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Original Contribution

Zinc supplementation decreases the development of atherosclerosis in rabbits

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Abstract

Developing atherosclerotic plaques in cholesterol-fed rabbits are enriched in iron but depleted in zinc. In order to examine further the role of zinc, New Zealand White rabbits were fed a high-cholesterol 1% (w/w) diet with zinc (1 g/kg) supplementation for 8 weeks. After the 8-week period, the average atherosclerotic lesion cross-sectional areas in the aortas of the animals fed with the zinc supplement were significantly decreased (1.0 mm^2) compared with lesion areas of the animals fed only on the high-cholesterol diet (3.1 mm^2). Using nuclear microscopy, a technique for mapping and measuring trace elements in tissue sections, lesion zinc levels (24 ppm) were observed to be unchanged in the zinc-fed rabbits compared to controls. However, average lesion Fe levels in the zinc-fed group were measured at 32 ppm, whereas in the control group the average Fe levels were significantly higher at 43 ppm ($P = 0.03$). Our data support the concept that zinc may have an antiatherogenic effect by decreasing iron levels in the lesion, possibly leading to inhibition of iron-catalyzed free radical reactions.

Keywords: Atherosclerosis; Zinc supplement; Lesion area; Iron; Zinc; Nuclear microscopy; Free radicals

Introduction

The development of atherosclerosis involves multiple events, including dysfunction of the vascular endothelium, penetration of circulating low-density lipoproteins (LDL) into the vessel wall, oxidation of these LDL, movement of circulating monocytes into the subendothelial space, differentiation of these monocytes into macrophages and transformation into foam cells, smooth muscle cell migration and proliferation, and artery wall degeneration [1–4].

Previous work has indicated that iron-dependent oxidative damage plays a part in atherosclerosis development [1–4], and Sullivan [5] has proposed that iron depletion protects against ischemic heart disease. We have found previously that when rabbits are fed a high-cholesterol (1%) diet, atherosclerotic lesions develop in the aorta after around 6 weeks, and these early lesions exhibit iron concentrations around seven times higher than the adjacent artery wall [6]. In addition, we found that venesection to induce mild anemia before feeding cholesterol decreases the progression of atherosclerosis in the aortas of cholesterol-fed rabbits, in parallel with decreased lesion iron content [7]. Further evidence that iron is linked to atherosclerosis development is provided by the observation that treatment with the antioxidant iron chelator, desferrioxamine (Desferal) [8] produced a significant decrease in lesion area after 12 weeks [9]. In this previous work we observed a high variability of lesion development not only from animal to animal but also along the length of the aorta and around the inner surface of the aortic wall from the same animal. Using nuclear microscopy, we observed that for each individual animal, the localized lesion iron concentrations were positively correlated with the atherosclerotic lesion depth, whereas the localized zinc concentrations were

Abbreviations: LDL, low-density lipoproteins; HCD, high-cholesterol diet.
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inversely correlated with lesion depth [10]. This work is consistent with current theories that iron plays a role in atherosclerosis through iron-dependent lipid peroxidation and hydroxyl radical formation [1–4,11], and suggestions that zinc may have antioxidant actions in vivo [12–24].

We have extended our work by investigating the effect of zinc on atherosclerotic lesion development. In the present study, we have measured the lesion development of rabbits fed a high-cholesterol diet supplemented with zinc, and monitored the trace element content of these lesions using nuclear microscopy.

Material and methods

Eighteen New Zealand white male rabbits weighing on average 2.5 kg were obtained from the Laboratory Animal Centre (Sembawang, Singapore) and divided randomly into three groups of 6. The first group was fed a normal diet; the second group was fed with high-cholesterol diet (HCD) SF00-221 (modified guinea pig and rabbit + 1% cholesterol); the third group was fed a zinc-supplemented diet SF03-017 (modified guinea pig and rabbit + 1% cholesterol + 1000 ppm (1 g/kg) zinc as zinc carbonate). One rabbit in the Zn-supplemented group died before the experiment was completed. These diets were purchased from Glen Forrest Stockfeeders, Western Australia. The food intake and animal body weights were monitored, and all rabbits gained weight during the experimental period of 8 weeks. No significant difference in the food intake was observed between the animals fed on the HCD (control group) and the group fed on high-fat diet plus zinc (test group).

During the 8-week feeding period, hemoglobin and blood zinc levels were monitored every 2 weeks (Table 1), and at the end of the 8-week animal feeding period a range of blood parameters was measured (Table 2).

The rabbits were sacrificed 8 weeks later by iv injection of Hypnorm (0.3 ml/kg). The aortic arch was removed and cut into three segments A, B, and C as described in our previous paper [10]. Segments were flushed with deionized water to remove residual blood from the inner artery wall and flash-frozen in liquid nitrogen.

Sections of aortic arch were taken using a Leica CM3050S cryostat. Sections for nuclear microscopy measurements were picked up on pioloform-coated nuclear microscopy target holders, for elemental mapping and concentration analysis.

Table 2
Rabbit blood parameters measured at the end of 8 weeks (standard deviation in parentheses) and significant differences between HCD and HCD with Zn-supplemented groups

	(i) Normal control	(ii) HCD	(iii) HCD with Zn supplement	*T* test significance between group (ii) and group (iii)
Hb (g/dl)	13.08 (0.63)	7.68 (1.42)	9.64 (1.32)	0.043
Total cholesterol (mmol/L)	1.34 (0.14)	36.36 (2.83)	28.76 (7.04)	0.072
Triglyceride (mmol/L)	1.43 (0.51)	3.18 (2.49)	1.37 (1.17)	0.155
HDL (mmol/L)	0.72 (0.16)	6.90 (2.86)	2.61 (1.36)	0.013
LDL (mmol/L)	0.05 (0.03)	31.49 (3.23)	25.54 (5.51)	0.086
Total white cells ($\times 10^9$/L)	7.51 (1.57)	10.33 (2.87)	10.10 (2.41)	0.892
Red blood cells ($\times 10^{12}$/L)	5.90 (0.67)	3.04 (0.74)	4.07 (0.56)	0.027
Platelets ($\times 10^9$/L)	266.20 (67.18)	137.33 (26.2)	153.00 (33.03)	0.417

(i) Rabbits on normal diet, (ii) rabbits on diet plus 1% cholesterol, and (iii) rabbits on diet plus 1% cholesterol + 1000 ppm (1 g/kg) zinc as zinc carbonate.

Serial sections were picked up on gelatin-coated slides and stained with H&E (Hematoxylin and Eosin). Lesion area measurements were carried out on the stained sections using the Carl Zeiss Axiophot 2 image analyzer utilizing the KS400 (version 3.18) analysis software. As in previous studies [10], the artery segment which exhibited the largest lesion area according to the area analysis was chosen for nuclear microscopy analysis. Serial sections of aortic arch were taken, one set for H&E staining, while serial unstained aortic artery sections were used for elemental analysis.

The nuclear microscopy scans were carried out at the Centre for Ion Beam Applications (CIBA) using a 2.1 MeV proton beam. A HVEE Singletron accelerator provided the proton beam, and the beam was focused to typical operating proton beam sizes of 1 μm at beam currents of around 300–400 pA using an Oxford Microbeams OM2000 focusing system. PIXE

Table 1
Hemoglobin (g/dl) and zinc (μmol/L) levels of rabbits measured every 2 weeks during the 8-week experimental period (standard deviations in parentheses)

	(i) Normal control		(ii) HCD control		(iii) Zn-supplemented HCD	
	Hemoglobin (g/dl)	Zn in blood (μmol/L)	Hemoglobin (g/dl)	Zn in blood (μmol/L)	Hemoglobin (g/dl)	Zn in blood (μmol/L)
2 weeks	12.58 (0.52)	181 (52.0)	11.37 (1.72)	149 (27.2)	11.37 (1.09)	132 (19.6)
4 weeks	12.15 (1.19)	146 (4.6)	9.83 (1.24)	147 (9.6)	10.72 (1.29)	147 (11.8)
6 weeks	12.40 (1.34)	158 (15.2)	9.00 (0.91)	148 (7.6)	10.50 (1.06)	167 (11.6)
8 weeks	13.08 (0.63)	168 (17.9)	7.68 (1.42)	153 (6.7)	9.64 (1.32)	171 (2.3)
P value	0.472	0.252	0.001	0.917	0.161	<0.001

(i) Rabbits on normal diet, (ii) rabbits on diet plus 1% cholesterol (HFD control), and (iii) rabbits on diet plus 1% cholesterol plus 1000 ppm (1 g/kg) zinc as zinc carbonate. Significance values (*P* values) indicate whether or not there has been a significant change in the hemoglobin or blood zinc levels for each group, and were calculated using one-way ANOVA.

data, backscattered protons (RBS), and protons scattered forward at 15° to the incoming beam (off-axis STIM) were recorded simultaneously using the OMDAQ data acquisition system in list mode [10].

This study was approved by the National University of Singapore local Animal Care and Use Committee.

Results

During the 8-week feeding period, hemoglobin and blood zinc levels were monitored every 2 weeks (Table 1). These results indicate that during the feeding period, whole blood zinc levels remained constant for the animals on their normal diet and those on the HCD, but increased significantly ($P<0.001$) for the zinc-supplemented HCD animals (from 132 ± 20 to 171 ± 2 μmol/L). There was a wide variation in blood zinc levels between animals. Hemoglobin levels in the normal controls (12.6 to 13.1 g/dl) remained constant throughout the feeding period, whereas hemoglobin levels progressively decreased ($P = 0.001$) in the HCD animals (11.4 to 7.7 g/L) but not ($P = 0.161$) in the zinc-supplemented HCD animals (11.4 to 9.6 g/L).

The results of measurements of other relevant blood parameters are summarized in Table 2. At the end of the 8-week period the levels of total cholesterol, as expected, are greatly increased in the animals fed on a HCD. The zinc supplementation did not produce a significant change in total cholesterol, triglyceride, or LDL ($P > 0.05$). However, a significant difference was observed in the HDL levels ($P = 0.013$), with the HCD group exhibiting HDL levels of 6.9 mmol/L compared with 2.6 mmol/L for the Zn-fed group. As observed in a previous study [10], a high-fat diet appears to cause anemia in rabbits after several weeks. In our present study, hemoglobin (Hb) levels decreased in the HCD animals compared with the normal diet controls at 8 weeks from 13.1 to 7.7 g/dl, whereas the Zn-supplemented HCD rabbits showed a marginally significant amelioration (9.6 g/dl) in the fall in Hb ($P = 0.043$). We also observed a reduction in the red blood cell count of the HCD animals, less marked in the zinc-supplemented HCD rabbits ($P = 0.027$).

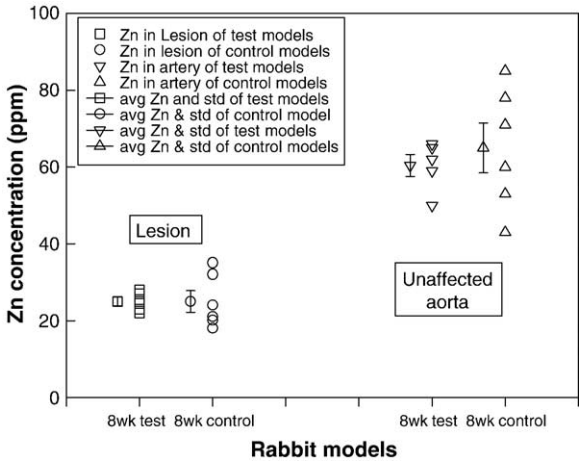

Fig. 2. Zinc concentrations (parts per million) in aortic lesion and unaffected aorta of the control and test rabbits. Test, HCD + zinc; control, HCD only.

Fig. 1 shows lesion areas from sections of aorta from rabbits in the HCD group and the HCD group plus zinc. The results show that the zinc supplement has a marked effect ($P = 0.0045$) on the lesion area; the HCD with zinc group has an average lesion area of 1.0 mm^2 compared with the HCD alone where there was an average lesion area of 3.1 mm^2. Fig. 2 shows that the zinc concentrations in the lesions are similar in both the test and the control cases, with an average of 24 ppm. In the adjacent unaffected vessel wall the zinc concentrations are also similar between the test and the control group, averaging 63 ppm. The decreased zinc concentration between the lesion and the adjacent healthy tissue has been observed in our previous work [10]. Fig. 3 shows iron concentrations for the lesions in the test and control groups, together with iron concentrations in the adjacent healthy aortic tissue. In the Zn-supplemented HCD group, the average Fe lesion levels are 32 ppm, whereas in the HCD group the

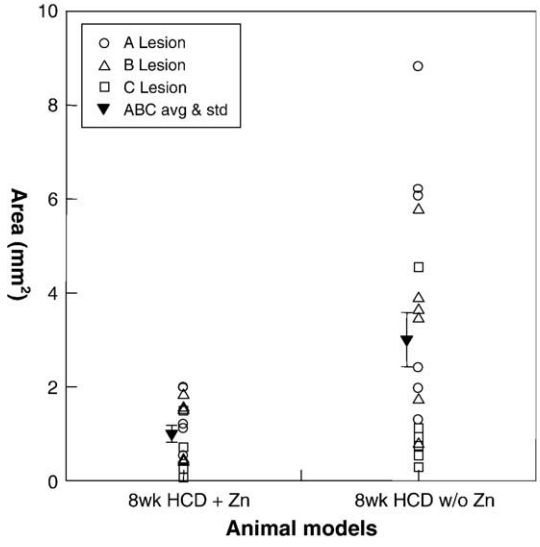

Fig. 1. Lesion area measured from A, B, and C segments of all the rabbits in control and test groups.

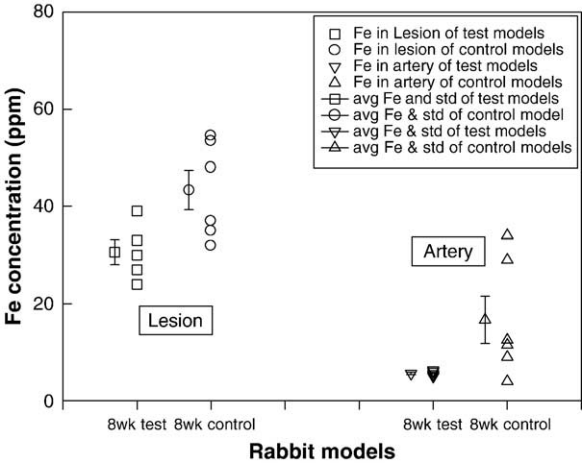

Fig. 3. Iron concentrations (parts per million) in lesion and unaffected aorta of the control and test rabbits. For further details see the legend to Fig. 2.

average Fe levels are higher at 43 ppm, a significant difference ($P = 0.03$). For the adjacent healthy artery wall, the iron levels were lower at (as expected [10]) 6 ppm in the Zn-supplemented HCD group and 15 ppm in the HCD only group. Given the variation in the control values, the difference does not reach significance ($P = 0.07$).

Discussion

Our results show that there is no significant difference in total cholesterol and LDL levels for the HCD and HCD plus zinc groups at the end of the 8-week period, although the HDL levels appear to be decreased in the Zn-fed HCD group. Since HDL is antiatherosclerotic, it seems unlikely that the protective effects of zinc are mediated by altering plasma lipid levels, in agreement with [21]. The zinc administration raised zinc levels in the whole blood (Table 1) but not in plasma (data not shown), suggesting an increase in cellular levels. During the feeding period of 8 weeks, hemoglobin levels progressively decreased in the HCD animals (11.4 → 7.7 g/L), indicating a deleterious effect of a prolonged high-fat diet in these animals. This decrease was smaller in the Zn-fed HCD group. However, preexisting anemia in rabbits decreases atherosclerosis [6], so it seems unlikely that zinc is protecting by maintaining hemoglobin levels. Other evidence that Zn is influencing Fe metabolism is that the red blood cell count dropped in the HCD animals from 5.90 to 3.04 × 10^{12}/L, whereas in the Zn-supplemented HCD animals the drop was smaller, from 5.90 to 4.07 × 10^{12}/L.

We infer from our data in Fig. 1 that zinc supplementation markedly retards atherolesion development in rabbits fed on a high-cholesterol diet. Although the blood zinc levels increased in the Zn-fed rabbits (Table 1), it appears that there was no extra zinc in the lesions at the time our measurements were made (Fig. 2). Of course, we cannot rule out a transient rise in the zinc content of the vessel walls at earlier times. Nevertheless, zinc administration seems to lead to a reduction in iron concentrations in the lesion and possibly (to a lesser extent) in the adjacent vessel wall (Fig. 3).

Zinc may thus play a role in inhibiting lesion formation through the indirect prevention of iron-mediated free radical damage, in that it decreases the iron content of the lesion. Indeed, we found that the increased formation of F_2-isoprostanes and cholesterol oxidation products in the aortas of animals on the HCD was almost completely ameliorated by zinc administration (Jenner, Ren, Rajendran, Ning, Tan, Watt, and Halliwell, in preparation). However, zinc does not appear to act by accumulating within the lesions, at least at the time points that we measured. Further work is underway to elucidate the mechanisms involved.

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