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Oral zinc supplementation does not improve oxidative stress or vascular function in patients with type 2 diabetes with normal zinc levels☆,☆☆

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ABSTRACT

Objective: There is considerable controversy about what constitutes optimal zinc intakes in patients with type 2 diabetes mellitus. Several studies suggest that higher zinc intakes improve vascular function and decrease oxidative damage. We aimed to assess the effects of zinc supplementation using a range of reliable biomarkers of oxidative damage and vascular function in patients with type 2 diabetes.
Methods: Forty male type 2 diabetic patients were supplemented either with 240 mg/day of zinc as zinc gluconate ($n=20$) or with placebo ($n=20$) for 3 months. Blood and spot urine samples were taken at baseline, days 3 and 7, months 1, 2 and 3 during supplementation and 1 month after cessation. Serum zinc, reliable biomarkers of oxidative damage (F_2-isoprostanes, neuroprostanes, cholesterol oxidation products, allantoin) as well as hydroxyeicosatetraenoic acid products and vascular-related indices (augmentation index, pulse wave velocity and aortic pressure) were measured.
Results: Despite significantly higher levels of serum zinc in the treatment group, markers of oxidative damage, levels of hydroxyeicosatetraenoic acid products and vascular indices were unchanged by zinc supplementation during the four-month study period.
Conclusion: Improving the zinc status in patients with type 2 diabetes with normal zinc levels did not have any impact on oxidative damage and vascular function, and such supplementation may not be generally beneficial in these individuals.

1. Introduction

Diabetes mellitus is widely reported to be accompanied by increased levels of oxidative damage, which are associated with hyperglycemia, hyperinsulinemia, hypercholesterolemia and insulin resistance [1,2]. The vast majority of cases of diabetes fall into two broad etiopathogenic categories: patients with type 1 diabetes have a deficiency of insulin secretion while those with type 2 diabetes have a combination of resistance to insulin action and an inadequate compensatory insulin secretory response [3]. Zinc appears to exert insulin-like effects by supporting the signal transduction response to insulin and by reducing the production of cytokines, which lead to beta-cell death during the inflammatory process in the pancreas [4]. Low serum or plasma zinc levels have been observed in some patients with diabetes mellitus, due to (in part) loss of zinc in urine [5], leading to several suggestions that oral zinc supplementation may be beneficial in these individuals [6]. At high levels, zinc could hypothetically decrease the extent of oxidative damage through several mechanisms: by displacing pro-oxidant metals (such as iron and copper) from membranes or lipoproteins, thus decreasing free radical production at the ligand-binding site; and through its role in the Cu–Zn superoxide dismutase enzyme [6]. In animals, zinc supplementation lowers elevated blood glucose in genetically obese mice [7] and reduces the extent of lipid peroxidation and atherosclerotic plaques in rabbits on a high-cholesterol diet even though these animals are not zinc-deficient [8].

In previous studies, oral zinc supplementation (30 mg/day) has been claimed to lower levels of oxidative damage (as monitored by plasma thiobarbituric acid-reactive substances, TBARs) and result in improved zinc status [9–11]. By contrast, a meta-analysis of controlled trials involving 14,238 subjects concluded that there is no effect of zinc supplementation on plasma lipoproteins [12]. A systematic review did not support a role of zinc supplementation in the prevention of type 2 diabetes [13]. In the Zenith study, no

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beneficial effect on oxidative stress and antioxidant defences was found in middle-aged and elderly subjects who received zinc supplementation [14]. In diabetic patients with normal baseline serum zinc, higher oral zinc doses (100 mg/day) are capable of increasing zinc to supranormal levels [15]. These studies, however, lacked accurate measurements of oxidative damage. Thiobarbituric acid, for example, reacts with a wide variety of chemical species that are unrelated to lipid peroxidation and is probably an unreliable measure of oxidative damage in humans [16,17]. Zinc is thought to have a putative protective role against the development of atherosclerosis. Previous studies have shown that dietary zinc intake correlates inversely with subclinical atherosclerosis [18] and zinc supplementation down-regulates the production of atherosclerosis-related cytokines/molecules in humans [19].

We performed a randomized placebo-controlled study to assess the effects of zinc supplementation on oxidative damage (using established and reliable biomarkers) and vascular function among patients with type 2 diabetes mellitus. F_2-isoprostanes (F_2-IsoPs), products of oxidative damage of arachidonic acid, are widely thought to be the best biomarker for assessing *in vivo* oxidative stress related to lipid oxidation [20,21]. F_2-IsoPs are present in an esterified form in phospholipids and are released in a free form through the activities of phospholipase A_2 (PLA_2) and platelet activating factor-acetylhydrolase (PAF-AH) [22]. Increases in F_2-IsoPs have been observed in diabetes mellitus, coronary artery disease, stroke and Parkinson disease [1,23–25]. Neuroprostanes (F_4-NPs), on the other hand, are oxidation products of docosahexaenoic acid (DHA) that are highly concentrated in neuronal membranes and have been associated with mild cognitive impairment and dementia [26–28]. Arachidonic acid can also be oxidized by free radicals, lipoxygenase and cytochrome P450 enzymes, to produce epoxyeicosatrienoic acid products (EETs) or hydroxyeicosatetraenoic acid products (HETEs) [29–31]. Numerous forms of HETE exist (5-, 8-, 9-, 11-, 12-, 15-, 20-HETE). Some of these isomers are thought to participate in the pathogenesis of cerebral vasospasm and tumor progression, and their levels are elevated during inflammation [32,33].

Our study also examined another biomarker of oxidative stress, allantoin, an oxidation product of uric acid [34,35]. Allantoin levels respond rapidly to changes in oxidative stress status [36]. We additionally measured cholesterol oxidation products (COPs), products of cholesterol oxidized by enzymatic cytochrome P450-dependent reactions (to give 24- and 27-hydroxycholesterol, and 7α-hydroxycholesterol) or by oxidative damage (to give 7β-hydroxycholesterol and 7-ketocholesterol) [37]. Several studies have shown changes in COPs in dengue fever, Parkinson disease and stroke [24,25,38].

2. Materials and methods

2.1. Study participants

Forty male type 2 diabetic patients, recruited from the outpatient clinic at the National University Hospital, Singapore, were randomized to receive two tablets of either zinc gluconate (GNC, USA) or placebo (99% microcystalline cellulose, 1% magnesium stearate) per day for 3 months. Each tablet, stated to contain 100 mg zinc in the gluconate form, consistently contained 120 mg elemental zinc (determined in every batch by graphite furnace atomic absorption spectrometer, Varian SpectraAA-220G). The choice of oral zinc at 240 mg/day for three month duration was made on the basis of previous findings that demonstrated safety at this dosage and potential efficacy for this duration in diabetic patients [15,39,40]. Subjects aged 21 years and above who fulfilled the American Diabetes Association criteria for the diagnosis of diabetes mellitus (fasting plasma glucose ≥7.0 mmol/L or plasma glucose ≥11.1 mmol/L following 75-g oral glucose tolerance test) were included [3]. We excluded those who had consumed over-the counter or prescription drugs, vitamin/mineral supplements or traditional Chinese remedies, suffered acute infection less than 30-days prior to the start of the study, had been diagnosed with active neuropsychiatric disease or hematological diseases, had hemoglobin less than 10 g/dL, had previous use of narcotic drugs or regular alcohol intake in excess of 14 units per week. Power calculations, performed *a priori* on the primary variables (i.e., F_2-IsoPs) derived from data of our pilot study, indicated that a minimum sample size of 19 was required in each treatment group to assess differences in biomarker levels over time. Study controls comprised age-matched non-diabetic healthy individuals from a previous study [41]. The study protocol was approved by the Domain-Specific Review Board, National Healthcare Group, Singapore. Each participant provided written informed consent prior to their study participation.

2.2. Supplementation and sampling of blood and urine

A hospital research pharmacist was engaged to randomize, blind the assigned treatment groups and count the remaining tablets so as to assess compliance. Blood and spot urine samples were taken following an 8-h fast at baseline (before supplementation), days 3 and 7, months 1, 2 and 3 during supplementation and 1 month following the intake of zinc or placebo (washout). An aliquot of blood was collected in plain or EDTA tubes for serum or plasma separation, respectively. Heparin zinc-free anticoagulants were used in this study.

2.3. Markers of oxidative damage

Blood samples collected into EDTA tubes were centrifuged, indomethacin and butylated hydroxytoluene added into the plasma, and fresh urine samples placed in polypropylene tubes. The prepared samples were stored at −80 °C until analysis [42]. Oxidative stress related biomarkers, F_2-IsoPs, F_4-NPs, COPs and allantoin, as well as HETEs, were measured by gas chromatography–mass spectrometry (GC–MS) using previously described methods [35,42–44]. Urinary creatinine levels were measured to standardize urinary F_2-IsoPs and HETEs (Sigma Diagnostic kit, USA). Urate was measured in plasma using high performance-liquid chromatography [35]. Phospholipase A_2 (PLA_2) and platelet-activating factor acetylhydrolase (PAF-AH) activities (Cayman, Ann Arbor, MI) were measured in serum.

2.4. Laboratory parameters

Blood glucose, HbA1c and insulin, serum high-sensitivity C-reactive protein (hs-CRP), cholesterol, LDL, HDL, triglyceride and iron were measured using the Cobas C111 analyzer (Roche Diagnostics, Switzerland). Serum zinc and copper levels were determined by flame atomic absorption spectrophotometer at the Referral Laboratory, Singapore General Hospital, Singapore [45]. White and red blood counts, hemoglobin, hematocrit concentrations, platelets, neutrophils and lymphocytes, were assessed using the Full Blood Count Analyzer (Sysmex, Japan).

2.5. Vascular indices

The technique of pulse wave analysis was used to determine the aortic pressure and augmentation index [46]. Pulse wave velocity recordings were made from the radial artery using a Millar tonometer, and data were collected and analyzed using the SphygmoCor (SphygmoCor 2000 v7.0, PWV Medical, Sydney, Australia),

which allows continuous recording of the radial artery pressure waveform. The augmentation index (defined as augmented pressure divided by pulse pressure and expressed as a percentage) is a composite measure of the magnitude of wave reflections and arterial stiffness, which affects timing of wave reflections. Because the augmentation index is influenced by changes in heart rate, it was also corrected accordingly. Plasma nitrate/nitrite was measured by GC–MS using methods adapted from [47].

2.6. Statistical analyses

Statistical analysis was performed using by GraphPad Prism version 5.0 for Macintosh (GraphPad Prism Software, CA, USA). All values are expressed as mean ± standard deviation. Differences between groups were assessed using Student's *t*-test and within group differences were assessed using ANOVA with Bonferroni adjustments for multiple comparisons. Statistical significance was considered when $p < 0.05$.

3. Results

All enrolled subjects completed the study and their compliance (determined by pill counting) to the medication regimen was above 95% in both groups. The mean age was 55 ± 8 years in the placebo group and 57 ± 9 years for zinc group. Their body mass indices (BMI) were 26 ± 8 and 25 ± 3, respectively. Both groups comprised a mixture of ethnicities: Chinese, Malays, Indians and Caucasian (placebo 16:2:1:1; zinc 13:5:2:0). Their vascular risk factors included hypertension (26/40), hyperlipidemia (41/40), ischemic heart disease (4/40) and previous ischemic stroke (10/40), which were comparable between treatment groups. The mean duration of diabetes was 8 ± 8 years (range, 1–40 years). Mild symptoms of gastrointestinal intolerance were observed in fifteen patients; these symptoms resolved within the first three days of zinc intake. The remaining subjects tolerated the study medications well. The dose 240 mg/day was chosen to ensure that sufficient zinc level was achieved and on the basis of the reported safety of high-dose zinc in previous studies [39,48].

The levels of iron, copper and hematological parameters were monitored as a safety measure in light of anecdotal reports that suggested the potential for high-dose zinc to suppress absorption of iron and copper, potentially leading to severe anemia [49]. These levels are shown in Fig. 1. The baseline zinc was 12.9 ± 1.4 μmol/L in the placebo group and 14.1 ± 2.1 μmol/L in the zinc group (Fig. 1A). None of the study participants had serum zinc levels below 10.7 μmol/L (normal, 10.7–20.2 μmol/L), a cut-off used (in our clinical laboratory and in previous studies [10,11]) to diagnose zinc deficiency. Zinc supplementation significantly increased serum zinc levels from 33% (day 3) up to 78% (month 3); zinc levels remained 21% higher than baseline levels even after 1 month washout. Placebo supplementation did not alter serum zinc level.

The baseline levels of copper were not different between placebo and zinc groups (Fig. 1B). Zinc supplementation appeared to lower copper level significantly at months 2 and 3, but to levels that were still within the normal reference range, 11.8–22.0 μmol/L. There were no changes in copper levels in the placebo group. Iron levels did not differ between placebo and zinc group at baseline and during subsequent study follow-up (Fig. 1C). Although there were no significant differences in baseline urinary albumin levels ($p = 0.20$), a greater loss of urinary albumin was observed in the zinc group (baseline, 31 ± 61 mg/L vs month 4, 70 ± 196 mg/L) as compared with the placebo group (baseline, 69 ± 135 mg/L vs month 4, 27 ± 20 mg/L). Serum glucose, insulin, hsCRP, cholesterol, LDL, HDL, triglyceride and PAF-AH and PLA_2 activities at baseline did not differ between the placebo and zinc

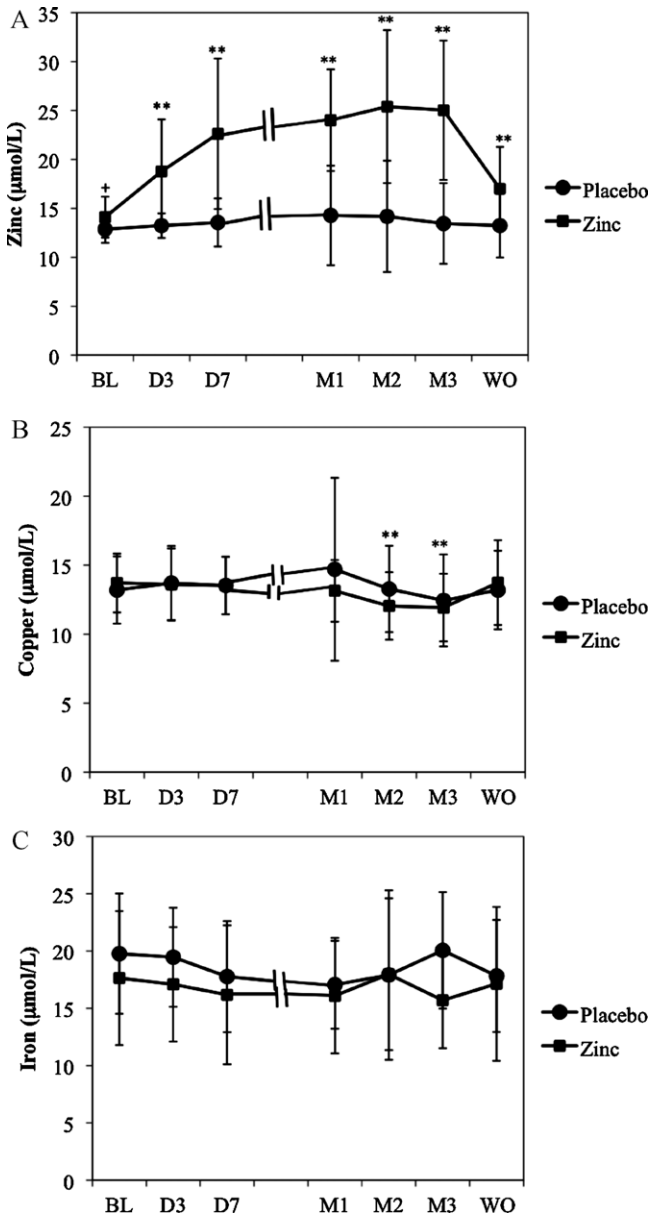

Fig. 1. Serum zinc, copper and iron levels of study participants. $^{+}$ denotes $p < 0.05$ between placebo and zinc groups; ** denotes $p < 0.01$ between baseline levels and subsequent time-points (using unpaired Student's *t*-test, $n = 20$ for each group). Error bars indicate standard deviation (SD). Abbreviations: BL, baseline; D, day; M, month; WO, washout.

groups. Similarly, glycosylated hemoglobin (HbA1c) did not differ between groups at baseline (placebo 8.2 ± 1.8%, zinc 7.7 ± 1.9%) and following washout (placebo 7.6 ± 1.7%, zinc 7.5 ± 1.5%). There was no significant effect of zinc or placebo on serum glucose, insulin, cholesterol, LDL, HDL, triglyceride and PAF-AH and PLA_2 activities (Table 1). A non-significant trend to a decrease in hs-CRP levels was observed in patients who received zinc supplementation (Table 1). There were comparable hematological profiles at baseline between groups. Zinc supplementation did not have any effect on these hematological parameters (Table 1).

We measured several reliable biomarkers of oxidative damage, namely oxidation products of arachidonic acid, docosahexaenoic acid, cholesterol and urate. The levels of these biomarkers were significantly higher in patients with diabetes mellitus compared with age-matched non-diabetic controls, except for urinary 2,3-dinor-5,6-dihydro F_2-IsoPs, 7-ketocholesterol, allantoin and

Table 1
Changes in biochemical and hematological parameters before, during and after placebo or zinc supplementation.

		Baseline	Supplementation					Washout	ANOVA[b]
		Day 1[a]	Day 3	Day 7	Month 1	Month 2	Month 3	Month 4	
Biochemical parameters									
Glucose (mmol/L)	P	9.0 ± 3.2	8.9 ± 3.0	8.8 ± 3.2	8.7 ± 3.0	8.5 ± 2.9	8.6 ± 3.2	8.0 ± 3.1	0.11
	Zn	8.3 ± 2.8	8.0 ± 2.9	8.0 ± 2.9	7.7 ± 2.8	8.0 ± 2.3	7.8 ± 2.1	8.4 ± 2.8	0.25
Insulin (mU/L)	P	15.9 ± 7.7	13.6 ± 10.1	15.7 ± 12.4	15.2 ± 11.7	15.1 ± 10.8	14.1 ± 9.3	15.2 ± 11	0.57
	Zn	13.4 ± 7.7	11.7 ± 5.6	13.1 ± 5.5	13.0 ± 5.4	11.7 ± 3.6	13.7 ± 8.4	13.6 ± 6.5	0.87
hsCRP (mg/L) (median, IQR)	P	0.80 (0.15–3.71)	0.92 (0.12–6.00)	0.82 (0.08–5.46)	0.74 (0.12–4.43)	0.71 (0.16–4.91)	0.77 (0.07–6.00)	0.87 (0.07–10.72)	0.96
	Zn	0.99 (0.23–4.82)	0.90 (0.21–5.38)	0.71 (0.23–4.32)	0.73 (0.20–2.31)	0.65 (0.15–5.86)	0.72 (0.11–2.87)	1.08 (0.14–7.33)	0.37
Cholesterol (mmol/L)	P	4.83 ± 1.41	4.94 ± 1.52	4.80 ± 1.37	4.85 ± 1.47	4.93 ± 1.38	4.97 ± 1.47	4.93 ± 1.34	0.88
	Zn	4.56 ± 0.82	4.72 ± 0.88	4.62 ± 0.78	4.63 ± 0.80	4.64 ± 0.80	4.53 ± 0.93	4.51 ± 0.87	0.87
LDL (mmol/L)	P	2.88 ± 1.10	3.18 ± 1.20	3.16 ± 1.31	3.24 ± 1.42	3.31 ± 1.37	3.34 ± 1.50	3.07 ± 1.25	0.07
	Zn	2.76 ± 0.72	3.11 ± 0.98	3.06 ± 0.98	3.10 ± 0.92	3.05 ± 0.88	3.01 ± 1.01	2.74 ± 0.79	0.22
HDL (mmol/L)	P	1.00 ± 0.21	1.00 ± 0.25	0.98 ± 0.21	0.99 ± 0.18	1.00 ± 0.25	1.01 ± 0.19	1.08 ± 0.23	0.89
	Zn	1.07 ± 0.27	1.01 ± 0.23	1.04 ± 0.25	0.97 ± 0.23[b]	0.98 ± 0.22	1.00 ± 0.24	1.11 ± 0.26	0.18
Triglyceride (mmol/L)	P	1.90 ± 1.43	1.93 ± 1.61	1.80 ± 0.98	1.87 ± 0.99	1.70 ± 0.66	1.90 ± 1.30	1.73 ± 0.64	0.86
	Zn	1.47 ± 0.52	1.57 ± 0.70	1.51 ± 0.57	1.65 ± 0.63	1.72 ± 0.67	1.56 ± 0.55	1.40 ± 0.49	0.28
PAF-AH (μmol/min/ml)	P	0.016 ± 0.006	0.015 ± 0.007	0.015 ± 0.007	0.016 ± 0.008	0.016 ± 0.008	0.016 ± 0.006	0.016 ± 0.006	0.89
	Zn	0.015 ± 0.004	0.013 ± 0.003	0.015 ± 0.004	0.015 ± 0.006	0.016 ± 0.006	0.015 ± 0.005	0.015 ± 0.005	0.08
PLA_2 (μmol/min/ml)	P	0.0067 ± 0.0009	0.0061 ± 0.0010	0.0063 ± 0.0009	0.0060 ± 0.0010	0.0062 ± 0.0008	0.0062 ± 0.0008	0.0061 ± 0.0009	0.06
	Zn	0.0063 ± 0.0012	0.0062 ± 0.0008	0.0064 ± 0.0008	0.0064 ± 0.0008	0.0065 ± 0.0010	0.0064 ± 0.0008	0.0065 ± 0.0012	0.82
Hematological parameters									
Hemoglobin (g/dL)	P	14.6 ± 1.2	14.5 ± 1.1	14.5 ± 1.3	14.3 ± 1.3	14.5 ± 1.5	14.6 ± 1.3	14.3 ± 1.1	0.70
	Zn	14.4 ± 1.5	14.4 ± 1.5	14.2 ± 1.5	13.9 ± 1.6	13.8 ± 1.6	14.0 ± 1.3	14.1 ± 1.6	0.83
Hematocrit (%)	P	43 ± 4	43 ± 4	43 ± 4	43 ± 5	43 ± 4	43 ± 4	43 ± 4	0.73
	Zn	43 ± 4	43 ± 4	42 ± 4	42 ± 4	42 ± 4	42 ± 3	42 ± 4	0.08
WBC ($\times 10^3/\mu L$)	P	7.1 ± 2.0	7.0 ± 2.0	6.9 ± 1.8	7.0 ± 1.9	6.9 ± 2.1	6.7 ± 1.8	6.9 ± 1.9	0.56
	Zn	6.6 ± 1.7	6.9 ± 1.6	7.1 ± 1.4	6.9 ± 1.8	6.8 ± 1.9	6.4 ± 1.5	6.5 ± 1.5	0.04
RBC ($\times 10^3/\mu L$)	P	5.1 ± 0.6	5.1 ± 0.6	5.1 ± 0.6	5.0 ± 0.6	5.1 ± 0.7	5.1 ± 0.6	5.1 ± 0.6	0.50
	Zn	5.1 ± 0.7	5.0 ± 0.7	5.0 ± 0.7	4.9 ± 0.6	4.9 ± 0.7	5.0 ± 0.6	5.0 ± 0.7	0.59
Lymphocytes ($\times 10^3/\mu L$)	P	2.2 ± 0.7	2.1 ± 0.7	3.2 ± 5.3	2.0 ± 0.6	2.1 ± 0.6	2.0 ± 0.6	2.0 ± 0.7	0.48
	Zn	2.0 ± 0.5	2.1 ± 0.6	1.9 ± 0.4	2.0 ± 0.5	2.0 ± 0.6	1.9 ± 0.4	2.0 ± 0.6	0.11
Platelets ($\times 10^3/\mu L$)	P	267 ± 45	260 ± 46	257 ± 43	267 ± 48	267 ± 63	265 ± 50	274 ± 47	0.48
	Zn	261 ± 50	267 ± 52	270 ± 49	268 ± 52	267 ± 56	258 ± 56	269 ± 71	0.56
Neutrophils ($\times 10^3/\mu L$)	P	4.2 ± 1.5	4.3 ± 1.4	4.3 ± 1.3	4.4 ± 1.5	4.2 ± 1.4	4.1 ± 1.3	4.3 ± 1.2	0.74
	Zn	5.2 ± 5.7	5.3 ± 5.5	4.5 ± 0.9	4.3 ± 1.4	4.2 ± 2.3	3.9 ± 1.1	3.9 ± 1.2	0.46

Values are expressed as mean ± standard deviation.
P, placebo group ($n = 20$); Zn, zinc group ($n = 20$).
Abbreviations: hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PAF-AH, platelet activating factor-acetylhydrolase; PLA_2, phospholipase A_2; WBC, white blood count; RBC, red blood count; ANOVA, analysis of variance; IQR, interquartile range.

[a] No significant differences in each parameter at the beginning of the study between placebo and zinc groups.

[b] No significant main effects for each variable, and no significant interactions between group and time.

Table 2
Comparison of biomarkers of oxidative damage between diabetic subjects and age-matched non-diabetic controls.

	Diabetic subjects ($n=40$)	Non-diabetic controls ($n=40$)	p Values[a]
Arachidonate (μg/ml)	76.5 ± 17.7	90.2 ± 23.8	0.006
Plasma F_2-IsoPs			
Total F_2-IsoPs (ng/ml)	1.32 ± 0.68	0.38 ± 0.14	<0.001
Esterified F_2-IsoPs (ng/ml)	1.23 ± 0.68	0.32 ± 0.14	<0.001
Free F_2-IsoPs (ng/ml)	0.09 ± 0.02	0.06 ± 0.02	<0.001
Total F_2-IsoPs/AA (pg/μg)	17.7 ± 8.7	4.4 ± 1.8	<0.001
Esterified F_2-IsoPs/AA (pg/μg)	16.6 ± 8.6	3.6 ± 1.7	<0.001
Urinary F_2-IsoPs			
Urinary F_2-IsoPs (ng/mg Cr)	1.02 ± 0.56	0.59 ± 0.32	<0.001
Urinary 2,3-dinor-5,6-dihydro F_2-IsoPs (ng/mg Cr)	5.56 ± 3.07	4.80 ± 2.85	0.775
Urinary 2,3-dinor F_2-IsoPs (ng/mg Cr)	5.08 ± 5.90	2.47 ± 2.17	<0.001
Plasma HETEs			
Total HETEs (ng/ml)	35.3 ± 13.8	25.4 ± 9.1	<0.001
Esterified HETEs (ng/ml)	33.4 ± 13.8	23.3 ± 9.2	<0.001
Free HETEs (ng/ml)	1.85 ± 2.03	2.08 ± 1.58	0.578
Total HETEs/AA (ng/μg)	0.79 ± 0.65	0.29 ± 0.09	<0.001
Esterified HETEs/AA (ng/μg)	0.76 ± 0.65	0.26 ± 0.09	<0.001
Urinary HETEs (ng/mg Cr)	14.5 ± 12.4		
Docosahexaenoate (μg/ml)	21.4 ± 6.6	17.8 ± 5.5	0.010
Plasma neuroprostanes			
Total neuroprostanes (ng/ml)	0.86 ± 0.44	0.45 ± 0.24	<0.001
Total neuroprostanes/DHA (pg/μg)	42.4 ± 22.2	26.4 ± 13.2	<0.001
Cholesterol oxidation products			
7β-Hydroxycholesterol (ng/mg cholesterol)	3.0 ± 2.0	2.2 ± 0.8	0.018
7-Ketocholesterol (ng/mg cholesterol)	11.9 ± 12.1	15.4 ± 6.2	0.104
7α-Hydroxycholesterol (ng/mg cholesterol)	11.9 ± 6.8	13.2 ± 8.2	0.451
24-Hydroxycholesterol (ng/mg cholesterol)	9.9 ± 4.8	8.2 ± 6.2	0.200
27-Hydroxycholesterol (ng/mg cholesterol)	14.5 ± 4.5	32.2 ± 9.2	<0.001
Allantoin (μM)	1.63 ± 0.94	3.0 ± 0.86	<0.001
Urate (μM)	345 ± 145	359 ± 119	0.475
Allantoin/urate	5.2 ± 3.6	7.9 ± 4.9	0.018

Values are expressed as mean ± standard deviation.
[a] Unpaired t-test between diabetic subjects and non-diabetic controls.

7α-hydroxycholesterol (Table 2). Zinc supplementation did not alter the extent of oxidative damage in patients with type 2 diabetes, as none of the measured biomarkers (F_2-IsoPs, F_4-NPs, COPs, urate and allantoin) showed significant differences as compared with baseline levels and between treatment groups. The levels of HETEs were similarly not changed with zinc supplementation (Table 3).

Larger values of augmentation index indicate increased wave reflections from the periphery and/or earlier return of the reflected wave as a result of increased pulse-wave velocity (due to increased arterial stiffness). In this study, no differences were observed in the augmentation index between the placebo and zinc groups at baseline and during supplementation. In addition, no difference was found in other vascular functions measured, such as central systolic and diastolic blood pressure and pulse wave velocity (PWV). Similarly, plasma nitrate/nitrite levels, as an index of vascular nitric oxide production, were unchanged between placebo and zinc groups (Table 4).

4. Discussion

Although the USA RDA for zinc in men is 11 mg [50], there have been suggestions that higher zinc levels through oral supplementation may be advantageous [9–11,51,52]. Some of these benefits include amelioration in the extent of oxidative damage and vascular dysfunction, as well as decreasing the incidence of infection and enhancing bone formation [9,10,51,52]. Different doses of oral zinc supplementation have been studied, from 25 mg/day up to 150 mg/day [9–11,15,39,40,48,53,54]. Excessively high levels of zinc, however, may manifest clinically as nausea, abdominal cramps, vomiting and diarrhea [49]. Toxic zinc levels could potentially lead to copper deficiency, impaired immune function and deranged lipid profiles [49]. In this study, three in four patients developed mild features of gastrointestinal intolerance at oral zinc dose of 240 mg/day, which spontaneously resolved within the first week of treatment. In keeping with data from previous studies, none of these patients had abnormal changes in serum copper, iron, lipid and hematological parameters [15,39,40]. Despite achieving a two-fold increase in zinc levels, we did not observe changes in markers of oxidative damage and vascular function among patients receiving zinc supplementation.

These findings appear to contrast with observation in previous studies that observed lower TBARs and antioxidant enzymes (superoxide dismutase and glutathione peroxidase) after six months of low-dose zinc supplementation (30 mg/day) [10,11]. In these studies, a 15% reduction in TBARs was observed after six months of 30 mg/day zinc supplementation, although there were no changes found after three months. Antioxidant levels were unchanged. These studies included a cohort of patients with a longer duration of diabetes, poorer glycemic control and higher body mass index; close to a third of whom had serum zinc levels below 10.7 μmol/L [10,11]. As alluded to earlier, TBARs are prone to artefactual results and are unreliable as markers of oxidative damage *in vivo* [16,17].

We measured the 'gold standard marker' of oxidative stress, F_2-IsoPs, in both plasma and urine and also other biomarkers of oxidative stress [20,21]. PLA_2 and PAF-AH enzymatic activities catalyze the release of the free form of F_2-IsoPs, some of which are subsequently excreted in the urine [22]. The enzymatic activities of PLA_2 and PAF-AH were unaffected by zinc supplementation (Table 1). Despite reports in the literature of the profound effects of zinc on cells of the immune system among patients with diabetes mellitus [52,55], we did not observe any association between serum zinc and white blood cells and their differential counts. Mixed reports are found on the effect of zinc on glycemic control among

Table 3
Changes in markers of oxidative damage before, during and after placebo or zinc supplementation.

		Baseline	Supplementation					Washout	ANOVA[b]
		Day 1[a]	Day 3	Day 7	Month 1	Month 2	Month 3	Month 4	
Arachidonate (μg/ml)	P	80.1 ± 20.3	78.1 ± 17.6	79.2 ± 16.6	78.6 ± 16.8	82.8 ± 20.5	78.3 ± 18.23	79.5 ± 17.1	0.74
	Zn	72.4 ± 17.0	74.7 ± 17.7	74.3 ± 19.3	77.5 ± 22.4	79.1 ± 21.35	83.9 ± 25.4	80.7 ± 20.4	0.60
Total F_2-IsoPs (ng/ml)	P	1.40 ± 0.70	1.13 ± 0.42	1.12 ± 0.53	1.09 ± 0.56	1.07 ± 0.53	1.28 ± 0.63	1.21 ± 0.46	0.10
	Zn	1.19 ± 0.50	1.11 ± 0.48	1.13 ± 0.51	1.09 ± 0.34	1.25 ± 0.55	1.10 ± 0.51	1.21 ± 0.52	0.74
Esterified F_2-IsoPs (ng/ml)	P	1.31 ± 0.71	1.04 ± 0.43	1.04 ± 0.52	1.00 ± 0.55	0.99 ± 0.53	1.19 ± 0.62	1.12 ± 0.05	0.11
	Zn	1.15 ± 0.65	1.04 ± 0.47	1.05 ± 0.50	1.01 ± 0.34	1.22 ± 0.67	1.02 ± 0.50	1.13 ± 0.52	0.52
Free F_2-IsoPs (ng/ml)	P	0.09 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.21
	Zn	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.08 ± 0.02	0.27
Total F_2-IsoPs/AA (pg/μg)	P	18.1 ± 9.2	15.5 ± 7.6	14.9 ± 8.4	14.8 ± 8.8	13.5 ± 7.0	18.1 ± 11.6	16.0 ± 7.6	0.10
	Zn	17.8 ± 8.4	16.2 ± 9.5	15.4 ± 7.5	15.2 ± 6.9	17.0 ± 8.9	14.0 ± 8.2	15.8 ± 7.9	0.33
Esterified F_2-IsoPs/AA (pg/μg)	P	17.0 ± 9.2	14.3 ± 7.4	13.8 ± 8.2	13.7 ± 8.6	12.4 ± 6.8	16.9 ± 11.2	14.8 ± 7.5	0.10
	Zn	16.6 ± 8.2	15.1 ± 9.1	14.3 ± 7.3	14.0 ± 6.6	15.9 ± 8.6	13.0 ± 7.9	14.7 ± 7.7	0.36
Urinary F_2-IsoPs (ng/mg Cr)	P	0.90 ± 0.50	1.00 ± 0.49	0.88 ± 0.53	0.90 ± 0.59	0.79 ± 0.68	0.91 ± 0.60	0.93 ± 0.60	0.83
	Zn	1.08 ± 0.60	1.01 ± 0.57	0.91 ± 0.55	0.96 ± 0.92	0.86 ± 0.43	1.06 ± 0.91	0.95 ± 0.55	0.87
Urinary 2,3-dinor-5,6-dihydro F_2-IsoPs (ng/mg Cr)	P	3.52 ± 2.08	4.07 ± 2.66	3.76 ± 2.62	4.06 ± 3.20	3.78 ± 3.32	4.32 ± 3.48	4.17 ± 3.60	0.80
	Zn	4.06 ± 2.56	3.50 ± 2.46	3.08 ± 2.60	3.99 ± 3.93	3.87 ± 3.00	3.18 ± 2.77	3.69 ± 3.62	0.59
Urinary 2,3-dinor F_2-IsoPs (ng/mg Cr)	P	5.32 ± 3.42	5.35 ± 3.53	6.60 ± 5.25	5.96 ± 4.69	4.86 ± 3.22	6.58 ± 6.13	5.15 ± 4.37	0.61
	Zn	5.70 ± 3.07	6.82 ± 4.93	7.39 ± 5.21	8.16 ± 7.05	7.24 ± 5.10	6.89 ± 4.52	6.59 ± 4.82	0.58
Total HETEs (ng/ml)	P	33.8 ± 10.4	35.4 ± 13.8	38.6 ± 12.6	35.2 ± 10.1	35.6 ± 11.7	33.9 ± 12.8	38.0 ± 11.5	0.60
	Zn	36.7 ± 16.9	35.6 ± 14.3	34.5 ± 11.8	32.4 ± 12.8	32.1 ± 11.6	33.0 ± 15.7	32.7 ± 16.0	0.66
Esterified HETEs (ng/ml)	P	32.0 ± 11.0	33.8 ± 13.8	37.0 ± 13.4	33.3 ± 10.2	32.8 ± 11.9	32.4 ± 13.0	35.8 ± 11.6	0.59
	Zn	34.9 ± 16.7	33.8 ± 14.8	32.7 ± 11.6	30.0 ± 12.7	30.2 ± 11.3	32.3 ± 16.1	30.3 ± 16.0	0.61
Free HETEs (ng/ml)	P	1.85 ± 2.51	1.63 ± 1.24	1.59 ± 1.83	1.97 ± 1.50	2.80 ± 2.74	1.51 ± 1.52	2.23 ± 1.88	0.16
	Zn	1.82 ± 1.70	1.84 ± 1.59	1.74 ± 1.45	2.39 ± 2.18	1.90 ± 1.69	1.69 ± 1.80	2.37 ± 2.16	0.76
Total HETEs/AA (ng/μg)	P	0.98 ± 0.81	1.25 ± 1.28	1.16 ± 1.01	1.08 ± 1.12	1.56 ± 2.00	0.97 ± 1.03	1.19 ± 1.30	0.26
	Zn	0.67 ± 0.49	0.59 ± 0.34	0.64 ± 0.41	0.58 ± 0.36	0.55 ± 0.34	0.58 ± 0.44	0.59 ± 0.40	0.62
Esterified HETEs/AA (ng/μg)	P	0.98 ± 0.81	1.25 ± 1.28	1.16 ± 1.01	1.08 ± 1.12	1.56 ± 2.00	0.97 ± 1.03	1.19 ± 1.30	0.26
	Zn	0.69 ± 0.51	0.64 ± 0.44	0.70 ± 0.56	0.78 ± 1.11	0.75 ± 1.19	0.78 ± 1.19	0.59 ± 0.40	0.96
Urinary HETEs (ng/mg Cr)	P	16.8 ± 15.5	24.9 ± 23.6	23.8 ± 20.8	13.55 ± 10.9	23.4 ± 18.4	21.1 ± 15.2	17.5 ± 11.5	0.12
	Zn	12.1 ± 9.9	21.7 ± 18.3	15.4 ± 10.9	19.8 ± 18.1	18.3 ± 17.2	21.5 ± 15.9	15.7 ± 15.3	0.10
Docosahexaenoate (μg/ml)	P	22.3 ± 7.7	22.1 ± 7.6	22.1 ± 9.2	23.0 ± 10.5	22.1 ± 8.3	22.4 ± 7.0	22.8 ± 8.3	0.97
	Zn	20.6 ± 5.9	20.0 ± 5.4	20.4 ± 5.6	20.2 ± 5.9	22.1 ± 6.5	20.5 ± 6.1	22.0 ± 7.2	0.48
Total neuroprostanes (ng/ml)	P	0.93 ± 0.57	0.86 ± 0.36	0.87 ± 0.42	0.81 ± 0.39	0.90 ± 0.45	0.84 ± 0.44	0.88 ± 0.43	0.83
	Zn	0.81 ± 0.23	0.94 ± 0.54	0.91 ± 0.62	0.81 ± 0.43	0.75 ± 0.38	0.68 ± 0.43	0.88 ± 0.66	0.14

Esterified neuroprostanes (ng/ml)	P	0.79 ± 0.56	0.77 ± 0.36	0.77 ± 0.41	0.67 ± 0.36	0.78 ± 0.42	0.73 ± 0.44	0.75 ± 0.38	0.77
	Zn	0.71 ± 0.28	0.87 ± o.52	0.79 ± 0.61	0.73 ± 0.39	0.65 ± 0.36	0.60 ± 0.43	0.80 ± 0.68	0.16
Free neuroprostanes (ng/ml)	P	0.14 ± 0.21	0.10 ± 0.11	0.10 ± 0.12	0.14 ± 0.14	0.12 ± 0.11	0.11 ± 0.10	0.13 ± 0.14	0.37
	Zn	0.10 ± 0.10	0.08 ± 0.09	0.12 ± 0.19	0.09 ± 0.09	0.10 ± 0.11	0.08 ± 0.08	0.08 ± 0.09	0.29
Total neuroprostanes/DHA (pg/μg)	P	44.8 ± 28.2	43.5 ± 29.1	45.3 ± 28.3	40.3 ± 24.6	41.8 ± 17.6	39.3 ± 21.1	41.4 ± 24.4	0.77
	Zn	41.7 ± 16.0	46.6 ± 19.6	43.5 ± 19.7	41.9 ± 25.3	36.1 ± 17.7	34.0 ± 18.3	40.7 ± 25.0	0.17
Esterified neuroprostanes/DHA (pg/μg)	P	37.9 ± 26.4	38.5 ± 28.7	39.8 ± 26.6	32.6 ± 22.2	35.9 ± 16.5	33.8 ± 19.3	35.0 ± 19.8	0.59
	Zn	37.0 ± 17.6	42.6 ± 19.2	37.1 ± 19.4	37.1 ± 22.9	31.3 ± 17.9	30.3 ± 18.6	36.3 ± 22.7	0.21
7β-Hydroxycholesterol (ng/mg cholesterol)	P	5.9 ± 8.4	5.5 ± 6.4	4.8 ± 6.1	4.9 ± 6.1	4.3 ± 4.5	4.4 ± 5.1	4.7 ± 7.1	0.38
	Zn	4.3 ± 4.1	4.7 ± 5.2	4.2 ± 3.7	4.5 ± 4.7	5.3 ± 6.1	5.5 ± 6.9	5.0 ± 5.4	0.44
7-Ketocholesterol (ng/mg cholesterol)	P	12.5 ± 15.9	13.7 ± 15.8	14.1 ± 13.2	14.0 ± 13.2	13.3 ± 17.5	11.9 ± 14.4	10.1 ± 8.5	0.30
	Zn	11.4 ± 7.2	10.2 ± 4.9	11.6 ± 6.2	10.9 ± 7.4	11.5 ± 7.4	11.5 ± 7.4	9.7 ± 4.0	0.84
7α-Hydroxycholesterol (ng/mg cholesterol)	P	13.5 ± 6.7	13.9 ± 7.2	12.2 ± 3.7	14.3 ± 7.5	14.5 ± 7.2	12.4 ± 4.4	12.1 ± 6.2	0.66
	Zn	10.4 ± 6.9	10.5 ± 4.6	10.6 ± 4.9	10.0 ± 4.9	11.8 ± 5.9	12.7 ± 6.1	12.1 ± 7.2	0.33
24-Hydroxycholesterol (ng/mg cholesterol)	P	10.2 ± 5.5	10.4 ± 5.6	10.1 ± 5.0	10.3 ± 6.9	9.8 ± 6.4	10.9 ± 7.7	10.7 ± 0.8	0.96
	Zn	9.4 ± 4.5	9.8 ± 7.5	11.5 ± 5.8[b]	9.3 ± 4.2	10.7 ± 6.7	10.8 ± 6.5	11.9 ± 6.6	0.42
27-Hydroxycholesterol (ng/mg cholesterol)	P	14.0 ± 5.5	12.0 ± 4.1	14.6 ± 5.4	13.7 ± 3.5	13.2 ± 4.3	13.5 ± 5.3	13.4 ± 5.0	0.70
	Zn	14.8 ± 3.3	13.9 ± 5.2	14.0 ± 3.4	13.8 ± 3.3	13.2 ± 3.4	13.8 ± 4.0	14.6 ± 6.3	0.74
Allantoin (μM)	P	1.62 ± 0.94	1.55 ± 0.92	1.44 ± 0.78	1.48 ± 0.69	1.38 ± 0.52	1.54 ± 0.67	1.58 ± 0.65	0.77
	Zn	1.50 ± 0.87	1.64 ± 0.79	1.46 ± 0.82	1.71 ± 0.86	1.52 ± 0.99	1.57 ± 0.88	1.59 ± 0.76	0.77
Urate (μM)	P	393 ± 155	391 ± 176	385 ± 148	399 ± 197	387 ± 190	400 ± 192	408 ± 174	0.97
	Zn	324 ± 137	307 ± 136	332 ± 119	333 ± 136	304 ± 78	345 ± 156	307 ± 101	0.59
Allantoin/urate	P	4.2 ± 2.0	4.3 ± 2.7	5.0 ± 6.4	4.4 ± 2.8	4.3 ± 2.9	4.7 ± 3.9	4.4 ± 2.2	0.91
	Zn	5.2 ± 3.9	5.7 ± 3.2	4.7 ± 3.3	5.4 ± 3.1	5.3 ± 3.1	5.1 ± 3.1	5.5 ± 2.9	0.71

Values are expressed as mean ± standard deviation.
P, placebo group ($n = 20$); Zn, zinc group ($n = 20$).
Abbreviations: F_2-IsoPs, F_2-isoprostanes; HETEs, hydroxyeicosatetraenoic acid products; AA, arachidonate; DHA, docosahexaenoate; Cr, creatinine; ANOVA, analysis of variance.

[a] No significant differences in each parameter at the beginning of the study between placebo and zinc groups.

[b] No significant main effects for each variable, and no significant interactions between group and time.

Table 4
Changes in vascular indices before, during and after placebo or zinc supplementation.

		Baseline	Supplementation					Washout	ANOVA[b]
		Day 1[a]	Day 3	Day 7	Month 1	Month 2	Month 3	Month 4	
Nitrite (μM)	P	2.71 ± 0.85	2.71 ± 0.65	2.53 ± 0.76	2.80 ± 0.76	2.43 ± 0.93	2.48 ± 0.67	2.56 ± 0.79	0.17
	Zn	2.91 ± 0.66	2.85 ± 1.10	2.75 ± 0.71	2.89 ± 1.86	2.40 ± 0.88	2.47 ± 0.71	2.73 ± 0.79	0.20
Nitrate (μM)	P	66.9 ± 26.4	73.9 ± 25.3	74.5 ± 29.3	81.3 ± 49.8	90.6 ± 60.8	74.2 ± 25.6	90.8 ± 43.5	0.28
	Zn	79.7 ± 42.9	75.3 ± 29.4	66.1 ± 30.2	83.9 ± 40.9	66.7 ± 24.0	68.9 ± 28.2	73.5 ± 30.3	0.20
Total nitrate + nitrite	P	69.6 ± 26.6	76.6 ± 25.4	77.1 ± 29.6	84.1 ± 50.1	93.0 ± 60.9	76.7 ± 25.6	93.4 ± 43.6	0.30
	Zn	82.6 ± 43.0	78.1 ± 29.8	68.9 ± 30.1	86.8 ± 40.6	69.1 ± 24.2	71.4 ± 28.5	76.3 ± 30.6	0.18
SBP (mm Hg)	P	116 ± 20	115 ± 19	119 ± 20	117 ± 20	120 ± 18	116 ± 15	113 ± 15	0.33
	Zn	120 ± 19	123 ± 15	120 ± 11	119 ± 14	120 ± 13	117 ± 14	120 ± 14	0.53
DBP (mm Hg)	P	76 ± 8	76 ± 11	77 ± 11	74 ± 11	77 ± 12	74 ± 9	73 ± 9	0.33
	Zn	77 ± 9	79 ± 9	76 ± 5	77 ± 8	77 ± 9	76 ± 9	77 ± 9	0.63
Augmentation index (%)	P	17.0 ± 11.5	14.3 ± 12.9	13.8 ± 13.0	16.6 ± 12.8	17.7 ± 10.1	17.4 ± 11.6	16.7 ± 10.5	0.06
	Zn	21.8 ± 7.4	24.1 ± 7.0	21.4 ± 8.9	22.7 ± 9.5	23.3 ± 7.9	22.0 ± 8.3	22.0 ± 6.5	0.44
Pulse wave velocity (m/s)	P	8.6 ± 1.3	8.9 ± 1.5	8.7 ± 1.4	8.8 ± 1.1	9.1 ± 1.0	9.2 ± 1.1	9.1 ± 1.3	0.14
	Zn	8.2 ± 1.6	8.4 ± 1.7	8.4 ± 1.5	8.8 ± 1.1	8.8 ± 0.8	8.7 ± 1.1	8.9 ± 1.1	0.09

Values are expressed as mean ± standard deviation.
P, placebo group ($n = 20$); Zn, zinc group ($n = 20$).
Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; ANOVA, analysis of variance.
[a] No significant differences in each parameter at the beginning of the study between placebo and zinc groups.
[b] No significant main effects for each variable, and no significant interactions between group and time.

patients with type 2 diabetes. Zinc supplementation has been reported to decrease [53] or have no effect on glycemic parameters such as Hb1Ac and glucose levels [10,11,40,56]. Despite improving zinc status, we did not observe differences in the glycemic control among patients with type 2 diabetes treated with oral zinc as compared with placebo. Serum glucose (measured throughout the study) and HbA1c indices at baseline and washout period (when serum zinc was still elevated) were not affected by zinc supplementation. The non-significant decrease in hs-CRP levels in patients who received zinc supplementation is consistent with findings of previous studies, highlighting the potential for zinc to exert anti-inflammatory effects *in vivo* [6,19].

Vascular function is (in part) determined by the endogenous generation of nitric oxide. Nitric oxide is normally produced by endothelial nitric oxide synthase (eNOS) in the vasculature, which is a crucial mediator of endothelium-dependent vasodilatation. The generation of superoxide will react with nitric oxide to give peroxynitrite that can eventually uncouple eNOS and consequently impair endothelial function and insulin signaling [57]. Abou-Seif et al. showed high serum nitrite and approximately 50% lower plasma zinc level in patients with type 2 diabetes compared with healthy volunteers [58]. By contrast, at normal serum zinc level, nitrate was higher in our cohort of diabetic patients as compared with levels in a healthy cohort (111 μM vs 74 μM) (data not shown).

Zinc is bound principally (up to 70%) to albumin and is tightly regulated to keep zinc concentration in a narrow range. This is achieved through: complex interactions between zinc sensors (such as metal responsive element-binding transcription factor-1) and cell signaling machinery; the trafficking of zinc through the cell by metallothionein (MT); and the synthesis and/or degradation of proteins that bind zinc with high affinity [6,59]. Despite this, serum zinc levels nearly doubled in the supplemented group who received 240 mg zinc/day. The rise in serum zinc is consistent with previous observations that zinc levels are increased following its supplementation [9–11,15,39,48,53,54]. In this study, greater loss of urinary albumin was observed in the zinc group compared with the placebo group, which suggests that less albumin might be available for binding to zinc. These data indicate that oral zinc, at these doses, could overwhelm the natural elimination mechanisms and tight homeostatic mechanisms in humans.

Our study has several limitations. Patients were not randomized according to zinc levels and, although there were no gender restrictions, we were only able to recruit men into the study. As such, these findings cannot be generalized to women and diabetic subjects with suboptimal zinc levels. It is not known whether the function of zinc cotransporters (such as the zinc transporter 8, ZnT8), which regulate beta cell insulin processing and secretion [60], vary in our patients and possibly explain the lack of zinc effects. To conclude, high-dose zinc supplementation did not have any impact on oxidative damage and vascular function in patients with type 2 diabetes mellitus with normal zinc levels. Although it remains possible that a longer period of supplementation may produce different results and oral zinc may be advantageous in patients whose zinc levels are suboptimal, our findings do not support a beneficial effect of high-dose zinc supplementation in diabetic subjects who are not previously demonstrated to be zinc-deficient.

Conflict of interest

None.

Acknowledgments

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