNA extracted from thoracic aortae. The control and zinc-supplemented groups did not differ in their expression of this gene.

Liver transcription factor activities. Zinc deficiency significantly increased the DNA binding activity of the proinflammatory transcription factor NF-κB in liver tissues (Table 3). Control and zinc-supplemented mice did not differ in DNA binding activity of this transcription factor. On the other hand, the anti-inflammatory transcription factor PPAR_γ was most activated in the livers of control mice, and the lowest DNA binding levels occurred in zinc-deficient mice (Table 3).

TABLE 3

Zinc-dependent changes in liver transcription factors and aortic mRNA levels of genes involved in inflammation of mice fed control, Zn-deficient, or Zn-supplemented diets for 4 wk¹

Component	Zn-deficient	Zn control	Zn-supplemented
DNA binding ²			
NF-κB	117.5 ± 19.5 ^a	70.8 ± 12.3 ^b	66.1 ± 9.2 ^b
PPAR _γ	7.7 ± 2.1 ^c	23.3 ± 4.4 ^a	13.5 ± 2.6 ^b
mRNA ³			
VCAM-1	11.5 ± 2.5 ^a	4.6 ± 1.4 ^b	5.9 ± 2.0 ^b
PPAR _γ	15.0 ± 5.1 ^a	5.1 ± 1.6 ^b	6.8 ± 1.3 ^{ab}
Glutathione reductase	24.6 ± 3.5 ^a	9.8 ± 0.7 ^b	8.9 ± 0.9 ^b

¹ Values are means ± SEM, *n* = 11. Means in a row without a common letter differ, *P* < 0.05.² Values are arbitrary units obtained by radiography.³ Values are arbitrary units obtained by RT-real time PCR. Data shown were normalized to β-actin expression.

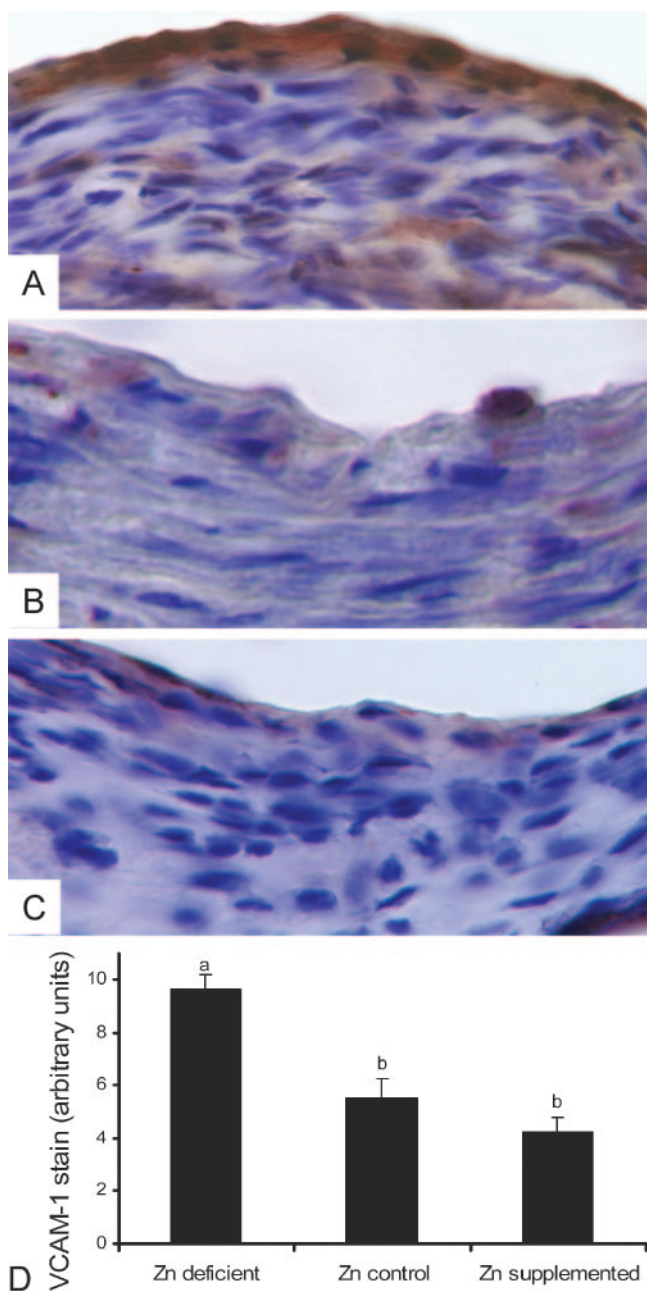


FIGURE 1 VCAM-1 protein in thoracic aortae of mice fed Zn-deficient (A), control (B), or Zn-supplemented (C) diets for 4 wk. VCAM-1 expression was detected in aortic roots by immunohistochemistry. Pictures are representative examples. Nuclei can be seen in blue and VCAM-1 staining on the endothelium in red. Quantitative data (D) are means \pm SEM, $n = 6$. The arbitrary units are VCAM-1 expression as determined by ranking slides according to the staining intensity. Means without a common letter differ, $P < 0.05$.

VCAM-1 mRNA and protein. VCAM-1 mRNA expression was significantly greater in thoracic aortae of mice fed a zinc-deficient diet compared with the other 2 groups (Table 3). VCAM-1 expression was confirmed by immunohistochemistry of aortic roots (Fig. 1). Tissues of zinc-deficient mice exhibited a significantly higher amount of immunostaining for VCAM-1 than tissues from control or zinc-supplemented mice.

PPAR γ mRNA expression. PPAR γ mRNA was significantly greater in thoracic aortae of mice fed the zinc-deficient

diet than in those of mice fed the control diet (Table 3). PPAR γ mRNA expression values from mice in the zinc-supplemented group did not differ from those of the other groups.

DISCUSSION

Although zinc deficiency is more common in Third World countries, dietary zinc intake is still below recommendations among certain population groups in industrialized countries (22). Zinc deficiency has been associated with growth retardation, impaired immune functions, delayed wound healing, and skin and taste abnormalities (23). The role of zinc deficiency in atherosclerosis is not well defined; however, epidemiologic studies suggest that in some population groups, low serum concentrations of zinc are associated with coronary artery disease (2). The results of the current study add evidence to the importance of zinc in preventing atherosclerotic events in LDL-R^{-/-} mice fed a moderate-fat diet.

As expected, femur zinc was significantly modified by both zinc deficiency and supplementation. On the other hand, we did not detect changes in the liver zinc concentrations, indicating a mobilization of zinc stored in bone to replenish soft tissue zinc pools.

The effect of zinc on plasma lipid concentrations remains a controversial subject. Other investigators studying different model systems found increased concentrations of cholesterol, phospholipids, VLDL, and intermediate density lipoproteins during zinc deficiency (24). On the other hand, it was reported that zinc deficiency decreased plasma and VLDL triacylglyceride concentrations in rats (25). However, this effect was attributed mainly to the lower food intake resulting from zinc deficiency. The effect of zinc deficiency has never been studied in a mouse model appropriate for studying atherosclerosis. In this study, we utilized LDL-R^{-/-} mice, which have a lipoprotein profile similar to that of humans (14). Here, we observed a striking difference in cholesterol, triacylglycerides, and phospholipids in the lipoprotein fractions between zinc-deficient and zinc-supplemented mice. The lipid-lowering effect of zinc was also shown in humans (26). The mechanism for the effect of zinc on lipoproteins is not clear. It was suggested that zinc is required for enzymes involved in lipid synthesis and lipoprotein excretion (27). For example, Porsch-Ozcurumez et al. (28) reported that zinc finger protein 202 (ZNF202) inhibited the expression of ATP-binding cassette transporter A1 and G1, both responsible for the efflux of cholesterol and phospholipids.

The upregulated expression of glutathione reductase in the thoracic aortae of zinc-deficient mice could be the result of increased oxidative stress with resulting upregulation of genes involved in the defense against free radicals. Furthermore, we observed increased plasma isoprostane concentrations in mice fed a zinc-deficient diet, also indicating an increase in oxidative stress due to zinc deficiency (data not shown). Isoprostanes are prostaglandin-like compounds that are formed from the peroxidation of arachidonic acid; they can be used as reliable and quantifiable markers of oxidative stress (29).

Oxidative stress can induce inflammation and contribute to the progression of atherosclerosis. We and others reported previously that inflammatory pathways, such as the NF- κ B pathway, are sensitive to oxidative stress. We showed in endothelial cell cultures that zinc deficiency can induce NF- κ B DNA binding activity [reviewed in (30)]. Here, we provide in vivo evidence for the zinc deficiency-mediated induction of NF- κ B activity in liver extracts. NF- κ B has a key role in the transcription of inflammatory genes that induce endothelial

cell dysfunction and thus mediate monocyte recruitment. VCAM-1 is a very important example of a NF- κ B target gene that is involved in leukocyte attachment to the endothelial cells and diapedesis through the endothelium (31). The fact that VCAM-1 mRNA and protein expression were upregulated in aortic tissues of mice fed a zinc-deficient diet indicates that NF- κ B was most likely activated not only in livers but also in the vasculature. In fact, we previously observed a marked induction of NF- κ B in cultured endothelial cells during zinc deficiency (32).

NF- κ B activation and VCAM-1 expression were reported to be inhibited by PPAR α and γ agonists (12,13). PPARs are nuclear receptors expressed in a wide variety of tissues, including vascular endothelial cells (13). We reported recently that zinc is required for the anti-inflammatory properties of PPAR α and γ agonists against tumor necrosis factor-induced endothelial cell activation (10). In the current study, we showed that PPAR binding activity in liver extracts can be modified by nutritional zinc intake. Because zinc concentrations in liver tissues did not change in response to various amounts of dietary zinc, the link between zinc and PPAR DNA binding activity remains unresolved. Previous data obtained from cell culture studies, however, showed that PPAR α and γ agonists could not induce PPAR binding activity in zinc-deficient endothelial cells (10).

We have provided in vivo evidence that zinc deficiency induces atherosclerotic events in LDL-R $^{-/-}$ mice. We showed a significant upregulation of VCAM-1 in aortic tissues and increased plasma lipids in zinc-deficient mice. Furthermore, zinc deficiency appeared to induce oxidative stress and modify PPAR expression and activity. Zinc supplementation decreased plasma lipids below control levels but did not significantly improve other markers compared with the control diet. Because dietary zinc intake of certain population groups is still below intake recommendations (22), these data underline the importance of zinc for the prevention of cardiovascular diseases such as atherosclerosis.

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