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The protective role of iron chelation and zinc supplements in atherosclerosis induced in New Zealand white rabbits: A nuclear microscopy study

M.Q. Ren [a,*], R. Rajendran [a], N. Pan [b], B.T.K. Huat [c], B. Halliwell [b], F. Watt [a]

[a] *Department of Physics, Centre for Ion Beam Applications, National University of Singapore, Singapore 117542, Singapore*
[b] *Department of Biochemistry, National University of Singapore, Singapore 117542, Singapore*
[c] *Department of Pharmacology, National University of Singapore, Singapore 117542, Singapore*

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Abstract

The protective properties of iron chelator *desferal* and zinc supplements on atherosclerosis induced in New Zealand white rabbits were investigated using nuclear microscopy, incorporating particle induced X-ray emission (PIXE), Rutherford backscattering spectrometry (RBS) and scanning transmission ion microscopy (STIM).

Firstly, we examined the effects of the desferal (desferrioxamine – a chelator which forms a stable complex with ferric iron) on atherosclerosis progression and lesion iron content in cholesterol-fed New Zealand white rabbits. Rabbits were fed with a 1% w/w cholesterol diet (HFD – high fat diet) for either 8 weeks (with the last 5 weeks injected daily with desferal), or for 12 weeks (with the last 9 weeks injected with desferal). Controls were injected with saline. A significant reduction in average lesion area ($p = 0.038$) was observed in the 12-week treated animals as compared with the 12-week controls. The average lesion iron level of the 12 week treated animals (58 ppm dry weight) was significantly lower ($p = 0.03$) than for the 12-week control animals (95 ppm dry weight). No reduction in lesion area or iron content was observed in the 8 week treated animals compared with controls, and no change in lesion zinc concentration was observed for either group. This data is consistent with the concept that iron contributes to the early stages in the development of atherosclerosis and that removal of iron from the lesion retards the progression of the disease.

Secondly, the effect of zinc supplements on atherosclerotic lesion growth was examined. The rabbits in the test group received a 1% w/w cholesterol diet with Zn supplements for 8 weeks and the rabbits in the control group were fed only with a 1% w/w cholesterol diet for the same period of time. Lesion area analyses using light microscopy showed that the average lesion area was 1.0 mm^2 for the test models compared with 3.0 mm^2 for the control group models ($p = 0.0045$). Elemental analysis of the lesion and adjacent artery wall showed that the average zinc level remained the same for both the lesion and the artery wall for both test and control models, whilst the iron levels are reduced from 43 ppm to 31 ppm

* Corresponding author. Tel.: +65 68742638; fax: +65 67776126.
E-mail address: phyrenmq@nus.edu.sg (M.Q. Ren).

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(in the lesion) and from 17 ppm to 6 ppm (in the artery wall). This raises the possibility that zinc may act as an endogenous protective factor against atherosclerosis by reducing iron levels.

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Keywords: Atherosclerosis; Iron chelator desferal; Zinc supplements; Lesion area; Iron; Zinc; Nuclear microscopy; Free radicals

1. Introduction

Reactive oxygen species (ROS) are thought to play an important role in the pathogenesis of atherosclerosis causing lipid peroxidation, endothelial cell damage and eventual arterial degeneration [1].

Iron is a transition metal that catalyzes the formation of ROS by the Fenton reaction [2], and several epidemiologic studies have investigated the role of iron as a potential risk factor in coronary heart disease [3–9]. We have found previously that mild anaemia decreases the progression of atherosclerosis in cholesterol-fed rabbits in parallel with decreased lesion iron content [10]. In apo E-deficient mice, vascular iron deposition is closely related to progression of atherosclerosis and LDL oxidation [11].

The iron chelator, *desferal*, is a trihydroxamate-containing siderophore produced by *Streptomyces pilosus*. It forms a stable complex with ferric iron, decreasing its availability for the production of reactive oxygen species. Desferrioxamine is a powerful inhibitor of iron-dependent lipid peroxidation [12,13] and hydroxyl radical formation [13]. Desferal has been found to protect against tissue damage in vivo, in several model systems, probably through its ability to inhibit iron-dependent free radical reactions [14].

Endothelial dysfunction, preceding the appearance of structurally evident atherosclerosis, has been recognized as an important early functional abnormality in atherogenesis [15] and is accepted as a surrogate marker of vascular pathology leading to atherosclerosis [16]. Studies also indicate that zinc is vital to vascular endothelial cell integrity and zinc deficiency causes severe impairment of the endothelial barrier function [17–20]. Zinc is believed to have specific antiatherogenic properties by inhibiting oxidative stress-responsive transcription factors that are activated during an inflammatory response in atherosclerosis.

In this study, the protective properties of iron chelator *desferal* and zinc supplements on the progression of atherosclerosis in New Zealand White rabbits were investigated using nuclear microscopy.

2. Materials and methods

2.1. Materials

Desferrioxamine mesylate (Desferal^{R}, 500 mg/vial dry active substance for injection) was purchased from Novartis Pharma AG, Basle, Switzerland, and dissolved in sterile saline solution at a concentration of 250 mg/ml. For the desferal studies, the desferal was administered three weeks into the diet; test rabbits received daily injections of desferal subcutaneously in the back near the neck of the animal (72 mg/kg/day, 5 days/week), while control animals received saline. For the zinc supplement study, zinc (1 g/kg) was added to the rabbit diet as zinc carbonate. All rabbit diets were purchased from Glen Forrest Stockfeeders, Western Australia.

2.2. Animal treatment

Forty two male New Zealand white rabbits obtained from the Laboratory Animal Centre (Sembawang, Singapore) weighing on average 2.5 kg, were divided randomly into seven groups of 6. The zinc studies (group 1–3) and the iron chelation studies (group 4–7) were carried out separately.

Group 1: fed on normal standard guinea pig and rabbit (GPR) diet (normal control models);

Group 2: fed on zinc-supplemented diet SF03-017 (standard GPR + 1% cholesterol + 1000 ppm (1 g/kg) zinc as zinc carbonate) for 8 weeks (8-week zinc test models);
Group 3: fed on SF00-221 (standard GPR + 1% cholesterol) (8-week zinc control models);
Group 4: fed on SF00-221 (standard GPR + 1% cholesterol) diet for 8 weeks with the last 5 weeks injected with desferal (8-week desferal test models);
Group 5: fed on SF00-221 (standard GPR + 1% cholesterol) diet for 8 weeks with the last 5 weeks injected with saline (8-week desferal control models);
Group 6: fed on SF00-221 (standard GPR + 1% cholesterol) diet for 12 weeks with the last 9 weeks injected with desferal (12-week desferal test models);
Group 7: fed on SF00-221 (standard GPR + 1% cholesterol) diet for 12 weeks with the last 9 weeks injected with saline (12-week desferal control models).

There was a loss of 6 rabbits due to unexpected complications during the experimental period. The surviving rabbits were sacrificed by i.v. 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 [21]. Segments were flushed with deionized water to remove residual blood from the inner artery wall, and flash frozen in liquid nitrogen.

This study was approved by the NUS local Animal Care and Use Committee.

2.3. Histochemistry and nuclear microscopy analysis

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. Serial sections were picked up on gelatin coated slides to be H and E (Hematoxylin and Eosin) stained for lesion area analysis carried out using the Carl Zeiss Axiophot 2 image analyzer utilizing the KS400 (version 3.18) analysis software.

The nuclear microscopy measurement was carried out at the NUS Centre for Ion Beam Applications (CIBA) using a 2.1 MeV proton beam focused to a 1-um spot size. Three techniques were simultaneously applied: (1) STIM (scanning transmission ion microscopy; (2) RBS (Rutherford backscattering spectrometry); (3) PIXE (proton induced X-ray emission). The area of interest was positioned using the structure and density information provided by STIM. Quantitative results are determined by RBS and PIXE. RBS provides information on matrix composition and incident charge; PIXE was used for measuring the concentrations of the elements from sodium upwards in the periodic table. X-rays of different elements were detected simultaneously using a lithium-drifted silicon X-ray detector placed at 90° to the beam axis and fitted with a filter designed for optimal detection of trace elements such as iron in biological specimens.

3. Results

3.1. H and E analysis of lesion area and artery wall

Table 1 shows a summary of the area of the artery wall and the developed lesion area determined from H and E stained sections.

Zinc supplement results: From Table 1 and Fig. 1, it is clear that the rabbit group fed on zinc supplements (group 2) has a lowest lesion area development compared with the control group 3. It should be noted that the rabbits in normal control group (group 1) have no developed lesions. The average lesion area for group 2 (1% high cholesterol diet + zinc supplements for 8 weeks) was $1.0 \pm 0.18\ mm^2$ compared with $3.0 \pm 0.58\ mm^2$ for group 3 (1% high cholesterol diet for 8 weeks). Using the student t test, this indicated a significant slowing of lesion progression with zinc supplements ($p = 0.0045$). In addition, a more detailed lesion area analysis taken from sections removed from aortic arch segments A, B and C from the groups 2–7 is shown in Fig. 1.

Desferal results: For the 8-week desferal groups (group 4 and 5), there was no significant reduction

Table 1
Artery wall area and developed lesion area (mm^2) (standard deviations in parentheses) were determined from H and E stained sections using the Carl Zeiss Axiophot 2 image analyzer utilizing the KS400 (version 3.18) analysis software

		Group 1 8 week normal Ctl ($n = 5$)	Group 2 8 week zinc suppl. ($n = 5$)	Group 3 8 week no zinc Ctl ($n = 6$)	Group 4 8 week desferal test ($n = 5$)	Group 5 8 week desferal Ctl ($n = 6$)	Group 6 12 week desferal test ($n = 5$)	Group 7 12 week desferal Ctl ($n = 4$)
A	Lesion	0.00	1.36 (0.28)	4.46 (1.03)	5.02 (1.19)	3.23 (0.85)	3.22 (0.94)	5.08 (1.42)
	Artery	7.25 (0.53)	5.08 (0.36)	6.85 (0.77)	8.28 (0.74)	7.22 (0.80)	4.68 (0.50)	5.55 (0.60)
B	Lesion	0.00	1.14 (0.30)	3.20 (0.60)	2.71 (0.56)	3.58 (0.68)	1.51 (0.48)	3.75 (1.19)
	Artery	3.61 (0.27)	2.44 (0.30)	3.15 (0.35)	3.93 (0.33)	3.92 (0.20)	2.10 (0.18)	3.06 (0.50)
C	Lesion	0.00	0.50 (0.28)	1.36 (0.55)	1.01 (0.19)	1.56 (0.29)	0.81 (0.43)	2.24 (1.27)
	Artery	2.68 (0.35)	2.21 (0.23)	2.23 (0.15)	3.13 (0.26)	3.11 (0.11)	1.44 (0.33)	3.01 (0.33)
	Average lesion	0.00	1.00 (0.18)	3.01 (0.58)	2.91 (0.60)	2.79 (0.46)	1.84 (0.44)	3.69 (0.76)

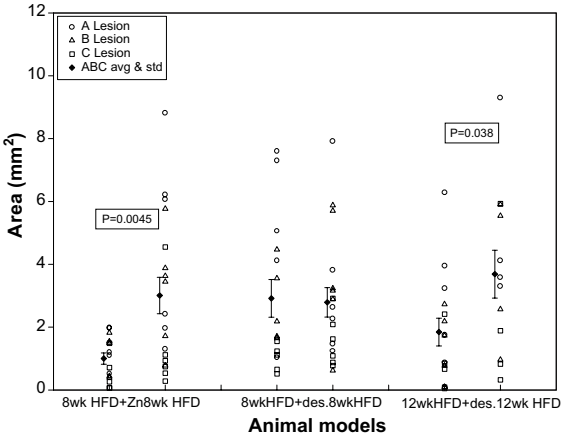

Fig. 1. Lesion area for the 6 groups: 8-week HFD + Zn; 8-week HFD; 8-week HFD + desferal; 8-week HFD; 12-week HFD + desferal and 12-week HFD.

in the average lesion areas of the 8-week desferal-treated animals as compared with the control (see Fig. 1). However, a significant change ($p = 0.038$) was observed in the 12-week desferal-treated animals (group 6), with a substantial reduction in mean lesion area (1.84 ± 0.44 mm^2) compared with the control group 7 (3.69 ± 0.76 mm^2).

3.2. Elemental analysis

Based on the lesion area analysis, for nuclear microscopy studies we chose the segment that has the relatively largest lesion area for each rabbit [21], which corresponds to the highest localized progression of the disease. Table 2 shows a summary of the average concentrations (in parts per million) of selected major and trace biological elements in lesion and artery wall of the aorta arch for the animal groups (2–7). The results show that phosphorus concentrations in the lesion is always higher than that in artery wall, which makes identification of the lesion boundaries possible by mapping the phosphorous elemental distribution. STIM mapping was also used to confirm the identification of the lesion and artery wall boundary. Calcium concentrations are generally higher in the lesion than in the artery wall except one group (group 5: 8 week desferal control group). The zinc levels are similar in the lesion and artery wall within each group. No difference was observed in iron levels of the unaffected adjacent arterial walls between 12-week desferal test and 12-week desferal control.

The lesion iron content results are shown in more detail in Fig. 2. In the Zn supplement group 2, the average iron in lesion is 31 ppm, whereas in the 8-week control group 3 the average lesion iron content is 43 ppm representing a significant difference ($p = 0.03$). For the adjacent artery wall, the iron levels (shown in Table 2) are 6 ppm and 17 ppm with $p = 0.07$. It was observed that there was a marginally significant ($p = 0.03$) reduction of average lesion iron level in the 12-week desferal test group compared with the 12-week desferal

Table 2
Average elemental concentrations (ppm, standard deviations in parentheses) of selected biological elements in lesion and artery wall of the aorta arch for the 6 groups animals

		8 week zinc suppl.	8 week no zinc Ctl	8 week desferal test	8 week desferal Ctl	12 week desferal test	12 week desferal Ctl
Lesion	P	3500 (569)	4946 (456)	6119 (605)	5338 (353)	4707 (493)	4904 (336)
	S	2472 (147)	2823 (156)	2970 (341)	2906 (292)	2870 (204)	3174 (370)
	Cl	2654 (96)	3112 (187)	2550 (269)	2600 (227)	3150 (196)	2510 (971)
	K	1392 (142)	1509 (173)	1464 (227)	1544 (131)	1246 (192)	1266 (539)
	Ca	517 (235)	1213 (510)	786 (171)	400 (84)	710 (228)	628 (200)
	Fe	31 (3)	43 (4)	67 (8)	47 (4)	57 (13)	93 (14)
	Zn	25 (1)	25 (3)	27 (6)	26 (1)	21 (3)	24 (4)
Artery	P	1867 (271)	2550 (296)	3321 (602)	2736 (268)	2396 (293)	2420 (315)
	S	3807 (412)	4838 (601)	5915 (824)	5383 (625)	5046 (459)	5141 (438)
	Cl	4102 (197)	5005 (459)	5344 (498)	4993 (509)	5310 (586)	3670 (1185)
	K	1637 (206)	2093 (347)	2054 (352)	2105 (366)	1769 (311)	1464 (577)
	Ca	439 (44)	605 (92)	689 (158)	492 (25)	565 (81)	466 (55)
	Fe	6 (0.2)	17 (5)	23 (5)	10 (1)	20 (7)	27 (10)
	Zn	60 (3)	65 (6)	81 (13)	67 (6)	63 (14)	68 (8)

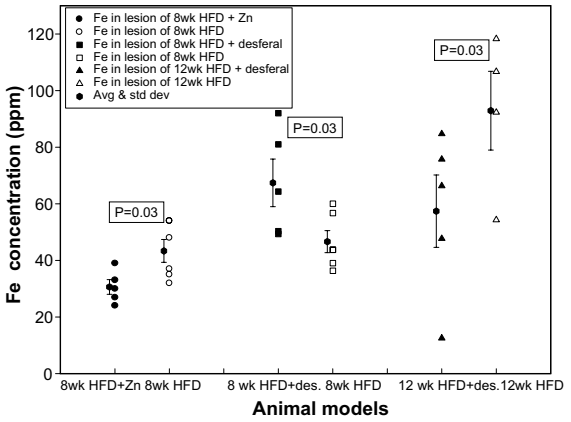

Fig. 2. Lesion iron content (parts per million) for the 6 groups: 8-week HFD + Zn; 8-week HFD; 8-week HFD + desferal; 8-week HFD; 12-week HFD + desferal and 12-week HFD.

controls. Surprisingly, there was a marginally significant *increase* in lesion iron levels ($p = 0.04$) between the desferal treated animals and controls.

4. Discussion and conclusion

Our previous study showed that desferal-induced iron chelation applied at a period of 6 weeks of cholesterol feeding for a duration of 2 weeks did not slow down lesion development [21]. The results showed that there was a non-significant, short-term tendency for desferal treatment to increase lesion area. However, when applied for a period of 4 weeks, there was a non-significant trend ($p = 0.07$) for a reduction of lesion size [21]. In that study, the desferal was delivered to the rabbits using osmotic pumps implanted into the abdominal cavity. In the present study, we switched to desferal injection because of potential health problems of long-term pump implantation.

Our current data suggest that desferal treatment in the short-term fails to decrease the Fe content of the lesions or have an impact on lesion reduction. Indeed, there appears to be a short-term trend to raise lesion iron content (Fig. 2). Hence it is possible that desferal is bringing in iron from elsewhere or modulating vessel wall Fe metabolism [22]. However, for longer desferal treatment period as observed in the 12-week group in this study (9 weeks desferal treatment), there was a marginal but significant reduction in lesion Fe concentration accompanied by a decrease in lesion size.

It was also observed in our previous studies that lesion zinc levels vary between different animals and that the zinc levels in the lesion on average are a factor of 4 less than in the adjacent healthy artery tissue [21]. In our present study we measured a 3 times reduction in the average Zn concentrations between the lesions and the adjacent

artery walls (24 ± 5 ppm in the lesion compared with 70 ± 8 ppm in the healthy artery wall). However, there was no significant difference when comparing the mean zinc concentrations between zinc supplement and control groups. In addition, in the desferal-treated and control groups, desferal does not appear to be acting by modulating zinc content.

In summary, our results have shown that zinc supplementation appears to reduce lesion development in conjunction with reduced iron concentrations in the lesion and adjacent smooth muscle wall, although no change in the lesion average zinc levels were observed. This suggests that zinc has an antiatherosclerotic role by indirectly reducing lesion iron levels, which indicates that a possible mechanism may be through the inhibition of iron mediated free radical damage. Further studies are required to elucidate this mechanism. Our results also show that iron chelation also has an effect on the progression of the disease. Nine weeks after desferal treatment, both the lesion area and iron content are reduced, although over a shorter term period of five weeks there is no difference in lesion progression.

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