Effect of Interventions Started Early in Life on the Development of Spontaneous Atherosclerosis (40929)

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Abstract. A long-term study of the effect of interventions started early in life (3 months of age) on the age-related changes leading to spontaneous atherosclerosis in White Carneau pigeons was carried out. Of the four interventions tried, dietary restriction and cholestane-triol administration significantly decreased the incidence of atherosclerosis. Both of these interventions significantly reduced plasma cholesterol levels, aortic cholesterol content, and percentage distribution of oleic acid in cholesteryl esters. Cholestane-triol, in addition, decreased the content of hydroxyproline in the aorta. Ileal bypass surgery had no significant effect on the severity of atherosclerosis. Estrogen administration, on the other hand, caused an increase in aortic cholesteryl ester content and a trend toward an increase in the severity of atherosclerosis. These studies clearly demonstrate that some interventions started early in life can successfully prevent spontaneous atherosclerosis.

Epidemeological and pathological studies of atherosclerosis have suggested that this disease process is age related (1, 2). The progression of atherosclerosis is thought to involve a sequence with (a) fatty streaks appearing during the first two decades of life, (b) occurrence of fibrous plaques by the third decade, and (c) occurrence of complicated lesions during fourth and fifth decades. Therefore, it is of primary interest to determine whether interventions started early in life can prevent the age-related progression leading to complicated atherosclerotic lesions. Whether such an intervention started early in life can prevent or decrease the naturally occurring disease and which type of intervention (surgical, medical, or dietary) is more effective has not been adequately documented in any animal model. In animals fed cholesterol, however, it has been shown (3, 4) that the atherosclerotic lesions can be regressed following withdrawal of cholesterol. In order to demonstrate inhibition of progression of naturally occurring atherosclerotic disease one needs to have (a) an animal model that has 100% incidence of spontaneous atherosclerosis and (b) demonstrate age-related arterial changes leading to atherosclerosis without cholesterol feeding or artificial endothelial injury.

White Carneau pigeon is an animal model that fulfills these criteria with a 100% incidence of atherosclerotic lesions (5) and shows intimal cushion at birth, lipid accumulation at 9 to 12 months of age, and definite atherosclerotic plaques by 3 years of age (6–8). Using this animal model experiments were carried out to determine whether interventions started early in life can prevent or decrease the incidence of atherosclerosis by inhibiting the age-related arterial changes. Four interventions were chosen for the study: (i) ileal bypass surgery, previously shown (9) to regress well-established atherosclerotic lesions in this model; (ii) administration of estrogens, previously shown (10) to decrease the severity of atherosclerosis in adult pigeons; (iii) dietary restriction, previously shown (11) to decrease the accumulation of cholesterol in aorta in pigeons, and (iv) cholestane-triol, a compound shown (12) to decrease plasma cholesterol in this species. This report describes the effect of these four interventions started at 3 months of age on the incidence of atherosclerotic lesions after 33 months.

Materials and methods. One hundred White Carneau pigeons (3 months old) were obtained from the Palmetto Pigeon Plant, Sumter, South Carolina, and were given a cholesterol-free grain diet (Purina Pigeon Chow with (g/kg) 150 protein, 30 fat, and 60

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crude fiber) throughout the experimental period unless otherwise specified. A group of 15 pigeons of mixed sexes was randomly assigned to a control or an experimental group. Unlike other species, pigeons do not show any sex differences in the incidence of atherosclerosis (5). Group 1 was the controls; group 2, ileal bypass; group 3, sham surgery; group 4, dietary restriction; group 5, cholestane-triol; and group 6, estrogen treatment.

One group was subjected to ileal bypass surgery (13), as described by Gomes *et al.* (9). Ileal bypass was accomplished by side-to-side anastomosis of the small intestine and the cloaca (the cloaca in pigeons is equivalent to the colon in mammals). The products of digestion were excluded from the bypassed segment by a ligature. The other group was subjected to a sham operation (abdominal exploration) and was used as a control. Two pigeons with ileal bypass died during the first week after surgery and these birds were not replaced.

Conjugated equine estrogen (Premarin) in doses of 0.25 mg/kg (10) daily was administered by dissolving it in drinking water for a period of 33 months. Cholestane-$3_{\beta}5_{\alpha},6_{\beta}$-triol (purchased from Bioclinical Laboratories, Bohemia, N.Y.) was mixed with the diet in an amount to provide 0.5% of the diet by weight. This dosage has been previously shown to cause a definite reduction in plasma cholesterol (12).

For dietary restriction studies, the pigeons were kept in individual metabolism cages during the entire study (33 months). The dietary intake of the control group of pigeons (*ad libitum* group) was carefully monitored initially. The experimental group (dietary-restricted group) was then given 40% of the dietary intake of the *ad libitum* group. The dietary intake of the control group was monitored weekly, and the intake of the dietary-restricted group was adjusted to 40% of that intake. However, the adjustment needed was found to be minimal because dietary intake of the *ad libitum* group did not change significantly during the study period (29.2 to 35.1 g/day). The pigeons were killed 33 months after the study began.

At the end of 33 months, samples of blood were taken and the pigeons were killed by an overdose of barbital. The aortas were dissected quickly, and the area in between 2-mm points below and above the coeliac branch of the aorta (site of spontaneous atherosclerotic lesions (14) in these pigeons) was cut out and placed on top of a transparent plastic sheet lying on graph paper. The aortas were then photographed and the color slides obtained were projected on a screen. The area of atherosclerotic lesion (yellow) was traced on paper and estimated by planimentry, as previously described (9, 15). The area of the lesion at the celiac branch of the aorta was used for chemical studies.

Chemical analysis was carried out on three separate pools of aortas (each pool comprising three or four aortas) from each group. Total lipids from each pool of aortas were extracted with 20 columns of chloroform:methanol (2:1), as described by Folch *et al.* (16). Various lipids were separated by thin-layer chromatography on silica gel using a solvent system of heptane:isopropyl ether:acetic acid (65:40:4, v/v/v), as described previously (17). In this solvent system all the phospholipids remain at the origin. Bands corresponding to standards of free cholesterol and cholesteryl ester were marked and scraped into vials. Both sterols and steryl esters were eluted from the silica gel G by three extractions, each with 4 ml of chloroform. The extracts were pooled and evaporated to dryness. The steryl ester fraction was saponified with 1 *N* NaOH in 20% ethanol (14). The sterols were then quantitated by gas–liquid chromatography.

The sterols (both free and esterified) were converted to their trimethylsilyl derivatives and separated by gas–liquid chromatography using 5α-cholestane as an internal standard (11). The sterols were quantitated on the basis of the internal standard peak areas.

Sterols were analyzed using a Packard Model 409 gas chromatograph equipped with 4-ft 4-mm i.d. glass columns containing 3.8% W-98 packings. Column conditions were as follows: oven, 230°; injector, 270°; detector, 300°; and carrier gas,

TABLE I. EFFECT OF EARLY INTERVENTION ON AORTIC ATHEROSCLEROSIS, BODY WEIGHT, AND PLASMA CHOLESTEROL IN PIGEONS (MEAN ± SEM)

Group	Body weight (g)		Plasma cholesterol (mg %)		Aortic atherosclerosis (mm²)
	INITIAL	FINAL	INITIAL	FINAL	
Control (15)	567 ± 23	585 ± 19	268 ± 10	308 ± 14	8.8 ± 0.9
Ileal bypass (13)	523 ± 15	554 ± 39	261 ± 17	284 ± 19	7.1 ± 0.3
Dietary restriction (14)	541 ± 17	447 ± 10[a]	297 ± 12	242 ± 26[b]	0.4 ± 0.01[c]
Cholestane-triol (14)	527 ± 14	579 ± 9	304 ± 15	267 ± 10[b]	2.3 ± 0.4[c]
Estrogen (15)	607 ± 12	597 ± 17	305 ± 12	282 ± 19	12.0 ± 2.7

[a] $P < 0.01$ for difference from the control group.
[b] $P < 0.01$ for difference from the control group.
[c] $P < 0.01$ for difference from the control group.

helium, 50 ml/min. Sterols were identified by their retention times as compared with those of authentic standards. The plasma cholesterol level was measured by the method of Levine and Zak (18). The steryl ester fatty acid composition was analyzed following methylation, as described previously (14). The content of aortic hydroxy proline (as a measure of collagen concentration) was estimated by the procedure of Martin and Axelrod (19).

Results. Table I shows the data on the body weight, plasma cholesterol level, and aortic atherosclerosis index in control and in experimental groups of pigeons subjected to various interventions early in life. Body weights of pigeons from various intervention groups (except dietary restriction) did not change significantly during the study period. As expected, the body weights of pigeons subjected to dietary restriction showed a significant reduction ($P < 0.01$). The plasma cholesterol level of the control group increased significantly by the end of the study. Pigeons with ileal bypass and those treated with estrogens did not show significant differences in plasma cholesterol levels when compared to their initial levels. The plasma cholesterol levels of the dietary restricted and cholestane-triol-treated groups were significantly lower ($P < 0.01$) when compared to the control group. The plasma cholesterol levels of the ileal bypass and estrogen-treated groups did not differ significantly from the control pigeons at the end of study. The degree of aortic (coeliac) atherosclerosis was significantly lower ($P <$ 0.01) in dietary restricted and cholestane-

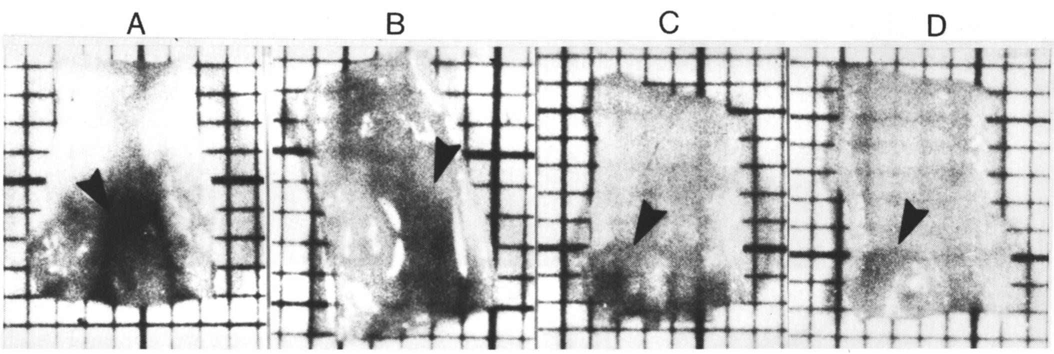

FIG. 1. Gross appearance of coeliac branch of aorta from control (A), estrogen-treated (B), cholestane-triol-treated (C), and dietary restricted (D) pigeons (all 3 years of age). Note marked reduction of atherosclerotic lesions in dietary restricted and cholestane–triol groups. The arrow indicates the position of atherosclerotic lesions (black).

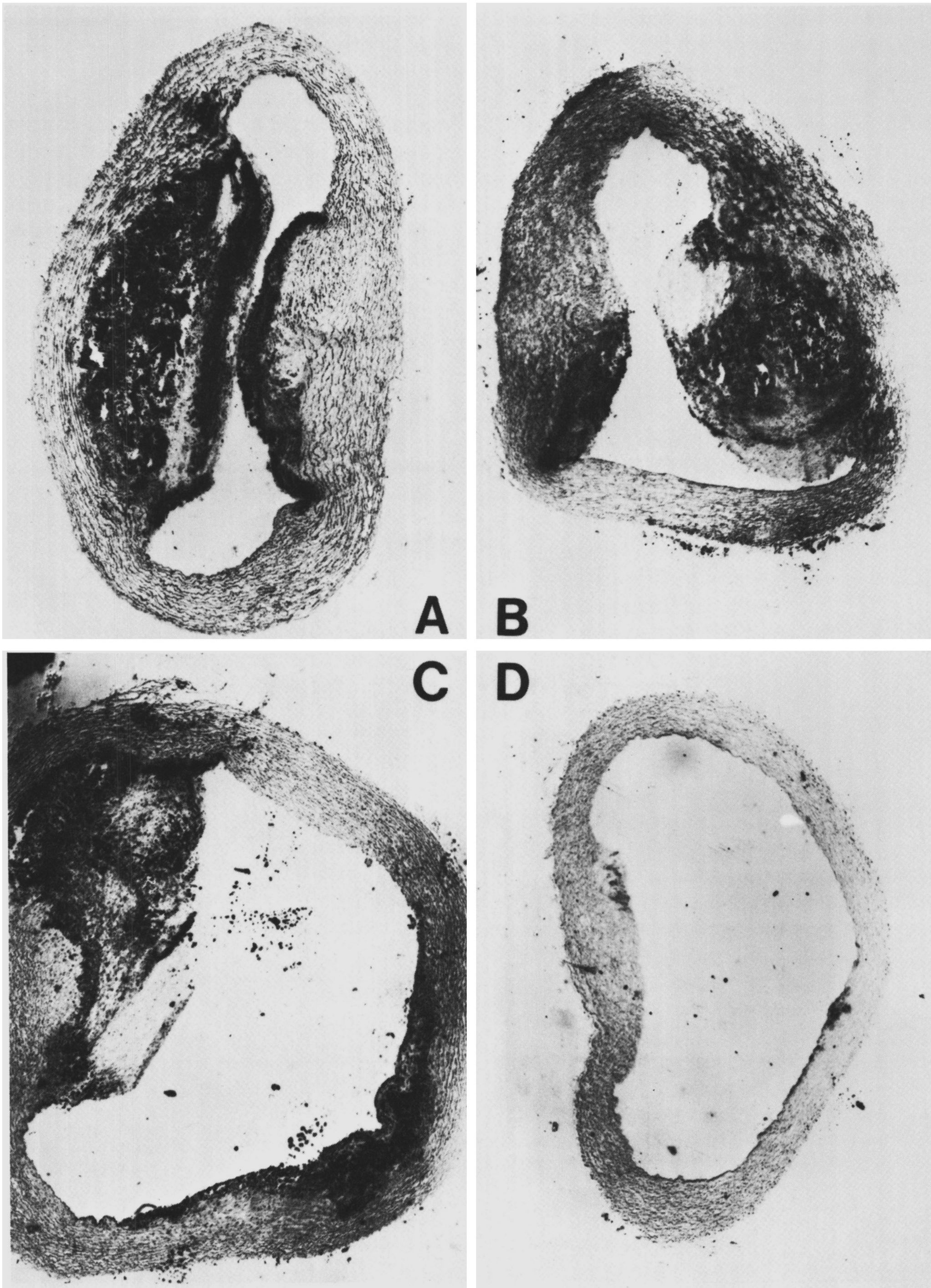

FIG. 2. Cryostat sections from the coeliac bifurcation of aorta from control (A), estrogen-treated (B), cholestane-triol-treated (C), and dietary restricted (D) pigeons (all 3 years of age). Note massive accumulation of lipids and lumen narrowing in A and B, while considerable decrease in lumen occlusion is seen in C and D (oil red O stain; fat particles seen as black spots; original magnification, ×40).

triol-treated groups. The group treated with estrogen showed a slight trend toward increase in the degree of atherosclerosis. Figure 1 shows photographs of representative gross aortas (around the coeliac bifurcation) from various groups of pigeons. As can be seen from the figure, the dietary restricted group showed a marked decrease (virtually traces) in atherosclerotic lesions, while the cholestane-triol group showed a moderate decrease in the severity of atherosclerosis.

Light microscopic examination of oil red O-stained sections from coeliac bifurcation of aortas from various groups of pigeons essentially confirmed the gross observations (Fig. 2). While aortas from control and estrogen-treated pigeons show marked narrowing of the lumen, in aortas from cholestane-triol and dietary restricted pigeons a substantial decrease in lumen occlusion is seen. Also, the intensity of staining with oil red O was minimal in the dietary restricted group and reduced in the cholestane-triol-treated group.

Table II shows the content of cholesterol and cholesteryl esters and hydroxyproline in the aorta of pigeons subjected to various interventions. The content of both free and esterified cholesterol in the dietary restricted and cholestane-triol-treated pigeons was significantly lower ($P < 0.01$) when compared to control pigeons, while aortic cholesteryl ester content was about 30% higher in estrogen-treated pigeons. The content of aortic hydroxyproline was significantly ($P < 0.05$) lower only in the cholestane-triol group. The fatty acid composition of aortic cholesteryl ester from various intervention groups (Table III) showed a significant decrease in the percentage composition of oleic acid in the dietary restricted and cholestane-triol-treated groups. There was a significant increase in the concentration of linoleic acid in the cholestane-triol-treated group. The group subjected to dietary restriction showed a complete absence of arachidonic acid in the cholesteryl ester fraction.

Discussion. The results of this study have clearly shown that early intervention in age-related arterial changes can successfully decrease the incidence and severity of atherosclerosis in pigeons. Of the four interventions tried, dietary restriction and cholestane-triol were successful in decreasing the severity of atherosclerosis. Surprisingly, ileal bypass (9) and estrogen treatment (10), which were successful in the regression of established atherosclerotic lesions in pigeons, did not prevent the age-related arterial changes leading to atherosclerosis. In fact, estrogen-treated pigeons showed a trend toward an increase in atherogenesis. This suggests that administration of excessive estrogens early in life has a detrimental effect on the arterial wall. This observation is important in view of the reports (20, 21) of increased incidence of thrombotic and atherosclerotic complica-

TABLE II. EFFECT OF EARLY INTERVENTION ON AORTIC CHOLESTEROL AND HYDROXYPROLINE CONTENT IN PIGEONS (MEAN ± SEM)

Group	Aortic cholesterol (mg/g dry wt) Free	Esterfied	Total	Aortic hydroxyproline (mg/g dry wt)
Control	37.8 ± 8.5	34.8 ± 4.3	72.6 ± 4.8	27.3 ± 1.0
Ileal bypass	31.5 ± 1.8	32.0 ± 5.0	63.6 ± 6.7	26.0 ± 0.5
Dietary restriction	6.3 ± 0.5[a]	2.9 ± 0.3[b]	9.2 ± 0.8[c]	25.1 ± 2.2
Cholestane-triol	10.0 ± 0.8[a]	6.8 ± 0.9[b]	16.9 ± 1.7[c]	18.4 ± 1.0[d]
Estrogen	35.5 ± 2.8	44.5 ± 3.2[d]	80.0 ± 6.0	25.9 ± 1.1

[a] $P < 0.01$ for the difference from the control group.
[b] $P < 0.01$ for the difference from the control group.
[c] $P < 0.01$ for the difference from the control group.
[d] $P < 0.05$ for the difference from the control group.

TABLE III. EFFECT OF EARLY INTERVENTION ON CHOLESTERYL ESTER FATTY ACID COMPOSITION IN PIGEON AORTA (Mean ± SEM)

Group	Fatty acid composition (%)							
	14:0	16:0	16:1	18:0	18:1	18:2	20:4	Others
Control	0.3 ± 0.06	9.6 ± 1.0	1.7 ± 0.3	1.1 ± 0.2	62.4 ± 4.1	20.9 ± 3.8	2.1 ± 0.4	1.9 ± 0.3
Ileal bypass	0.3 ± 0.06	11.0 ± 0.8	1.2 ± 0.3	1.4 ± 0.3	51.1 ± 6.2	30.8 ± 5.1	2.0 ± 0.3	2.2 ± 0.4
Dietary restriction	3.3 ± 0.9	26.2 ± 2.1	0.7 ± 0.1	11.0 ± 1.1	25.8 ± 4.6[a]	28.7 ± 4.8	—	4.3 ± 0.8
Cholestane-triol	0.3 ± 0.1	13.9 ± 0.3	0.3 ± 0.1	3.4 ± 0.2	36.0 ± 2.5[a]	41.7 ± 2.0[b]	1.4 ± 0.03	3.0 ± 0.5
Estrogen	1.4 ± 0.5	12.2 ± 4.0	0.3 ± 0.2	5.5 ± 1.8	40.7 ± 6.1	36.7 ± 4.0	0.4 ± 0.2	2.8 ± 0.4

[a] $P < 0.01$ for the difference from the control group.
[b] $P < 0.01$ for the difference from the control group.

tions in young women taking oral contraceptives. Perhaps the high concentration of estrogens in oral contraceptives might enhance atherogenesis. A study of the effect of estrogen administration early in life on atherogenesis in female White Carneau pigeons is currently under way.

Of the two interventions that were successful in decreasing the severity of atherosclerosis, dietary restriction showed a pronounced effect. Pigeons subjected to dietary restriction had practically no spontaneous lesions. Previous studies by Koletsky and Puterman (22, 23) in genetically obese rats with hyperlipidemia and by Griminger *et al.* (24) in chicks have noted a decrease in atherosclerosis following dietary restriction. The decrease in atherosclerosis and other degenerative diseases following dietary restriction might have been responsible for the noted (25) increase in life span of rats under such regimens.

It is interesting to note that cholestanetriol, which acts primarily by inhibiting cholesterol absorption and decreasing plasma cholesterol levels (12), was successful in decreasing the severity of atherosclerosis. The result of this study thus favors the concept that a reduction in serum cholesterol maintained from early life, perhaps can decrease the incidence of atherosclerosis in adult life.

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