Influence of chronic carbon disulphide intoxication on the development of experimental atherosclerosis in rats*

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ABSTRACT Rats fed on atherogenic diets containing 2% cholesterol and 0.5% cholic acid with or without 0.15% thiouracil were exposed to carbon disulphide (CS₂) vapours (1 mg/l of air), five hours a day, six days a week for 6-10 months. Serum and aorta lipid contents were determined, and the extent of atherosclerotic changes was investigated. The following effects of chronic exposure to CS₂ were found: (1) slower gain in body weight when rats were fed on atherogenic diet; (2) greater increase in serum cholesterol content (after thiouracil supplemented diet); (3) moderate increase in total cholesterol content in the aorta wall with a significantly increased esterified cholesterol fraction but none in phospholipid level in this tissue; and (4) more advanced lipid infiltrates of coronary arteries and endocardium, the latter predominantly in the aortic valves. These results together with data from previous studies indicate that metabolism of arterial lipids participates in the process of atheroma formation after chronic exposure to CS₂ vapours.

There is a considerable amount of evidence on carbon disulphide induced vasculopathy including increases of lipid infiltration in arterial walls,¹⁻¹¹ but the nature of CS₂-induced changes in lipid metabolism is still far from clear. The extensive biochemical studies carried out in this laboratory on rats chronically exposed to CS₂ have shown an increased rate of liver cholesterol synthesis¹²⁻¹⁵ and a parallel decrease of liver cholesterol degradation,¹⁶ an impaired plasma lipolytic activity^{17,18} with resulting raised plasma lipid concentrations,^{13-15,19} disturbance of lipid metabolism in the aortic tissue including decreased lipolytic activity,^{18,20} and an increased rate of ¹⁴C-cholesterol influx from serum and a raised rate of cholesterol synthesis²¹ in the aorta wall.

Despite these profound disturbances in the lipid metabolism of rats chronically exposed to CS₂, the histopathological signs of atherosclerosis were not seen during the above investigations (unpublished observations). We have assumed, therefore, that the

initial metabolic disturbances caused by CS₂ precede the morphological arterial changes that may develop eventually after long-term exposure to CS₂ in suitable experimental conditions. The present work has been undertaken to evaluate the effect of CS₂ alone or in combination with hyperlipaemic diets on the development of biochemical and morphological changes in the arterial wall of rats exposed to CS₂.

Material and methods

Eighty-four female Wistar albino rats (180 g initial body weight) were divided into groups according to their diets and the duration of CS₂ exposure:

- I 1 standard chow*/basal diet/—non-exposed controls (BD)
2 standard chow and CS₂ exposure for 12 to 15 months (BD + CS₂)
- II 1 atherogenic diet-1—non-exposed controls (AD-1)
2 atherogenic diet-1 and CS₂ exposure for 10 months (AD-1 + CS₂)
- III 1 atherogenic diet-2—non-exposed controls (AD-2)
2 atherogenic diet-2 and CS₂ exposure for 6 months (AD-2 + CS₂).

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*Laboratory chow for rats manufactured by LSM-Lowicz.

Rats were exposed to CS₂ in the inhalation chambers at a concentration of 1 mg CS₂/l of air for five hours daily six days a week. Both atherogenic diets consisted of ground wheat meal 48%, casein 25%, arachid oil 22%, salt mixture 4%,* and vitamins 1%† supplemented (at the expense of wheat) with: cholesterol 2%, cholic acid 0.5%, and choline chloride 0.25%. AD-1 was supplemented with 2% cholesterol only, AD-2 with 2% cholesterol and 0.15% thiouracil. The daily intake of diet in control and CS₂ intoxicated rats was maintained on uniform levels. Water was allowed ad libitum. The body weight of all rats was recorded once a month.

Biochemical investigations

Blood samples were collected from the tail vein and the following determinations were carried out: total cholesterol,²² triglycerides,²³ phospholipids,²⁴ and free fatty acids.²⁵ At the end of the experiment the animals were decapitated and aortas were removed for determination of lipid content. Total lipids were extracted from the homogenate using the method of Folch *et al.*²⁶ Individual classes of lipids separated by thin-layer chromatography were subsequently visualised with iodine vapour and extracted for chemical assay (DE Bowyer and JE King, unpublished observations). The procedure of Babson *et al.*²⁷ was used for free and esterified cholesterol determination; phospholipids were estimated by Usher's method.²⁴

Histological investigations

All hearts and additional small segments of aorta from two rats selected at random from each group were excised and fixed in formol-calcium immediately after death. Lipid staining in tissue blocks using isopropanol solution of Fat Red 7B was used (S Szendzikowski and T Wrońska, unpublished observations). The whole heart and specimens of aorta were impregnated with the dye solution and serial frozen sections were cut. Several of the sections were counterstained with Mayer's haematoxyline. After screening, selected sections were restained with haematoxyline and eosine, Lillie and Fulmer's elastic tissue stain, or Astra Blue for acid mucopolysaccharides. About 100 sections of each heart were examined in routine and polarised light.

*NaCl-70; CaHPO₄·2H₂O-410; potassium citrate, H₃O-423; MgSO₄·7H₂O-95; ferric citrate-15; KI-6.5; NaF-5.5; MnSO₄·5H₂O-1.7; CuI₂-0.5; AlK(SO₄)₂·12H₂O-0.92; ZnSO₄·7H₂O-0.50 (in grams).

†Vitamin B₁-0.12; vitamin B₂-0.40; inositol-8; vitamin B₆-0.08; calcium panthothenate-2.40; nicotinic acid-8; p-aminobenzoic acid-24; biotin-0.008; folic acid-0.08; vitamin B₁₂-0.002; choline-24 (in grams); potato starch—up to 1000 g, that is, 932.91 g.

Results

BODY WEIGHT

There were no differences in the body weights of the rats exposed to CS₂ and the non-exposed controls fed the basal diet. Rats exposed to CS₂ and control rats maintained on diet AD-1 gained 20% and 40% of their original body weight respectively. Loss of body weight observed in rats fed on diet AD-2 amounted to 15% in controls and 20% after six months of exposure to CS₂.

BLOOD LIPIDS

After 15 months of CS₂ intoxication a pronounced increase of cholesterol—total and esterified—was observed in the serum of rats fed on the basal diet (table 1). Both atherogenic diets had induced a noticeable increase in serum cholesterol content by the end of the first month's feeding. During the first six months of exposure no significant effect of CS₂ on the cholesterol content was found. At the end of the experiment effects of diets AD-1 and AD-2 were slightly intensified by CS₂ intoxication (tables 2 and 3).

AORTA LIPIDS

After feeding animals on diets AD-1 and AD-2 for ten and six months respectively, the concentrations of total cholesterol and cholesterol esters increased, but not to the same extent. Esterified cholesterol showed the most pronounced increase (by 174-374% in AD-1 and AD-2 groups respectively as compared to control rats fed on the basal diet—table 4). The 6-10 months of feeding on either atherogenic diet increased the content of total cholesterol by 10-38% compared with the controls. The increase in both total and esterified cholesterol induced by these diets was intensified by CS₂ (table 4).

In rats kept on basal diet a moderate enhancement of total cholesterol and a pronounced increase of cholesterol esters was noted after exposures of 12 and 15 months (table 4). In all groups of rats exposed

Table 1 Content of cholesterol in serum of control and CS₂-exposed rats fed on basal diet.* (Number of animals in parentheses)

Group	Cholesterol (mg/100 ml)	
	Total	Esters
Control	54.4 ± 12.5 (7)	36.7 ± 9.5 (8)
CS ₂	78.3 ± 24† (7)	58.1 ± 20.5† (8)

*Content of cholesterol in serum was determined after 15 months of exposure to CS₂ at conc 1.0 mg/l. Mean values ± SD are given.

†Statistically significant from controls, p < 0.05.

Table 2 Concentration of lipids in serum of rats exposed to CS₂ and fed atherogenic diet-1 (AD-1)*

Time of exposure (months)	Group of animals	Cholesterol (mg/100 ml)		Phospholipids (mg/100 ml)	Triglycerides (mg/100 ml)	Free fatty acids (mEq/l)
		Total	Esters			
1	C + AD-1	258.9 ± 55.8	234.9 ± 41.9	126.2 ± 13.7	—	—
	CS ₂ + AD-1	277.3 ± 71.6	236.4 ± 55.8	124.3 ± 30.5	—	—
3	C + AD-1	345.9 ± 85.5	286.0 ± 91.7	99.0 ± 31.7	86.6 ± 9	0.6 ± 0.17
	CS ₂ + AD-1	333.9 ± 113.7	295.4 ± 91.7	98.0 ± 21.5	83.3 ± 20.5	0.4 ± 0.17
6	C + AD-1	360.0 ± 70.6	299.6 ± 54.7	198.0 ± 21.7	—	0.27 ± 0.04
	CS ₂ + AD-1	345.2 ± 71	290.9 ± 59.8	160.6 ± 27.9	—	0.35 ± 0.05†
10	C + AD-1	366.4 ± 75.7	301.1 ± 65.4	230.7 ± 23.3	56.0 ± 15.7	0.75 ± 0.16
	CS ₂ + AD-1	404.6 ± 98.9	331.0 ± 80.1	190.2 ± 32.2	78.4 ± 16.1†	0.72 ± 0.2

*Each value is the mean ± SD, n = 12.

†Statistically significant from controls, p < 0.05.

Table 3 Concentration of lipids in serum of rats exposed to CS₂ and fed atherogenic diet-2 (AD-2)*

Time of exposure (months)	Group of animals	Cholesterol (mg/100 ml)		Phospholipids (mg/100 ml)	Triglycerides (mg/100 ml)	Free fatty acids (mEq/l)
		Total	Esters			
1	C + AD-2	705.2 ± 162.1	557.3 ± 130.9	171.9 ± 36.1	107.4 ± 27.8	0.64 ± 0.19
	CS ₂ + AD-2	654.8 ± 93.9	519.7 ± 101.1	160.7 ± 34.2	102.3 ± 37.8	0.96 ± 0.28
3	C + AD-2	585.0 ± 82.8	453.0 ± 72.3	263.0 ± 27.4	111.8 ± 24.7	0.20 ± 0.02
	CS ₂ + AD-2	516.6 ± 105.9	411.1 ± 99.2	230.7 ± 19.5	132.0 ± 20.8	0.27 ± 0.04
6	C + AD-2	406.2 ± 86.9	224.0 ± 77.0	314.2 ± 46.5	120.1 ± 47.8	0.40 ± 0.07
	CS ₂ + AD-2	536.7 ± 62.8†	300.6 ± 62.8†	327.0 ± 65.9	131.4 ± 40.9	0.50 ± 0.50

*Each value is mean ± SD, n = 12.

†Statistically significant from controls, p < 0.05.

Table 4 Effects of CS₂ exposure and atherogenic diets on cholesterol content in the aortic wall of rats

Type of diet	Group	Duration of exposure (months)	Cholesterol		Phospholipids (mg/g wet tissue)
			Esters (mg/g wt tissue)	Total	
Basal diet	Control	—	0.11	1.9	—
	CS ₂	12	0.17	2.1	—
Basal diet	Control	—	0.19	2.1	5.4
	CS ₂	15	0.44	2.6	5.5
Atherogenic diet-1	Control	—	0.52	2.9	6.0
	CS ₂	10	0.84	3.4	6.4
Atherogenic diet-2	Control	—	0.9	2.34	7.4
	CS ₂	6	1.4	3.4	7.7

Figures for cholesterol represent mean value for pooled aortas in each group (9-12 rats per group).

to CS₂ the relative contribution of the esterified fraction in the total cholesterol was greatly increased. No substantial changes in the concentration of aortic phospholipids were found in all groups of rats under investigation (table 4).

MORPHOLOGICAL INVESTIGATIONS

No lipid deposits were seen in the aorta, pulmonary artery, coronary vessels, and endocardium of control rats fed on basal diet. Occasional slight increases in the thickness of the walls of some coronary arterial branches within the muscle of the left cardiac ventricle was the only abnormal finding (fig 1). In the corresponding experimental group killed after 15

months of CS₂ exposure similar changes were more often noted, with the occasional positive Fat Red staining indicating deposition of lipids (fig 2).

No signs of gross atheromas were found in the aortas and hearts of rats fed on atherogenic diets. Microscopic lesions in the form of extra or intracellular, or both, lipid deposits were present in the endocardium of the valves of the heart, but ascending most prominently in the aortic valves, in the intima and inner media of the aorta, and in the intima and media of the coronary arteries. With a few exceptions changes in the coronary arteries were limited to the intramural arterial branches within the left ventricular wall and the papillary muscles. Aortic intima

Table 5 Combined grade of lipid deposits in coronary arteries, initial portion of aorta, and valvular endocardium in rats fed on atherogenic diets (AD-1, AD-2) and exposed to CS₂

Diet	Duration of exposure (months)	Control					CS ₂				
		0°	I°	II°	III°	IV°	0°	I°	II°	III°	IV°
Atherogenic I	10	0	2	3	5	0	0	0	1	9	0
Atherogenic II	6	0	3	2	1	2	0	0	1	2	5
Total		0	5	5	6	2	0	0	2	11	5

0° No discernible lipid deposits.
I° Minute lipid deposits either in initial portion of aorta and in valvular endocardium or in coronary arteries.
II° Small lipid deposits in coronary arteries, initial aortas, and valves; alternatively more advanced endocardial and aortic lesions while no lipid deposits in coronary vessels recorded.
III° Moderate lipid deposits in coronary arteries, initial aortas, and valves.
IV° Advanced atheromas of coronary vessels with regional narrowing; large lipid deposits in initial aortas and valves.

distant from the initial portion occasionally contained scattered, minute lipid deposits.

Extensive deposits of lipids were seen in the coronary arteries, ascending aorta, and the cardiac valves in animals fed on diet AD-2 at the sixth month of the experiment (figs 3 and 4). Prolonged feeding with the diet AD-1 (10 months' duration) resulted in advanced coronary lesions (fig 5) with a moderate grade of aortic and valvular involvement. In rats maintained on diet AD-2 and exposed to CS₂ small deposits of crystalline cholesterol were found occasionally within the coronary atheromas

(fig 6). They were accompanied by focal hyaline formation and minute calcium deposits in these lesions. Table 5 shows the results of comparative grading of lesions recorded in animals maintained on atherogenic diets, which indicate a distinct shift toward higher grades of abnormalities in the animals exposed to CS₂.

The frequencies of lipid deposits of grades III° and IV° against the grades 0°-II° in the exposed and non-exposed groups of rats fed on atherogenic diets were compared using the Fisher exact probability test. The difference is statistically significant (p = 0.05).

Fig 1 Branch of coronary artery within muscle of left cardiac ventricle of a control rat fed standard diet (15 months old). Slight thickening of vessel's wall; no accumulation of lipids. (Fat Red 7B and haematoxylin; magn × 300.)

Fig 2 Branch of coronary artery within muscle of left cardiac ventricle of a rat fed standard diet, exposed to CS₂ (1.0 mg/l) over 15 months. Unevenly thickened wall of vessel contains accumulated lipids. (Fat Red 7B stain and haematoxylin; magn × 300.)

Fig 3 Lipids deposited in wall of initial aorta of rat maintained for six months on atherogenic diet (AD-2) and exposed to CS₂. (Fat Red 7B; magn × 300.)

Fig 5 Coronary artery within papillary muscle of left cardiac ventricle after 10 months of feeding on atherogenic diet (AD-1) and CS₂ exposure. Large deposit of fat, narrowing of vascular lumen. (Fat Red 7B stain and haematoxylin; magn × 150.)

Fig 4 Coronary artery within left ventricular wall after six months on atherogenic diet (AD-2) and CS₂ exposure. Accumulation of fat in thickened intimal and medial layer with pronounced reduction of vascular lumen. (Fat Red 7B; magn × 300.)

Fig 6 Coronary artery within left ventricular wall after 10 months of feeding on atherogenic diet (AD-1) and CS₂ exposure. Crystalline cholesterol deposited in thickened arterial wall. (Polarised light; magn × 300.)

Discussion

In the present investigation there was a lack of any obvious gross and histological lesions within the aorta of the non-hypercholesterolaemic CS₂ intoxicated rats consistent with previous findings. Furthermore, it has been shown (as a confirmation and extension of previous observations) that chronic exposure to CS₂ may produce lipid metabolic dis-

turbances in the vascular walls manifested by a significant rise in total cholesterol content. Within the composition of this fraction, a pronounced shift towards cholesterol esters was observed. Total cholesterol content in serum was also increased.

Introduction of atherogenic diets resulted in a rise in serum cholesterol content in control animal groups with a further rise in total and esterified cholesterol content in CS₂-exposed rats. Statistically

significant differences between CS₂-exposed and control rats were obtained only when thiouracil was added to the atherogenic diet (AD-2). Therefore it seems that the combined effect of feeding with atherogenic diet and CS₂ intoxication was not sufficient to raise serum cholesterol to the concentration that results in a pronounced accumulation of lipids in the aorta wall. Except in the region of the aortic valves, where advanced lipid infiltration was encountered, no major intimal lesions occurred in the aorta. Our findings differ in this aspect from those of other investigators who observed a more advanced atheromathosis after similar atherogenic diets.²⁸⁻³⁰

In our studies a major rise of cholesterol content in the aortic tissue of non-exposed control animals fed on diets AD-1 and AD-2 was repeatedly found throughout the duration of the experiment. The effect of CS₂ intoxication follows the pattern of lipid changes within the aorta similar to those recorded in non-hypercholesterolaemic animals. It was observed that there was a moderate but significant increase in the content of total cholesterol. The increase in cholesterol was particularly pronounced within the esterified fraction. No significant changes in the content of vascular phospholipids were recorded. The above shift of the lipid spectrum bears a resemblance to the composition of lipids in the aorta wall shown at the plaque stage of experimental atherosclerosis³¹⁻³³ in contrast to the fatty streak stage, which is characterised by a predominant increase in free cholesterol with a minor increase of cholesterol esters.³⁴ This discrepancy requires further elucidation considering the absence of atherosclerotic gross lesions in our material.

A further confirmation of the biochemical findings is provided by the parallel histological examination of the heart. The morphological features of the coronary and endocardial lesions are consistent with data obtained in other laboratories for rats in similar atherogenic conditions.²⁸⁻²⁹⁻³⁵ As a result of long-term exposure to CS₂, noticeable alterations in the small coronary arterial branches were observed. CS₂ intensified arterial and endocardial lesions caused by atherogenic diets. These findings justify the conclusion that the endocardial and coronary arterial lesions are suitable experimental indicators of the vascular effects of CS₂.

Results obtained in this study seem to confirm those clinical and laboratory data that indicate the promoting influence of CS₂ on the development of atherosclerosis in general¹⁻⁸ and on the progress of the coronary heart disease in particular.⁹⁻¹¹ On the basis of data on metabolic lipid disturbances,¹²⁻¹⁶⁻¹⁹ especially those proving the impairment of lipid metabolism in the aortic tissue,¹⁸⁻²⁰⁻²¹ the present findings indicate that altered cholesterol metabolism

within the arterial wall play an important part in the vascular response of chronic CS₂ intoxication.

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