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Atherosclerosis and hypertension induction by lead and cadmium ions: An effect prevented by calcium ion

(drinking water/elements/cardiovascular disease/pigeon)

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ABSTRACT **In epidemiological studies, both positive and negative correlations have been found between cardiovascular disease and mortality and the presence of several inorganic ions in the drinking water. In an attempt to resolve this apparent disagreement, we exposed White Carneau pigeons to drinking water containing calcium (100 ppm), magnesium (30 ppm), lead (0.8 ppm), or cadmium (0.6 ppm) and used a 2^4-factorial design to measure the effects of these elements in atherosclerosis and hypertension. The results indicate that (*i*) lead and cadmium induced aortic atherosclerosis and hypertension, and (*ii*) calcium protects against the cardiovascular effects of cadmium. Furthermore, the effects of lead and cadmium were promoted by magnesium, and there were indications that magnesium antagonized the atherosclerotic protective effect of calcium. We suggest that, if these results with the pigeon can be applied to humans, the incidence of aortic atherosclerosis and hypertension should be significantly higher in areas where the drinking water contains magnesium, lead, and cadmium with a relatively low calcium concentration. Furthermore, if hard and soft water produce similar levels of lead and cadmium uptakes, the level of magnesium may be an additional factor in aortic atherosclerosis.**

Over the past 20 years, epidemiological and clinical evidence on the relationship between the quality of drinking water and the death rate from cardiovascular disease has been conflicting and inconclusive. Earlier studies correlated soft water with a higher cardiovascular death rate (1). Later studies showed a similar relationship but did not agree on the type of cardiovascular disease related to soft water. Various studies linked water softness to ischemic heart disease (2), to hypertension (3), to atherosclerosis (4), and to stroke (4). However, other studies do not confirm these observations (5, 6).

Several specific elements in drinking water have been implicated in epidemiological studies of cardiovascular mortality. Noting that the relative concentration of calcium is lower in soft water than in hard water, several investigators studied the relationship between drinking-water calcium concentration and cardiovascular mortality and observed an increase in mortality when the relative concentration of calcium was lower (7). Furthermore, some studies have shown a higher incidence of cardiovascular mortality associated with consumption of soft water with relatively high concentrations of lead and cadmium (8). Again, however, other studies fail to confirm these correlations (9).

The objective of the present studies was to determine the effects of calcium, magnesium, lead, and cadmium on the induction and progression of atherosclerosis and hypertension. We chose the male White Carneau pigeon (3 months of age) for these studies because, unlike many other experimental models, it develops spontaneous atherosclerotic lesions of both the aorta and coronary arteries (10).

METHODS

Male pigeons 3 months of age were housed indoors (in climate-controlled, uncrowded quarters) in stainless steel cages in a bacteria-free environment containing very low levels of cadmium and lead. They were given, ad lib, double-deionized distilled drinking water and a diet (from a single lot over the experimental period) containing cadmium at <0.27 ppb and lead at <0.63 ppb. We then added combinations of calcium (100 ppm), magnesium (30 ppm), lead (0.8 ppm), and cadmium (0.6 ppm) (each element was added in the chloride form) to double-deionized drinking water in a 2^4-factorial experimental design to investigate the effects of these elements on atherosclerosis and hypertension in the pigeon. These concentrations are within the ranges found in drinking water consumed by humans.

After exposing the pigeons to these elements for 6 months, we recorded aortic blood pressure by introducing a cannula [the cannula was attached to strain-gauge pressure transducer (Stratham, P23ID)] through the pectoral artery into the aorta of the pigeons (unanesthetized). After blood pressure was determined we obtained blood samples from which we isolated plasma lipoproteins and determined the protein and cholesterol associated with them by described methods (11). The effects of these elements on lipoprotein metabolism are included in these studies because previous investigators have suggested that changes in plasma lipoproteins may have implications for the induction and progression of atherosclerosis (12).

After the blood sample was removed, the pigeons were killed by an injection of air and the aorta and pectoral, coronary, and carotid arteries were removed. These tissues were fixed and stained for lipid deposition with Oil Red O. After the stained tissues were washed, we counted the number of lipid-stained plaques and determined the size of each plaque by measuring the square area it occupied. Each plaque was frozen and sectioned, and the sections were stained with Masson's trichrome or alizarin red. Sections to be evaluated for lipid droplet accumulation were counterstained with nuclear fast green. Histological procedures were used in these studies to confirm the presence of atherosclerotic plaques.

RESULTS

We observed no atherosclerotic plaques in the carotid artery. Those plaques observed in the thoracic aorta were characterized

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Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein.

by lipid infiltration (both intra- and extracellular), calcium deposition, smooth muscle cell infiltration of the intima, and collagen accumulation in the media near the intimal plaques. As shown below, cadmium and lead induced atherosclerosis in the aorta. The plaques isolated from the aorta of cadmium-treated pigeons showed diffuse lipid infiltration and thickening of the intima whereas, in the lead-treated pigeons, lipid infiltration, intima thickening, and an increase in the smooth muscle mass were characteristic. This latter finding suggests that lead induces smooth muscle cell proliferation. Preliminary studies with isolated smooth muscle cells from the pigeon aorta confirm this suggestion.

The results described in this report are based on measurements taken from 64 pigeons: 4 birds (replications) in each of the 16 (i.e., 2^4) combinations for the presence and absence of the four elements. The main effects of these elements are thus based on comparisons between groups of 32 birds, second-order effects involve comparisons between groups of 16 birds, and so on. In many instances the interpretation of main effects is complicated by the presence of higher-order interactions. This aspect of the data has implications for epidemiological studies, and a major emphasis in what follows is the description of opposing influences that various combinations of calcium, magnesium, lead, and cadmium have on atherosclerosis and hypertension in the pigeon. We will first consider the mean values for the individual cell (in this instance, the cage) for each experimental combination of the four elements. These initial observations will then be reviewed in light of results from an analysis of variance.

Mean Values of Groups. The mean values of eight measurements for each experimental combination of the four elements are shown in Table 1. These results represent the individual means from each treatment group. With the exception of the group treated with calcium alone, there was little difference in the low density lipoprotein (LDL) protein and cholesterol values. The high density lipoprotein (HDL) protein and cholesterol values also were not significantly altered by these elements.

Diastolic pressure was 25 mm Hg higher ($P < 0.01$) in pigeons treated with magnesium, lead, or cadmium than in calcium-treated pigeons. Systolic pressure was significantly higher in the cadmium-treated pigeons than in pigeons treated with calcium, magnesium, or lead. In pigeons treated with the combinations of magnesium, lead, and cadmium, systolic pressure was significantly lower in the presence of calcium. It thus appears that the presence of calcium tends to reduce diastolic pressure and reduce the increase in systolic pressure produced by (*i*) cadmium alone, (*ii*) lead in the presence of magnesium, or (*iii*) lead and cadmium in the presence of magnesium.

A decrease in the number of aortic plaques was a typical response to the presence of calcium, but there was an increase in the presence of lead. The effect of cadmium depended on the presence or absence of calcium and magnesium. The experiment with both calcium and cadmium indicated that calcium has a synergistic inhibitory effect on cadmium when magnesium is present and an additive inhibition when magnesium is absent. The mean size of aortic plaques was usually smaller when calcium was present and larger when cadmium was present and calcium was absent. We observed no substantial difference in plaque size resulting from the presence or absence of magnesium. The largest plaques were observed in those pigeons exposed to magnesium and cadmium; however, plaque size was substantially smaller when calcium was also present. These observations suggest that the effects of calcium, lead, and cadmium on atherosclerosis are influenced by the presence of magnesium in the drinking water.

In pigeons treated with lead or with lead plus either magnesium or cadmium, we observed atherosclerotic plaques in the pectoral arteries. This effect seemed to be antagonized by calcium because plaques were not observed in the pectoral arteries when calcium was present in the drinking water containing lead.

Although calcium reduced atherosclerosis in the aorta, we did observe coronary artheriosclerotic lesions in pigeons treated with calcium alone. In addition, the LDL protein in this group increased 4-fold. It has been suggested that LDL apoprotein is an important factor in the induction and progression of arteriosclerosis or atherosclerosis (13). The occurrence of such an effect in calcium-treated pigeons suggests that the coronary arteries are more susceptible than the aorta to the change in LDL protein. In support of this suggestion are the observations by Clarkson *et al.* (13) that the coronary arteries and aorta are independent of each other in susceptibility to atherosclerosis.

Table 1. Effects of various elements in drinking water on plasma lipoproteins, blood pressure, and aortic atherosclerotic plaques in male White Carneau pigeons

Treatment group	Lipoprotein component, mg/mg lipoprotein				Blood pressure, mm Hg		Aortic plaques	
	LDL protein	LDL cholesterol	HDL protein	HDL cholesterol	Systolic	Diastolic	Size, mm^2	No.
Control	0.9 ± 0.1	0.6 ± 0.1	2.1 ± 0.2	1.3 ± 0.2	170 ± 8	140 ± 6	1.1 ± 0.4	1.1 ± 0.1
Ca	4.2 ± 1.7	1.7 ± 0.4	2.0 ± 0.2	0.6 ± 0.1	180 ± 7	133 ± 13	0.8 ± 0.2	1.2 ± 0.4
Mg	0.8 ± 0.2	0.3 ± 0.1	1.4 ± 0.2	0.3 ± 0.02	190 ± 10	153 ± 6	0.9 ± 0.5	0.8 ± 0.5
Pb	0.9 ± 0.1	0.4 ± 0.02	2.7 ± 0.1	0.9 ± 0.1	190 ± 6	155 ± 4	2.4 ± 0.2	1.8 ± 0.2
Cd	0.6 ± 0.0	0.2 ± 0.03	1.6 ± 0.1	0.25 ± 0.1	203 ± 8	158 ± 6	2.9 ± 0.2	1.8 ± 0.5
Ca, Mg, Pb, Cd	0.9 ± 0.01	0.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.2	194 ± 10	158 ± 8	0.5 ± 0.3	0.8 ± 0.4
Ca, Mg	0.7 ± 0.03	0.4 ± 0.1	1.7 ± 0.1	1.4 ± 0.4	188 ± 3	146 ± 4	1.0 ± 0.0	1.2 ± 0.3
Ca, Pb	0.6 ± 0.1	0.8 ± 0.1	2.6 ± 0.8	1.3 ± 0.1	216 ± 15	157 ± 9	1.5 ± 0.3	1.7 ± 0.1
Ca, Cd	0.6 ± 0.1	0.9 ± 0.04	1.5 ± 0.2	1.3 ± 0.1	193 ± 7	150 ± 2	1.0 ± 0.4	1.5 ± 0.5
Ca, Mg, Pb	0.7 ± 0.1	1.9 ± 0.3	2.1 ± 0.3	1.2 ± 0.1	194 ± 2	155 ± 3	1.5 ± 0.3	2.2 ± 0.2
Ca, Mg, Cd	0.6 ± 0.1	0.6 ± 0.04	1.8 ± 0.13	1.1 ± 0.1	207 ± 8	150 ± 2	1.0 ± 0.4	1.5 ± 0.6
Ca, Pb, Cd	0.8 ± 0.1	1.9 ± 0.2	1.2 ± 0.0	1.1 ± 0.1	192 ± 5	151 ± 6	1.4 ± 0.5	1.8 ± 0.2
Mg, Pb, Cd	0.7 ± 0.1	0.7 ± 0.1	1.5 ± 0.3	1.1 ± 0.1	236 ± 9	186 ± 5	3.8 ± 0.4	2.4 ± 0.1
Mg, Pb	0.9 ± 0.1	0.8 ± 0.1	2.7 ± 0.6	1.4 ± 0.1	222 ± 9	153 ± 6	2.3 ± 0.8	1.7 ± 0.6
Mg, Cd	1.0 ± 0.04	1.3 ± 0.2	1.4 ± 0.01	0.5 ± 0.1	203 ± 4	160 ± 4	5.2 ± 1.1	1.5 ± 0.3
Pb, Cd	1.2 ± 0.1	0.3 ± 0.1	1.6 ± 0.2	0.8 ± 0.1	196 ± 6	150 ± 4	3.3 ± 0.3	2.3 ± 0.2

Pigeons were exposed to calcium (100 ppm), magnesium (30 ppm), lead (0.8 ppm), and cadmium (0.6 ppm) for 6 months before the tests were performed. Results are expressed as mean ± SEM for at least four pigeons per group. LDL, low density lipoprotein; HDL, high density lipoprotein.

Interactions. The overall first-order interactions of magnesium, lead, and cadmium on diastolic blood pressure indicated significant increases in the presence of these elements (Table 2). However, a significant third-order interaction involving these three factors implied that the interpretation of overall first-order interactions should also consider the joint presence of these three elements. Analyses of third-order interactions showed that, in the absence of magnesium, lead or cadmium appeared to increase diastolic pressure approximately 20 mm Hg, although the increase when they were both present was about 15 mm Hg. In the presence of magnesium, the increases observed in the presence of either lead or cadmium were again about 20 mm Hg; however, the increase for their joint presence was approximately 30 mm Hg. These values are based on averaging over calcium so that a difference for any two particular combinations of magnesium, lead, and cadmium has a SEM of $(MSE/4)^{1/2} = 6.6$. The large value for the magnesium, lead, cadmium combination (Table 1) essentially reflects the observations in pigeons not exposed to calcium and appears to be a substantial influence on the overall main effects of magnesium, lead, and cadmium.

The analysis of variance for systolic blood pressure also indicated significant overall first-order interactions of magnesium, lead, and cadmium (Table 2). No third- or fourth-order effects appeared to be significant, although second-order interactions between calcium and magnesium and between lead and cadmium were moderately significant (Table 3).

We noted a significant overall decrease in the number of aortic plaques when calcium was present and a significant overall increase when lead was present (Table 2). Also observed were significant second-order interactions between calcium plus cadmium and calcium plus lead. No third- or fourth-order effect proved to be significant, although the calcium, magnesium, cadmium combination effect was borderline ($P < 0.07$ and $P < 0.08$), thus confirming the suggestions that magnesium interacts with calcium and cadmium to block the effects of these two elements on plaque number.

For the measurements of mean size of aortic plaques, the presence of two significant third-order interactions—namely, magnesium, calcium, cadmium and magnesium, lead, cadmium—implied that the effects of calcium and cadmium should be interpreted in light of the joint presence or absence of magnesium and lead. The second-order effects of calcium plus cadmium and of lead plus cadmium (Table 3) were nonadditive in the presence of magnesium and additive in its absence. These results suggest that magnesium and cadmium are primarily responsible for these interactions.

Together, our findings on the number of aortic plaques and mean plaque size demonstrate the complex relationships of these four elements in their effects on blood pressure and atherosclerosis. Furthermore, when plasma lipoprotein or plasma lipoprotein cholesterol was considered with the number and size of aortic plaques, no correlation was apparent (correlation coefficients measuring linear association were all nonsignificant at 0.05). This suggests that, although these elements affect the levels of protein and cholesterol, the protein and cholesterol of these lipoproteins are not good indicators of aortic atherosclerosis.

DISCUSSION

Since Kobayashi (14) first observed a significant correlation between the quality of drinking water and human disease, many studies have been performed to determine if other human diseases could be correlated to the quality of drinking water. However, the results from these epidemiological studies have been conflicting. Evaluating the effects of drinking water quality independently from other potentially confounding variables such as sex, age, sample size, climate, diet, mineral content of drinking water, and urbanization has been a major problem. Furthermore, in most epidemiological studies, correlation coefficients have been used to measure the association of drinking water quality to cardiovascular disease. However, Neri *et al.* (15) have shown that the correlation coefficient is affected much more by chance variation than is the regression coefficient; thus, the sole use of the correlation coefficient for determining this association is questionable. The discrepancies that exist between the quality of drinking water and cardiovascular disease may be related to these variables and to the methods of analysis.

To clarify these epidemiological results, some investigators

Table 2. First-order interactions

		Lipoprotein component, mg/mg lipoprotein				Aortic plaques		Blood pressure, mm Hg	
Element	Level	LDL protein	LDL cholesterol	HDL protein	HDL cholesterol	No. per pigeon	Mean size, mm^2	Systolic	Diastolic
Ca	0	0.86	0.56	1.98	0.82	1.78	2.53	202	156
	1	1.11	1.14	1.87	1.24	1.25	1.52	202	151
	P	***	***	NS	***	**	***	NS	NS
Mg	0	1.19	0.86	1.98	0.94	1.47	2.03	194	149
	1	0.78	0.83	1.86	1.13	1.56	2.02	209	159
	P	***	NS	NS	***	NS	NS	**	**
Pb	0	1.15	0.76	1.70	0.83	1.09	2.00	195	149
	1	0.82	0.94	2.06	1.23	1.94	2.05	208	158
	P	***	*	***	***	***	NS	**	**
Cd	0	1.19	0.87	2.15	1.08	1.44	1.64	196	149
	1	0.78	0.82	1.61	0.99	1.59	2.41	207	159
	P	***	NS	***	*	NS	**	*	**
SEM (from analysis of variance)		0.021	0.562	0.042	0.027	0.138	0.162	3.31	2.32

Results for the one-way interaction for four elements in a 2^4-factorial analysis of variance; mean values based on the presence or absence of each element in the drinking water. Probability associated with the *F* test for each measurement: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not statistically significant.

Table 3. Second-order interactions

Elements	Level	Lipoprotein component, mg/mg lipoprotein				Aortic plaques		Blood pressure, mm Hg	
		LDL protein	LDL cholesterol	HDL protein	HDL cholesterol	No. per pigeon	Mean size, mm^2	Systolic	Diastolic
$Ca^{2+} + Mg^{2+}$	0 0	0.86	0.36	1.95	0.82	1.69	2.58	189	150
	0 1	0.85	0.75	1.84	0.83	1.88	2.55	215	163
	1 0	1.51	1.36	1.84	1.06	1.25	1.55	200	148
	1 1	0.72	0.92	1.89	1.42	1.25	1.49	203	154
	P	***	***	NS	***	NS	NS	*	NS
$Ca^{2+} + Pb^{2+}$	0 0	0.81	0.57	1.60	0.59	1.25	2.60	193	153
	0 1	0.90	0.54	2.19	1.06	2.31	2.46	211	160
	1 0	1.50	0.94	1.79	1.08	0.94	1.41	197	146
	1 1	0.73	1.34	1.94	1.40	1.56	1.64	206	157
	P	***	*	***	NS	NS	NS	NS	NS
$Ca^{2+} + Cd^{2+}$	0 0	0.86	0.54	2.19	0.98	1.44	1.62	193	150
	0 1	0.85	0.57	1.60	0.67	2.13	3.43	211	163
	1 0	1.53	1.20	2.11	1.17	1.44	1.65	200	148
	1 1	0.70	1.08	1.63	1.31	1.06	1.39	203	155
	P	***	NS	NS	***	**	***	NS	NS
$Mg^{2+} + Pb^{2+}$	0 0	1.54	0.88	1.79	0.85	1.13	1.74	190	146
	0 1	0.83	0.84	2.01	1.03	1.81	2.32	199	153
	1 0	0.77	0.63	1.60	0.82	1.06	2.27	201	153
	1 1	0.80	1.04	2.12	1.43	2.06	1.78	217	164
	P	***	**	*	***	NS	*	NS	NS
$Mg^{2+} + Cd^{2+}$	0 0	1.60	0.90	2.35	1.04	1.25	1.68	191	146
	0 1	0.77	0.82	1.45	0.84	1.69	2.37	198	152
	1 0	0.78	0.84	1.95	1.11	1.63	1.59	202	152
	1 1	0.78	0.83	1.77	1.14	1.50	2.46	216	166
	P	***	NS	***	**	NS	NS	NS	NS
$Pb^{2+} + Cd^{2+}$	0 0	1.62	0.76	1.80	0.91	0.88	1.24	185	144
	0 1	0.68	0.75	1.59	0.76	1.31	2.76	206	155
	1 0	0.76	0.98	2.49	1.24	2.00	2.03	208	154
	1 1	0.87	0.90	1.63	1.22	1.88	2.07	209	163
	P	***	NS	***	NS	NS	**	*	NS
SEM (from analysis of variance)		0.0296	0.0795	0.0592	0.0387	0.1951	0.2289	4.6744	3.2797

Results for the two-way interactions for four elements in a 2^4-factorial analysis of variance; mean values based on the presence or absence of elements taken in pairs. Probability associated with *F* test of second-order interactions: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not statistically significant.

have turned to experimental animals. However, conflicting reports have also appeared. For example, Neal and Neal (16) reported that rabbits drinking distilled water develop more atherosclerotic plaques than do those drinking hard water. In pigs raised in hard water and soft water areas of the United States and Europe, the incidence of atherosclerosis was higher (although not significantly) in those pigs raised in the soft water area (17). Puschner *et al.* (18) kept two groups of 10 pigs each under similar conditions from birth, except that one group was given hard water and the other group was given soft water. After 3 months' exposure to these waters, no significant differences in atherosclerosis in the pigs were observed at autopsy.

In the present study we attempted to explain these conflicting results in human and animal studies by exposing White Carneau Pigeons to elements that have been frequently associated with cardiovascular disease. Two of these elements have been negatively correlated (i.e., calcium and magnesium) and two have been positively correlated (i.e., cadmium and lead) with cardiovascular disease (1, 9, 19). The results from these studies confirm the cardiovascular effects of lead and cadmium. However, the atherosclerotic and hypertensive effects of cadmium depended on the presence or absence of calcium. For example, when calcium was present, the cardiovascular effects of cadmium were significantly reduced. Previous investigators have shown that calcium reduces the intestinal absorption of cadmium (20), which may explain the present interaction between calcium and cadmium. Nevertheless, calcium was ineffective in significantly reducing the cardiovascular effects of lead. In contrast, magnesium promoted the atherosclerotic (Table 3) and hypertensive (Table 1) effect of lead and cadmium.

The results of this study demonstrate that the cardiovascular effects of cadmium depend on the level of calcium in the drinking water. We suggest that, if these results apply to humans, the incidence of aortic atherosclerosis and hypertension should be significantly high in areas where there is a high intake of lead and cadmium and a low intake of calcium [the findings in Glasgow, Scotland, tend to support this suggestion (19)]. The lack of consistent results from the epidemiological and animal studies may be related to the relative level of calcium, lead, and cadmium found in the drinking water. For example, the concentration of calcium in drinking water considered to be hard ranges from 60 to 600 ppm (and higher in some areas). The ab-

sorption of cadmium may be significantly reduced by calcium at 100 ppm but not at 60 ppm. Furthermore, lead absorption may be significantly reduced by high level of calcium in the drinking water (i.e., >100 ppm). We have some evidence supporting this latter suggestion.

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