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Original Contribution

The iron chelator desferrioxamine inhibits atherosclerotic lesion development and decreases lesion iron concentrations in the cholesterol-fed rabbit

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Abstract

Several epidemiological studies have suggested that increased iron stores are associated with increased atherosclerotic events. In order to test the hypothesis that decreasing the vascular level of iron slows lesion growth, we examined the effects of the iron chelator Desferal (72 mg/kg/day, 5 days/week) on atherosclerosis and lesion iron content in cholesterol-fed New Zealand White rabbits. Rabbits were fed with a 1% w/w cholesterol diet for either 8 weeks (and for the last 5 weeks injected daily with Desferal) or 12 weeks (and for 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 compared with the 12-week controls. The average lesion iron level of the 12-week treated animals (58 ppm dry wt) was also significantly lower ($p = 0.030$) than in 12-week control animals (95 ppm dry wt), as measured using nuclear microscopy with the combination of scanning transmission ion microscopy, Rutherford back-scattering spectroscopy, and particle-induced X-ray emission. 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. Our data strengthen the concept that iron contributes to the early stages of the development of atherosclerosis.
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Keyword: Free radicals

It has been argued that iron-dependent free radical formation and consequent lipid peroxidation are related to atherosclerosis development [1–5], and Sullivan [6] has proposed that iron depletion protects against ischemic heart disease. We have found previously that mild anemia decreases the progression of atherosclerosis in cholesterol-fed rabbits in parallel with decreased lesion iron content [7]. In apo E-deficient mice, vascular iron deposition is closely related to progression of atherosclerosis and LDL oxidation [8].

The iron chelator desferrioxamine (marketed as Desferal as the methane sulfonate salt) 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 [9,10] and hydroxyl radical formation [10]. 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 [11].

We recently examined the effects of Desferal on newly formed atherosclerotic lesions in the cholesterol-fed rabbit [12]. These previous results showed that Desferal had a

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limited but not significant effect on atherosclerotic lesion development. This could have been due to insufficient iron removal, because the longest treatment period that our rabbits received was only 4 weeks. Previously we started the treatment 6 weeks into the cholesterol diet and continued only for a further 4 weeks. In the present study, using a new batch of animals, we have therefore extended the time interval of Desferal administration and also started the treatment earlier, at 3 weeks. In the present study, Desferal was administered by injection 3 weeks after the rabbits were placed on a high cholesterol diet and the period of the Desferal treatment was increased to 5 or 9 weeks.

Material and methods

Materials

Desferrioxamine mesylate (Desferal, 500 mg/vial dry active substance for injection) was purchased from Novartis Pharma AG (Basel, Switzerland) and dissolved in sterile saline solution at a concentration of 250 mg/ml.

Animal treatment

Twenty-four New Zealand White rabbits obtained from the Laboratory Animal Centre (Sembawang, Singapore), weighing on average 2.5 kg, were fed a diet of 1% w/w cholesterol-containing food (Glen Forrest Stockfeeders, Western Australia). 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), whereas control animals received saline only. Injection is necessary because Desferal cannot be absorbed by the gastrointestinal tract. Eleven animals were sacrificed after 5 weeks of Desferal treatment (8-week animals) and 9 animals were sacrificed after 9 weeks of Desferal treatment (12-week animals). The rabbits were sacrificed by iv injection of Hypnorm (0.3 ml/kg). The study was approved by the NUS local Animal Care and Use Committee.

Measurement of blood parameters

Whole blood hemoglobin measurement

Hemoglobin (Hb) was estimated by the SLS-Hb method, which employs the Sysmex XE-2100 system. Briefly, whole blood sample was diluted with a diluent, and the mixture then entered a flow cell where a sulfolyser, SLS, was added to hemolyse red blood cells, thus releasing Hb to form Hb-SLS. The concentration of SLS-Hb was measured as absorbance at 55 nm and calculated by comparison with the absorbance of the diluent measured before the addition of the blood sample.

For the measurement of serum total cholesterol, triglycerides (TG), and iron, the Dimension Clinical Chemistry System (Dade Behring, Newark, NJ, USA) was used.

Serum total cholesterol measurement

In this process, cholesterol esterase was added to the sample to hydrolyze cholesterol esters into free cholesterol, which together with free cholesterol present in the serum is then oxidized by cholesterol oxidase to produce hydrogen peroxide (H_2O_2). In the presence of horseradish peroxidase, H_2O_2 oxidizes an aminoantipyrine-containing chromagen substrate into a chromophore that absorbs at 540 nm. The absorbance is proportional to serum total cholesterol concentration.

Serum triglyceride measurement

The assay is based on an enzymatic procedure in which a combination of four enzymes (lipoprotein lipase (LL), glycerol kinase (GK), glycerol-3-phosphate oxidase, and peroxidase) is employed. Serum sample was preincubated with LL, an enzyme which converts TG into free glycerol and fatty acids. GK catalyzes the phosphorylation of glycerol by ATP to glycerol 3-phosphate, which is then oxidized by glycerol-3-phosphate oxidase to dihydroxyacetone phosphate and H_2O_2. The catalytic action of peroxidase forms quinoneimine from H_2O_2, aminoantipyrine, and 4-chlorophenol. The change in absorbance due to the formation of quinoneimine is directly proportional to the total amount of glycerol in the sample and was measured by using the bichromatic (510, 700 nm) endpoint technique.

Serum iron measurement

Iron bound to transferrin is released at acidic pH by adding ascorbic acid; the iron forms a blue complex with Ferene. The absorbance of the complex was measured using a bichromatic (600, 700 nm) endpoint technique and is proportional to the amount of transferrin-bound iron in the serum.

Analysis of lesion area and Fe content

The aortic arch was removed and cut into three segments, A, B, and C, as described in our previous paper [12]. Segments were flushed with deionized water to remove residual blood from the inner artery wall and flash frozen in liquid nitrogen.

Sections were taken using a Leica CM3050S cryostat. Sections of 25 μm thickness were picked up on gelatin-coated slides to be hematoxylin and eosin stained for lesion area analysis, which was carried out using a Carl Zeiss Axiophot 2 image analyzer utilizing the KS400 (version 3.18) analysis software. Fifteen-micrometer unstained sections were picked up on pioloform-coated nuclear microscopy target holders for elemental mapping and concentration analysis. Because our previous study [12] showed that the iron concentration was positively correlated with the depth of the lesion, the aortic segment which contained the largest lesion area from each rabbit was chosen to perform the elemental analysis. Unstained

aortic artery sections were used for elemental analysis to avoid possible contamination due to artifacts of the staining process. The elemental analyses were carried out at the NUS Centre for Ion Beam Applications using a 2.1-MeV proton beam focused to a 1-μm spot size. As in the previous study, three techniques were simultaneously applied: (1) STIM (scanning transmission ion microscopy, (2) RBS (Rutherford back-scattering spectrometry), and (3) PIXE (particle-induced X-ray emission). The areas of interest and the boundaries of the lesions were located in the unstained sections using structural mapping information provided by STIM. Because lesions are also characterized by elevated phosphorus concentrations, additional confirmation of lesion boundaries was obtained using elemental mapping. Quantitative analyses were determined using RBS and PIXE: RBS provided information on matrix composition and incident proton charge, whereas PIXE was used for measuring the elemental concentrations down to the parts per million level. 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.

Results and discussion

During the period of treatment, the body weights of all animals were monitored and it was observed that Desferal administration caused no significant change over control. The high cholesterol diet itself caused a fall in Hb levels, but this was not significantly altered by the Desferal. Desferal also had no effect on cholesterol, triglyceride levels, or serum iron (Table 1). This is not surprising because Desferal cannot remove iron from transferrin or hemoglobin [11].

Fig. 1 shows the lesion area analyses taken from sections cut from the A, B, and C aortic arch segments for the four animal groups (8-week test, 8-week control, 12-week test, and 12-week control). As observed previously [12], lesion depth varied widely around the artery wall and between rabbits. There was no significant change in the average lesion areas of the 8-week Desferal-treated animals compared with the control. However, a significant change ($p = 0.038$) was observed in the 12-week Desferal-treated animals, with a substantial reduction in mean lesion area compared with the control. The average area value for the A lesions (Desferal treated) was 3.2 mm^2 compared with the control of 5.1 mm^2. The average area value for the B lesions (Desferal treated) was 1.5 mm^2 compared with the control of 3.7 mm^2. The average area value for the C lesions (Desferal treated) was 0.8 mm^2 compared with the control of 2.2 mm^2. For all sections combined, the average was 1.83 mm^2 (test) compared with 3.67 mm^2 (control), and the difference is significant (0.038) using the Student t test.

Fig. 2 shows the iron levels in lesions and in the adjacent unaffected arterial walls of the four groups as determined by nuclear microscopy. For each animal, elemental analysis was carried out only on the aortic arch segment which exhibited the maximum lesion area, corresponding to the highest localized progression of the disease. It was observed that there was a significant ($p = 0.03$) reduction of average iron levels in the lesions of 12-week test animals compared with controls. Surprisingly, for the 8-week group, there was a less significant *increase* in lesion iron levels ($p = 0.04$) between the Desferal-treated animals and the controls. No difference was observed in iron levels of the unaffected adjacent arterial walls between 12-week test and 12-week control models (data not shown).

Our previous study showed that Desferal-induced iron chelation applied at 6 weeks of cholesterol feeding for a period of 2 weeks did not slow down lesion development [12]. These results showed that there was a nonsignificant, short-term tendency for Desferal treatment to increase lesion area. However, when applied for a period of 4 weeks, there was a nonsignificant trend ($p = 0.07$) for a reduction of lesion size [12]. 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 deleterious effects of long-term pump implantation.

Our data may throw light on the limited effect of Desferal administered over a short period on atherosclerosis progression; Desferal treatment in the short term fails to decrease the Fe content of the lesions or to have an impact on lesion reduction. Indeed, there seems to be a short-term trend to raise both lesion area and lesion iron content, and hence it is possible that Desferal is bringing in iron from elsewhere or modulating vessel wall iron metabolism [13].

Table 1
Total cholesterol, serum iron, hemoglobin, and triglyceride levels for the four groups

	Animal model	Total cholesterol (mmol/L)	Serum iron (μmol/L)	Hemoglobin (g/dl)	Triglycerides (mmol/L)
8-week	Control ($n = 6$)	38.2 ± 0.66	35.2 ± 2.98	7.6 ± 0.44	2.3 ± 0.83
8-week	Test ($n = 5$)	37.6 ± 1.03	43.4 ± 8.36	6.2 ± 0.74	3.6 ± 1.17
12-week	Control ($n = 4$)	38.0 ± 1.57	42.3 ± 7.22	6.9 ± 0.64	11.7 ± 5.91
12-week	Test ($n = 5$)	35.2 ± 2.01	44.6 ± 4.18	6.5 ± 1.11	10.2 ± 4.51

The four groups are 8-week control, 8 weeks on a 1% cholesterol diet; 8-week test, 8 weeks on a 1% cholesterol diet + Fe chelation for weeks 4–8; 12-week control, 12 weeks on a 1% cholesterol diet; and 12-week test, 12 weeks on a 1% cholesterol diet + Fe chelation for weeks 4–12. Hemoglobin levels at week 2 were 11.9 ± 0.3 g/dl and cholesterol levels were unchanged throughout in all the cholesterol-fed animals.

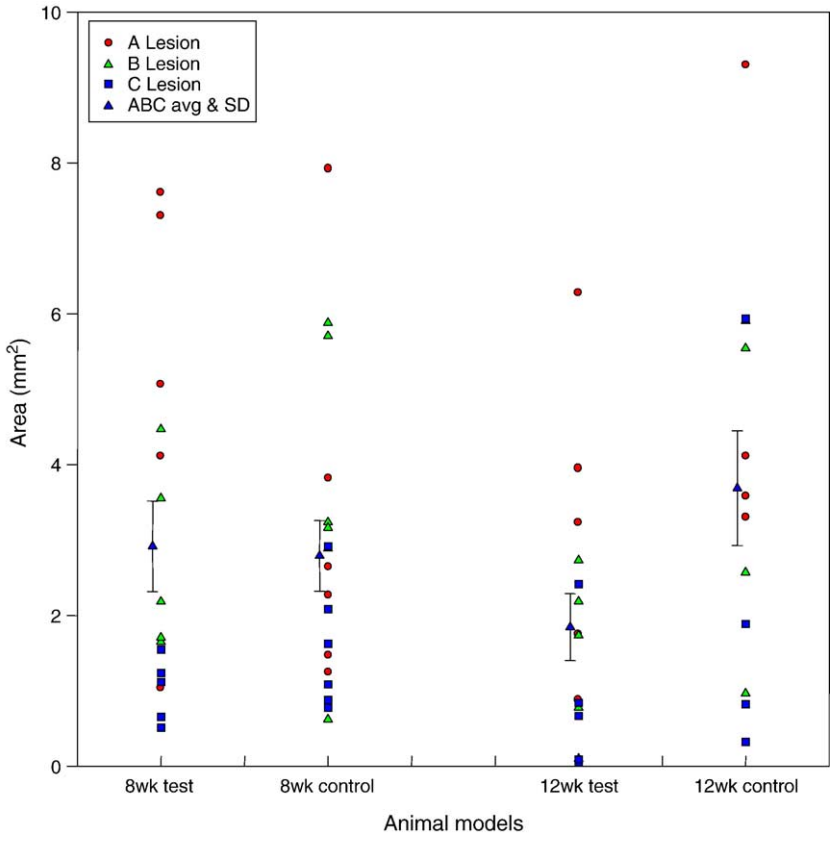

Fig. 1. Lesion areas for the four groups: 8-week test, 8 weeks on a 1% cholesterol diet + Fe chelation for weeks 4–8; 8-week control, 8 weeks on a 1% cholesterol diet; 12-week test, 12 weeks on a 1% cholesterol diet + Fe chelation for weeks 4–12; and 12-week control, 12 weeks on a 1% cholesterol diet.

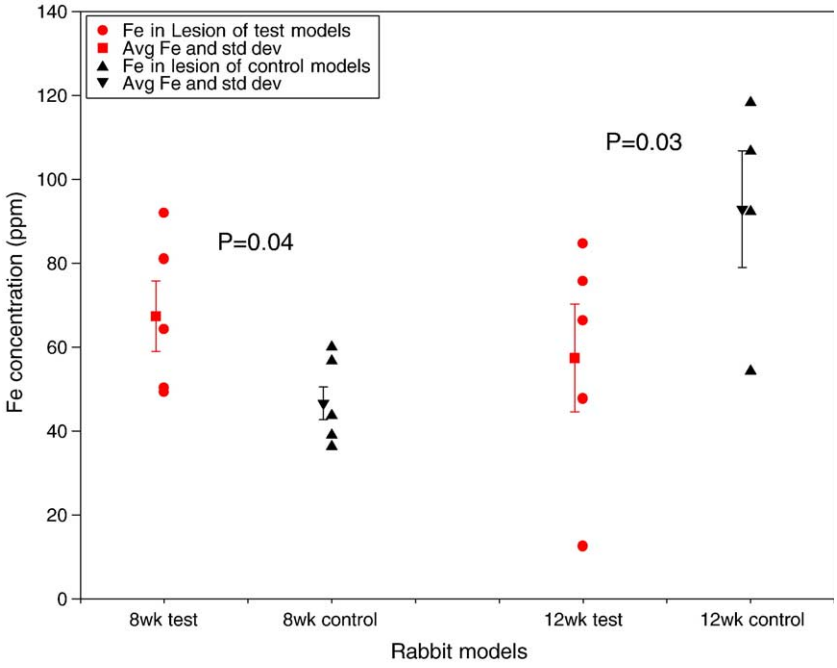

Fig. 2. Lesion iron levels (parts per million) of the four groups: 8-week test, 8 weeks on a 1% cholesterol diet + Fe chelation for weeks 4–8; 8-week control, 8 weeks on a 1% cholesterol diet; 12-week test, 12 weeks on a 1% cholesterol diet + Fe chelation for weeks 4–12; and 12-week control, 12 weeks on a 1% cholesterol diet. The aortic segment which contained the largest lesion area from each rabbit was chosen to perform the elemental analysis.

However, for longer Desferal treatment times as observed in the 12-week group (9 weeks of Desferal treatment), there was a significant reduction in lesion Fe concentration accompanied by a decrease in lesion size. In other words, Desferal has an antiatherosclerotic effect only when vascular iron levels are decreased.

It has been noted in our previous studies that zinc levels vary between different animals and that the zinc levels in the lesion on average are about four times lower than in the adjacent healthy artery tissue [12]. In our present study we measured a threefold 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, as can be observed in Fig. 3, there was no significant difference comparing the mean zinc concentrations between Desferal-treated and control groups. Hence, Desferal does not seem to be acting by modulating vascular zinc levels.

A recent study suggests that iron chelation may improve endothelial dysfunction, which is considered by many as the initial stage of the atherosclerotic process in humans [14]. Other studies suggest a role for iron in endothelial dysfunction in acute ischemic syndromes [15,16]. Our data support the growing view that Fe may play a role in the early stages of atherosclerosis.

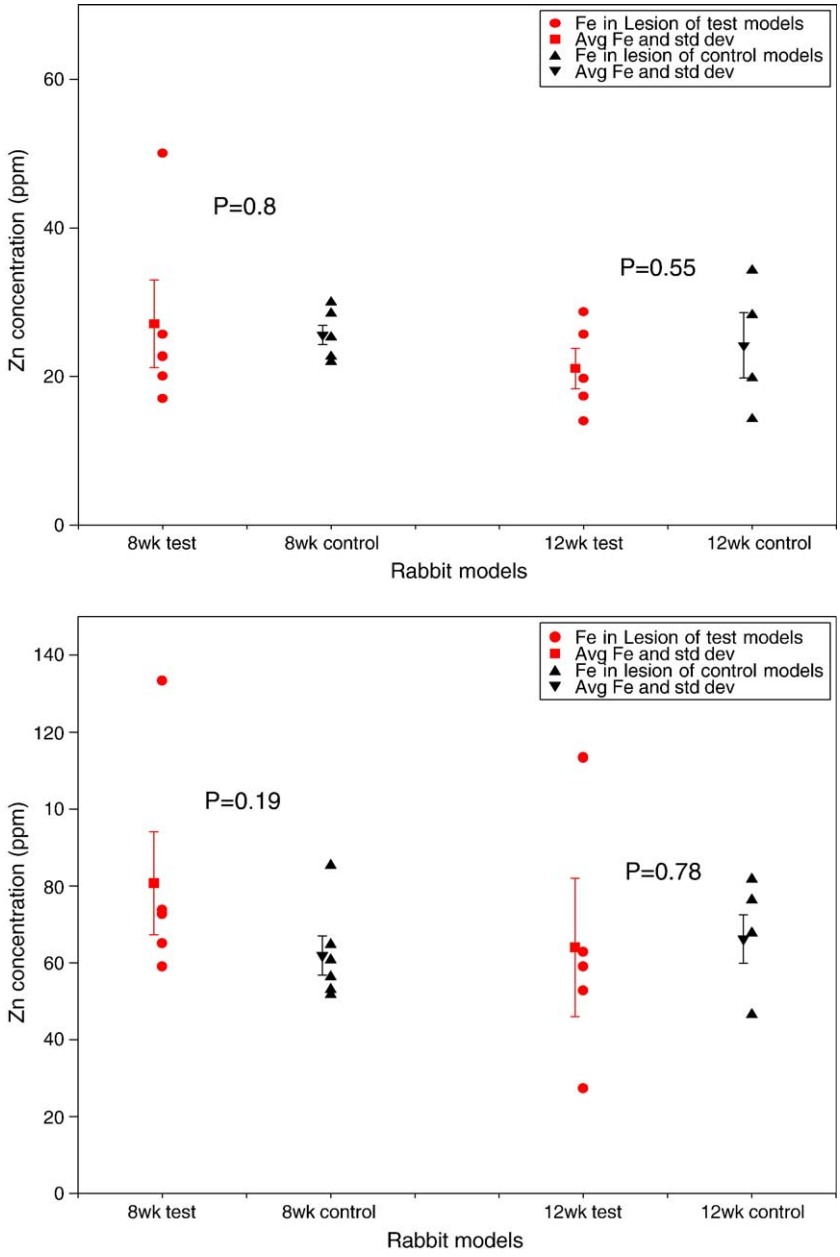

Fig. 3. Top: Lesion zinc levels (parts per million) for the four groups. Bottom: Adjacent artery zinc levels (parts per million) for the four groups. The aortic segment which contained the largest lesion area from each rabbit was chosen to perform the elemental analysis.

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