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Original Contribution

CORRELATION OF IRON AND ZINC LEVELS WITH LESION DEPTH IN NEWLY FORMED ATHEROSCLEROTIC LESIONS

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Abstract—Several studies have indicated a relationship between body iron content and cardiovascular disease, although other studies have not. There are also suggestions that zinc has an antioxidant and antiatherosclerotic effect. We have used Nuclear Microscopy, using the combination of Scanning Transmission Ion Microscopy (STIM), Rutherford Backscattering Spectrometry (RBS), and Proton Induced X-ray Emission (PIXE) to map and quantify iron and zinc levels in newly formed atherosclerotic lesions. Sixteen New Zealand White rabbits fed on a high cholesterol diet were divided into four groups of 4 rabbits each. Six weeks into the high cholesterol diet, two groups were treated with the iron chelating agent desferrioxamine, for 2 weeks and 4 weeks, respectively, by surgically implanting with Alzet osmotic pumps (Alza Corporation, Palo Alto, CA, USA) containing desferal (0.5 g/ml). The other two groups served as controls, and were surgically implanted with osmotic pumps containing saline. Tissue sections were taken from the aortic arch, flash frozen, and air-dried. Analysis of atherosclerotic lesions indicated a trend ($p = .07$) to a reduction in the progression of the lesion after 4 weeks of desferrioxamine treatment. For each of the control and desferrioxamine-treated animals however, the more extensive lesions contained a higher concentration of iron and a lower concentration of zinc. Our results are consistent with the view that early lesion formation may be accelerated by free radical production caused by increased iron levels, that zinc might antagonize such effects, and that more prolonged desferal treatment might have an antiatherosclerotic effect. © 2003 Elsevier Science Inc.

Keywords—Atherosclerosis, Lesion size, Iron, Iron chelator desferal, Nuclear microscopy, Free radicals

INTRODUCTION

Atherosclerosis is characterized by a buildup of fatty deposits on the inner walls of the affected arteries, beginning with fatty streaks containing lipid-engorged macrophages [1]. Although it is currently believed that oxidation of human LDL is an important early event in the genesis of atherosclerotic lesions [1–3], the mechanism by which LDL is oxidized in vivo is currently unknown [4]. Advanced atherosclerotic lesions contain metal ions (e.g., iron and copper), which may be catalytic for free radical reactions [5], although their role in early atherosclerosis development is uncertain [4]. Several epidemiological studies have suggested that elevated tissue iron levels may increase the risk for atherosclerosis [6–8], although there are some contrasting views [9,10]. Iron may be deleterious because of its ability to catalyze free radical reactions [11,12]. Indeed, we have found previously that rendering rabbits mildly anemic delayed the development of atherosclerosis induced by a high-cholesterol diet [13]. In contrast to iron, zinc has been reported to have antioxidant and antiatherosclerotic effects [14,15].

The original purpose of our present experiment was to examine the effect of an iron-chelating agent, desferrioxamine (desferal), on the development of atherosclerosis in cholesterol-fed rabbits. Desferrioxamine chelates iron by forming a stable complex that usually prevents the iron from entering into free radical reactions [12,16]. The iron chelate is soluble in water and passes easily through the kidney. Experimental work in animal models suggests that desferal can protect against tissue injury [6,12,16–19]. In our work, the desferrioxamine was administered after 6 weeks on a high cholesterol diet,

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which was the time deduced from our previous studies at which lesions were observed to be at an early stage of development. The original purpose, therefore, was to test whether or not the desferal treatment slowed, stopped, or reversed lesion development. During the course of this study, we observed a striking relationship between the rate at which lesions were developing on different parts of blood vessel walls and the lesion content of zinc and iron, in both controls and desferal-treated animals.

MATERIALS AND METHODS

Animal treatment

Sixteen New Zealand white rabbits obtained from the Laboratory Animal Centre (Sembawang, Singapore) weighing on average 2.5 kg, were fed a diet of 1% high cholesterol food (Glen Forrest Stockfeeders, Western Australia). Six weeks into the diet, the rabbits were each surgically implanted with Alzet osmotic pumps (Alza Corporation, Palo Alto, CA, USA) containing desferal (0.5 g/ml) for the test group (8 rabbits), and saline for the control group (8 rabbits). The administered dose per rabbit (50 mg/kg body weight) is based on the published dose of 20–60 mg/kg used for treating iron overload in humans [16]. Desferrioxamine (as desferal, desferrioxamine B methanesulphonate) was obtained from Novartis Pharma Ag (Basel, Switzerland). The procedure for the implantation into the abdominal cavity was as follows:

1. A vertical incision (about 2.5–3.0 cm) was made as close to the midline as possible.
2. Abdominal muscles were split longitudinally and the peritoneum opened carefully in a line with skin incision.
3. Two minipumps (2 ML 2, flow rate 5 μl/h) containing Desferal (or with normal saline for controls) were inserted into the abdominal cavity (8 week group). Four minipumps (2 ML 4, flow rate 2.5 μl/h) were inserted into the abdominal cavity (10 week group).
4. The incision was closed in layers with nylon suture and the wound cleansed with a sterilized solution.

Four test-group rabbits and 4 control-group rabbits were sacrificed 2 weeks after implantation (8 week group). Three test-group rabbits and 3 control-group rabbits were sacrificed 4 weeks after implantation (10 week group): 1 rabbit from each test and control 10 week group died before completion of the 10 week term. Animals were sacrificed by i.v. injection of sodium pentobarbitone (0.8 mg/kg). The aortic arch was removed and cut into three segments (A, B, and C, shown in Fig. 1). Segments were flushed with deionized water to remove residual blood from the inner artery wall, and flash frozen in liquid nitrogen.

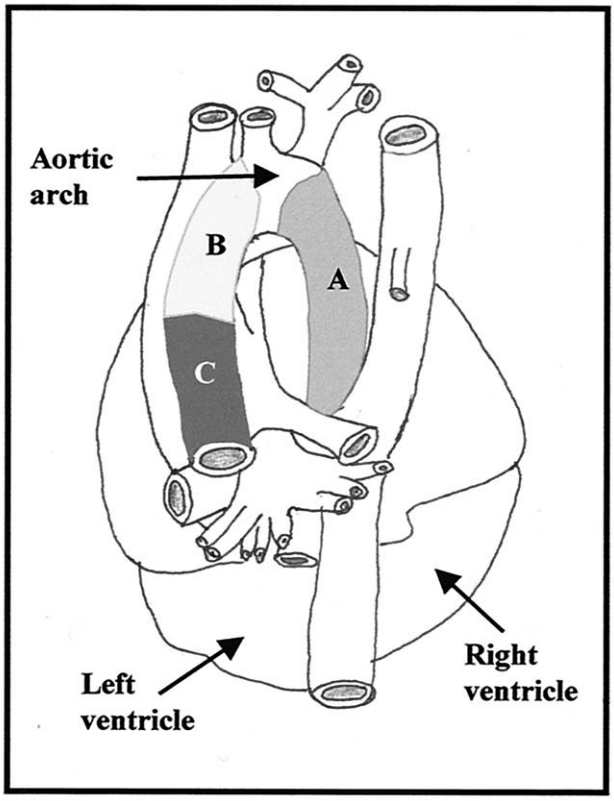

Fig. 1. A schematic drawing of the heart showing the three segments of aortic arch.

Blood and serum measurements

The blood and serum samples of all animals were used for the measurements of cholesterol, total iron-binding capacity (TIBC), and hemoglobin. TIBC was measured spectrophotometrically in nonhemolyzed serum samples as described in [20]. Colorimetric determination of hemoglobin concentration in whole blood was performed with Sigma kit product No.525 based on the method of Drabkin and Austin [21]. Serum total cholesterol level was quantified enzymatically as described in [22]. The weights and the food intake of all the animals were monitored throughout the experimental period. The study was approved by the N.U.S. Local Animal Care and Use Committee.

Analysis

Elemental analyses on unstained sections of tissue were carried out at the N.U.S. Research Centre for Nuclear Microscopy [23] using a 2.1 MeV proton beam focused to a 1-μm spot size, and utilized three comple-

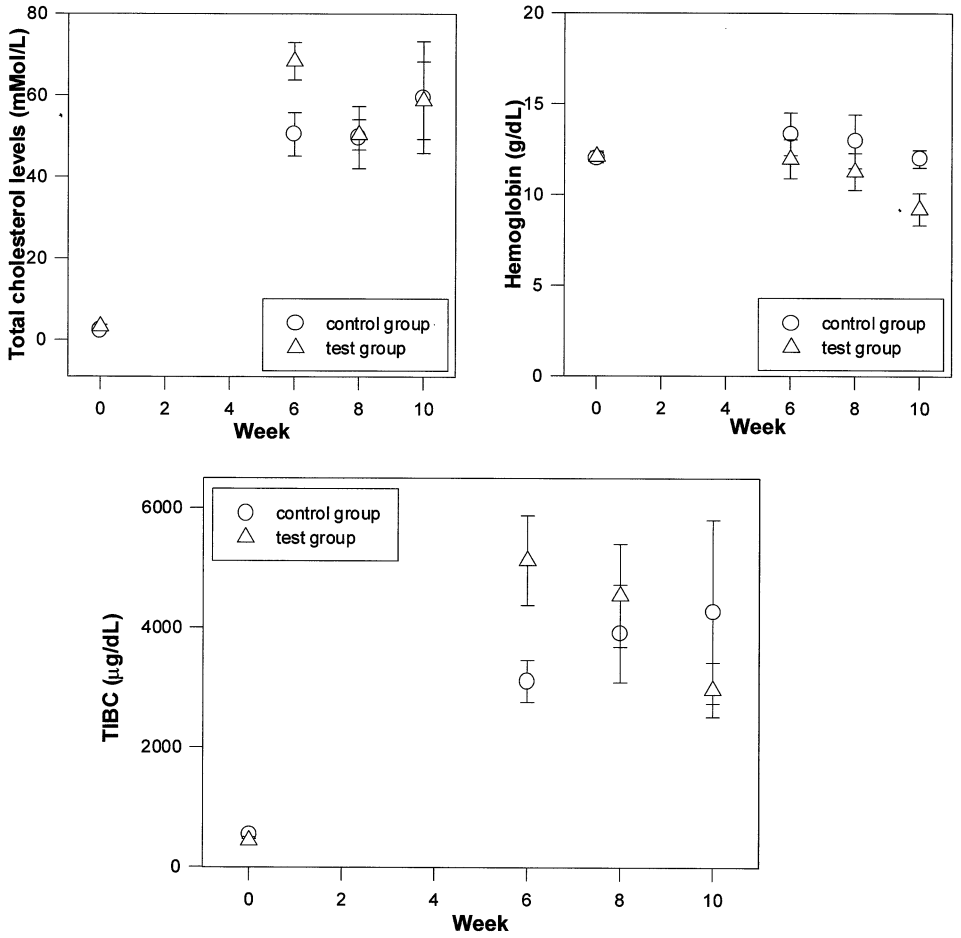

Fig. 2. Total cholesterol levels, hemoglobin levels, and TIBC levels monitored during the experiment.

mentary ion beam techniques simultaneously applied: (i) particle-induced X-ray emission (PIXE) was used for measuring the concentrations of elements such as sodium and above in the periodic table. X-rays were detected using a lithium-drifted silicon X-ray detector placed at 45° to the beam axis and fitted with a filter designed for optimal detection of trace elements such as iron in biological specimens; (ii) Rutherford backscattering spectrometry (RBS) provided information on matrix composition and incident charge used to extract quantitative results in conjunction with PIXE; and (iii) off-axis scanning transmission ion microscopy (STIM) provided information on the structure and density distribution of the sample and facilitated positioning of the unstained sections prior to analysis.

The lesion areas were determined from H and E stained tissue sections using the Carl Zeiss Axiophot 2 image analyzer utilizing the KS400 (version 3.18) analysis software.

RESULTS

Lesion area analysis

As expected, cholesterol feeding raised plasma cholesterol levels (Fig. 2), which led to the development of well-defined lesions. The lesion depth varies widely around the arterial wall (Fig. 3), as has been noted in many other studies. Lesion development begins at approximately 4 weeks and is substantial at 10 weeks (Fig. 4). Desferal had no effect on hemoglobin or cholesterol levels (Fig. 2). Lesion area analyses taken from sections removed from the A, B, and C aortic arch segments for the 4 groups (8 week test, 8 week control, 10 week test, and 10 week control) are shown in Fig. 4A. The average lesion area for the 8 week control group animals (cholesterol fed only) was 2.0 ± 0.5 mm^2 compared with 2.7 ± 0.5 mm^2 for the test animals (cholesterol fed + 2 week desferal), indicating no significant effect of desferal on lesion progres-

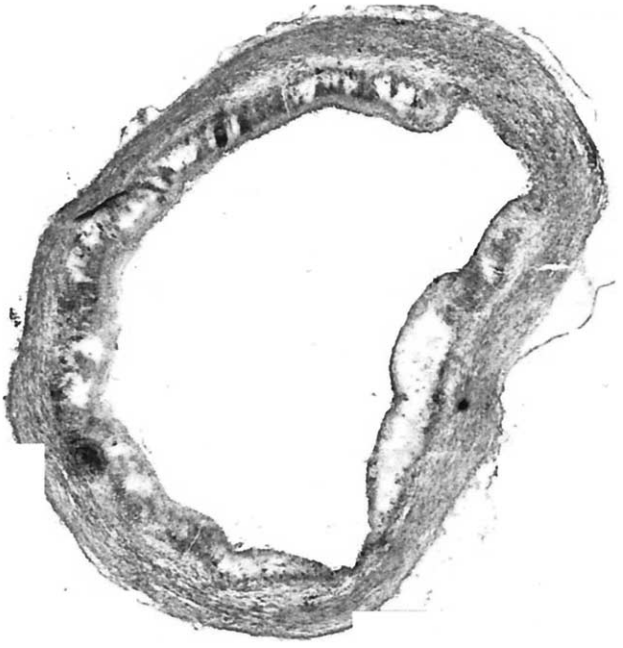

Fig. 3. H and E stained section of a rabbit aortic arch after 10 weeks on a high (1%) cholesterol diet showing atherosclerotic lesion and artery wall.

sion. The average lesion area for the 10 week control group animals (cholesterol fed only) was 5.4 ± 1.1 mm^2 compared with 3.5 ± 0.6 mm^2 for the test animals (cholesterol fed + 4 week desferal). A two sample Student's *t*-test yielded a nonsignificant difference in the 10 week controls compared with the 10 week test samples (C_p = 0.07). This limited effect may be because desferal is inefficient in chelating iron in non-iron-overloaded animals [16], as indicated by the lack of change in plasma Fe binding capacity (Fig. 2). The most striking aspect of our results, from the study of many samples, was that not only do the lesions vary in area around the inner artery wall in the same section, but also from section to section in the same animal and between different animals. This made it difficult to reach clear-cut conclusions.

Elemental analysis

The results of elemental iron concentrations (parts per million, dry weight) taken from unstained sections from the A, B, and C segments for the four groups are shown in Fig. 4B. The results show that for each group the iron levels are consistently higher in the lesion compared with the healthy artery wall. Further investigations were carried out to map the localized iron concentrations within the lesioned tissue around the inner artery wall. Sections taken from three animals from the 10 week test group and three animals from the 10 week control group were scanned using the nuclear microscope, and the iron concentrations were extracted for approximately 12 radial segments for each section of artery (see Fig. 5 for details). The lesion iron levels for each radial segment were plotted against the average depth of the lesion in that segment. Pearson correlation analysis of the data shows a strong significant correlation between iron levels and the depth of lesion for both the test groups and control groups (Fig. 6), but a clear inverse correlation for zinc except in one animal (Fig. 7). These graphs also further illustrate the high variability in iron and zinc levels and lesion areas between animals.

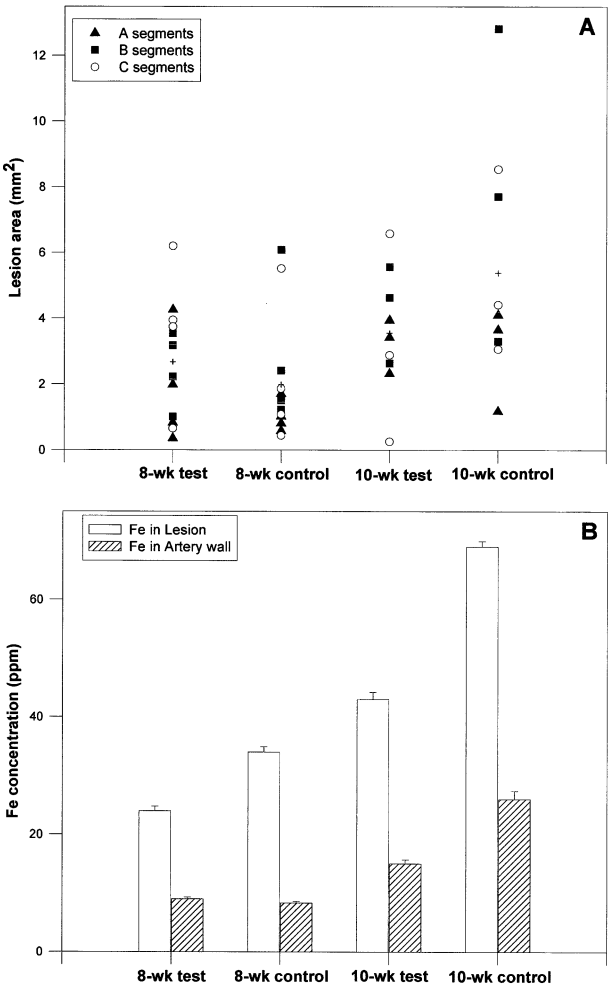

Fig. 4. (A) Graph of lesion area for the four groups: 8 week test (8 weeks on 1% cholesterol diet + chelation from 7 to 8 weeks), 8 week control (8 weeks on 1% cholesterol diet), 10 week test (10 weeks on a 1% cholesterol diet + chelation from 7–10 weeks), 10 week control (10 weeks on a 1% cholesterol diet). (B) Iron levels in lesion and the adjacent artery wall of control and test models.

DISCUSSION

Previous work has shown that New Zealand white rabbits fed a 1% cholesterol diet develop atheroscle-

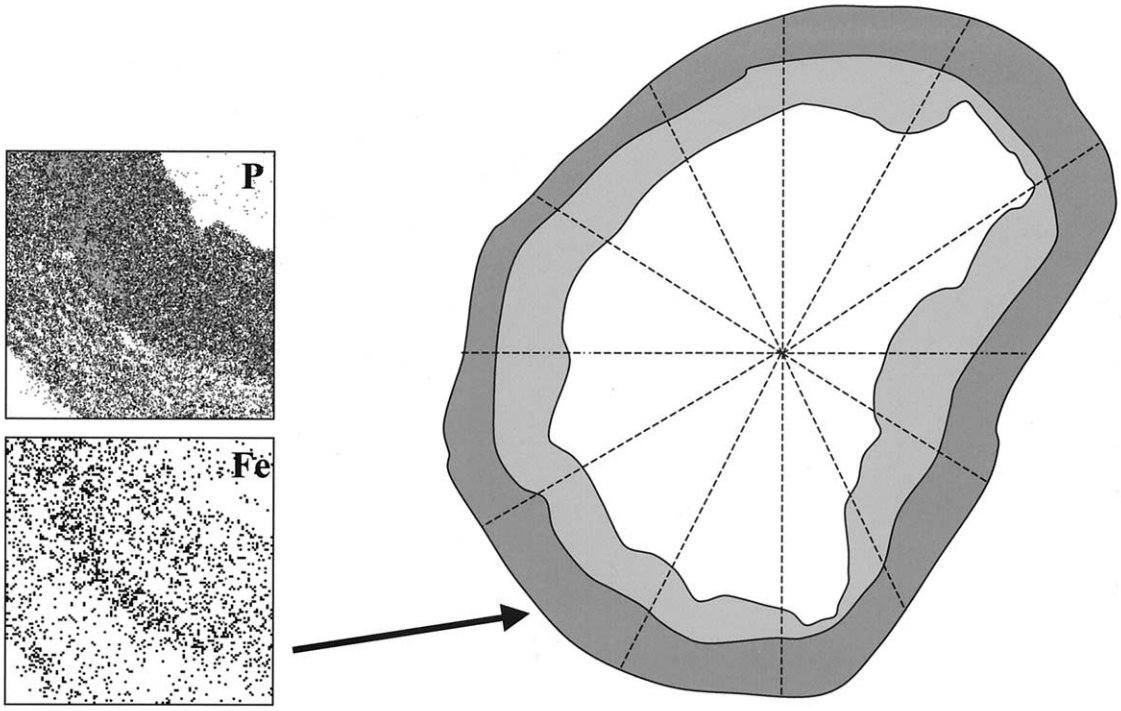

Fig. 5. Schematic diagram of iron concentration measurements. The lesion section is divided into approximately 12 segments, and elements (including iron) are mapped within these regions. Accurate concentrations can be extracted from these maps both for the lesion and the artery wall. Shown above is the iron map from one segment, together with the phosphorus map. The phosphorus map indicates the boundaries of the lesion since this region is rich in phospholipids. Where the lesion varied in depth over the segment, the average depth of the lesion is measured.

rotic lesions as early as 4 weeks, and that at 6 weeks the lesions are developing rapidly. Our previous results have shown that early atherosclerotic lesions contain up to 8 times the iron concentrations compared with healthy artery wall tissue, and that iron depletion induced by subjecting the rabbits to weekly bleeding both reduces the iron concentrations in early lesions and delays the onset of atherosclerosis [13]. We have observed here that desferal-induced iron chelation applied 6 weeks into the cholesterol diet for a period of 2 weeks does not slow the lesion development, but when applied for a period of 4 weeks indicates a possible trend ($p = .07$) to the reduction of lesion progression. This limited effect may be due to limited Fe chelation (Fig. 2) and raises the possibility that a further period of treatment might produce a significant reduction.

To show any systematic trend is not easy: observations on aortic arch tissue sections from New Zealand white rabbits that have been fed a high cholesterol diet show a high variability of lesion development not only from animal to animal, but also along the length of the artery and around the inner surface of the artery wall from the same animal. This may help to explain some of the contrasting reports in previous studies relating iron to atherosclerosis development. We have therefore decided to investigate the localized iron concentrations in the lesions around the artery wall using nuclear microscopy, which has the ability to accurately map iron concentrations down to the parts-per-million level (dry weight) in atherosclerotic lesions. Using tissue sections from three 10 week test rabbits (1% cholesterol diet, 4 weeks desferal chelation after 6 weeks), and three 10 week control rabbits (1% cholesterol diet only), we have shown that in all cases the iron concentrations within the lesion were highly correlated with the depth of the lesion in the artery wall for each individual animal. This result could imply that *local* elevated concentrations of iron present in early lesions may provide an accelerated process of atherogenesis in *specific* regions of the artery wall. From Fig. 6 it is also noted that although the lesion iron levels for each of the 6 animals individually are highly correlated with lesion depth, the average lesion iron levels differ from animal to animal. This could imply that for each animal there is a fraction of total lesion iron that is catalytic for free radical damage. Unfortunately, the nuclear microscope measures total iron and does not differentiate between Fe^{2+} and Fe^{3+} states. In addition, we have

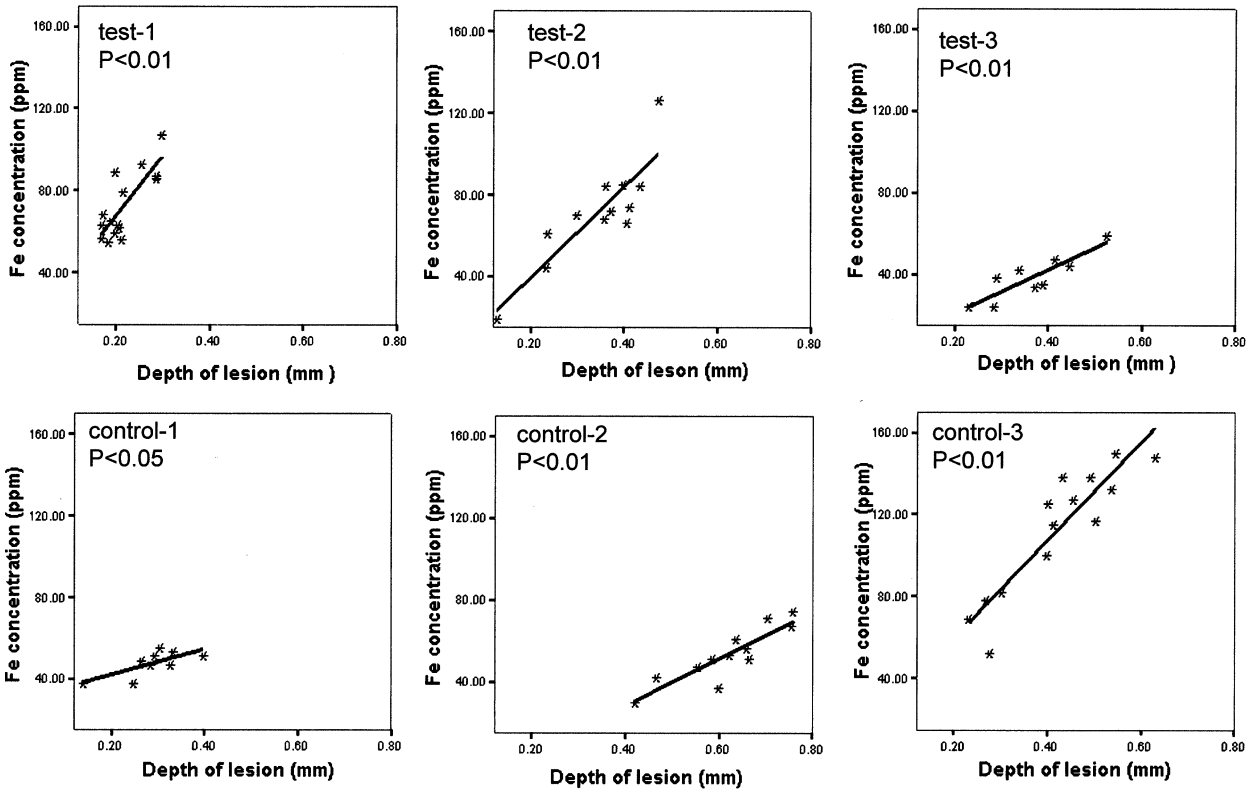

Fig. 6. Pearson correlation between the localized Fe concentration in the lesion and the depth of the lesion for three 10 week test specimens, and three 10 week control specimens.

also observed that zinc concentrations are inversely correlated with the depth of the lesion in the artery wall for each individual animal, consistent with suggestions of a possible antiatherosclerotic role for elevated zinc levels at microenvironments in the vessel wall [14,15].

Desferal does not penetrate into cells easily, and the rate at which it removes iron from various tissues is variable and can be slow [16]. Hence, it is possible that its limited effect in our study was due to insufficient iron removal, and this is suggested by Fig. 2, where statistically the TIBC levels at 6 weeks were not significantly different from levels at 10 weeks, both for the control and treatment groups. Nonetheless, it appears that, whether desferal is administered or not, the lower the local Fe level and the higher the local Zn level, the less developed is the atherosclerotic lesion in a particular region of the aortic arch. Of course our results cannot be taken to *prove* that iron is proatherosclerotic and zinc antiatherosclerotic, nor can nuclear microscopy identify the molecular form of these metals in the lesions. Nevertheless, our data add further support to the view that iron may be a promoter of atherosclerosis and zinc may have an antiatherosclerotic effect.

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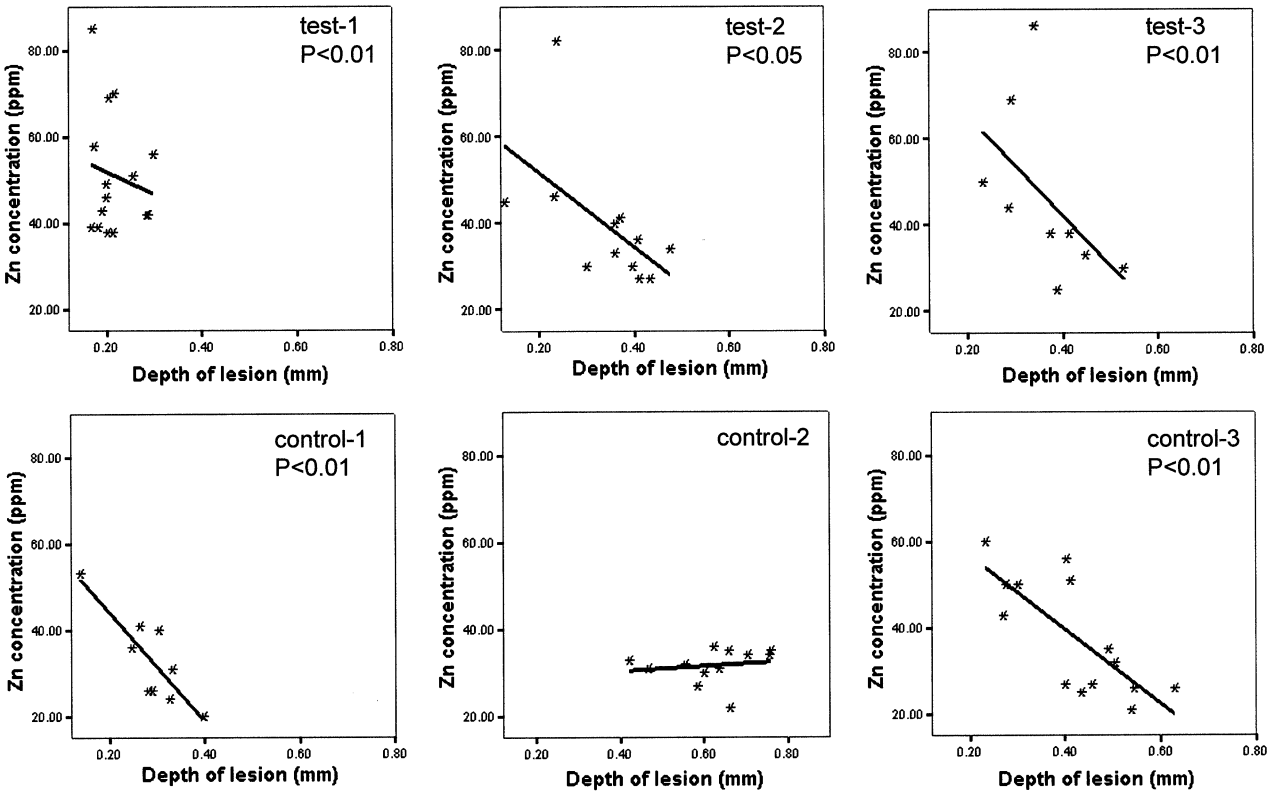

Fig. 7. Pearson correlation between Zn concentration (parts per million, dry weight), and the depth of the lesion.

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