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Long Term Lathyrism and Atherogenic Diet in the Rat

Protective Action of Pyridinol Carbamate

M.Th. Pieraggi[1]*, J. De Graeve[2], M. Julian[1]*, J.C. Thiers[1,3], H. Bouissou[1]

[1] Department of Anatomic Pathology (Pr. H. Bouissou) Université Paul Sabatier, Faculté de Médecine Rangueil, 31054 Toulouse Cedex France

[2] Laboratoire de Biochimie (Pr. P. Valdiguié) Université Paul Sabatier, Faculté de Médecine Rangueil, 31054 Toulouse Cedex France

[3] Unité 101 INSERM – Biochimie des Lipides (Pr. L. Douste-Blazy)

Summary. In the rat, prolonged administration (7 months) of Beta-Aminopropionitrile in association with a hyperlipidic diet caused the formation of widespread pronounced atheroma. The addition of Pyridinol Carbamate during the treatment minimized and retarded the appearance of lipid overload lesions.

The histological modifications were found together with an increase in the free cholesterol fraction. These two observations explain the protective role of Pyridinol Carbamate on the wall of the aorta.

Key words: Long term lathyrism – Atherogenic diet – Lipoproteins – Lipids – Aorta.

Introduction

In the weaning rat, Beta-Aminopropionitrile fumarate (BAPN) administration over 9 weeks, caused aortic lesions reminiscent of those encountered in human ageing (Julian et al., 1973; Julian et al., 1971a; Julian et al., 1971b; Julian et al., 1972). The rat, which is resistant to spontaneous and experimental atheroma, on receiving this treatment in association with an atherogenic diet, formed histological and biochemical modifications comparable to those observed in atheroma in man. The administration of Pyridinol Carbamate (PDC) over varying experimental periods protected the vascular wall, retarded and minimized the atheromatous lesions (Bouissou et al., 1974; Pieraggi et al., 1974).

The aim of the present investigation is to study the role of PDC during long term experimental atherogenesis induced by BAPN over 3, 5 and 7 months in association with a hyperlipidic or atherogenic diet (D) in order to investigate the protective role of PDC during continuous parietal agression.

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Material and Methods

330 three-week-old male Wistar rats were used at the start of experimentation. BAPN was administered orally at 1 g/kg/day.

The hyperlipidic or atherogenic diet (D) was the hypercholesterolemiant U.A.R. diet N° 214 B[1]. The PDC[+ +2] was given in the form of a gum suspension at 25 mg/kg/day. The animals were treated over 7 months. They were weighed each week and the doses of BAPN and PDC recalculated according to the weight.

Five groups of rats were formed:

- Group I (B+D): 85 animals simultaneously receiving BAPN and D.
- Group II (B+P+D): 75 animals receiving BAPN, PDC and D at the same time.
- Group III (D): 65 rats only receiving D throughout the experiment.
- Group IV (P+D): 65 rats receiving PDC and D at the same time.
- Group V (C): 40 control rats.

The treatments were carried out continuously for 7 months. Animals of each group were killed at 3, 5 and 7 months.

At each date, histological examination and parietal aortic cholesterol assay was performed.

a) Histological Examination. Sixty treated rats – 5 from each experimental group at 3, 5 and 7 months – were examined by optical and electron microscopy and compared with controls (12 in all) killed simultaneously.

For optical investigation samples of thoracic aorta were fixed in Duboscq's solution, embedded in parafin wax, sections were prepared, stained with Masson's blue trichrome, Verhoeff's iodated ferric haematoxylin and with Alcian blue-PAS using Mowry's method. A second sample was fixed in formaldehyde, sliced deep-frozen and stained with Sudan black.

The aortae analysed by electron microscopy were perfused with 4% glutaraldehyde, 1 cm long pieces were removed and soaked in the same fixing solution at 4° C for 3 h then post-fixed in cold 2% osmium tetroxide for 3 h. After dehydrating and embedding in Epon 812, slices, contrasted with uranyl acetate and lead citrate, were examined with a Hitachi HU 11A or OPL 75 electron microscope.

b) Biochemical Analysis. After each experimental period 5 animals were analysed individually after an 18-h fast.

The animals were killed after being anaesthetized with chloroform, the aorta was removed and immediately deep frozen in physiological saline.

Aorta lipid extraction was carried out using the method of Rose and Oklander (1965) with a 7/11 chloroform/methanol solvent mix. The total cholesterol was assayed on an aliquot of the total lipid extract with Liebermann's method.

The various lipids were separated by thin layer chromatography on Merck Silicagel (0.25 mm). Free and esterified cholesterol were separated with the solvent system: petroleum ether/diethyl ether/acetic acid (80/20/1). Each band was visualized with iodine, scraped off, and the level of cholesterol determined using Liebermann's method.

Results

1. Histological Study

Since the parietal aortic lesions induced by long-term BAPN treatment were described in a previous report (Bouissou et al., submitted Path. Biol.) they will

[1] U.A.R. Diet: 20% casein, 10.3% glucose, 13.4% starch, 35% animal and vegetable fats, 5% cellulose, 10% mineral components, 1% vitamine complex, 4.5% cholesterol, 1.8% sodium cholate

[2] The Pyridinol Carbamate (Angioxine) was kindly supplied by Roussel Uclaf Laboratories

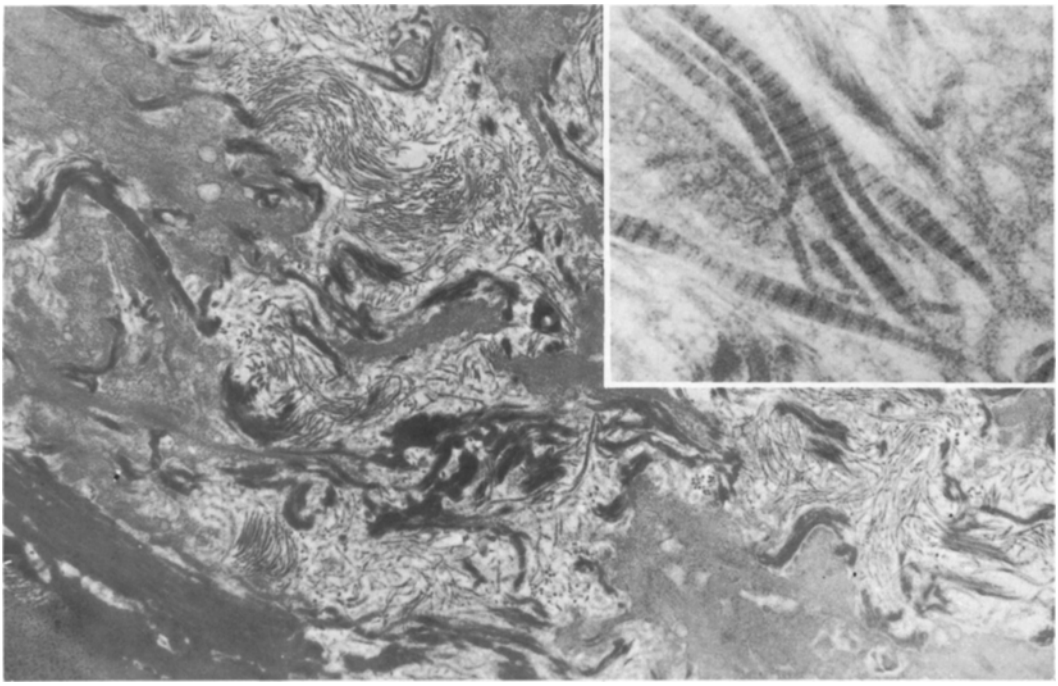

Fig. 1. 5 months continuous BAPN administration: disorders of aortic wall with disorganization of elastic framework. Uranyl acetate – lead citrate stain ×4,000. *Inset:* Appearance of abnormal collagen composed by large diameter fibres with a normal periodicity. Uranyl acetate – lead citrate stain ×23,000

not be dealt with in the present article. 5 months continuous BAPN administration caused aortic lesions identical to those noted after 9 weeks treatment. The seriousness of the parietal disorders does not change with the lenght of treatment. The only notable difference is the appearance of abnormal collagen composed of large diameter fibres which keep a normal periodicity – about 640 A°. (Fig. 1).

A summary of the different lesions is given in Table 1.

Group I (B+D). BAPN lesions were present; they involved the elastic lamellae and the interlamellar spaces. Lipid penetration was variable but always intense. Although there was no real formation of atherosclerotic plaques at 3 and 5 months penetration existed in all cases. After 7 months treatment, 5 animals out of 5 presented a real atheroma (strong lipid penetration into the internal $^2/_3$ of the media in association with marked angio-lathyrism lesions). The atheromatic plaque affected almost the whole of the wall of the vessel (Fig. 2).

Electron microscopy shows the constant presence of BAPN lesions. Lipid penetration was seen to be very intense from the third month. After 5 and 7 months, it could be seen through out the whole thickness of the wall (lipids were seen in the outermost space). The lipids were extracellular in the form of pools or intracellular blistering the myocytes. From the 5th month the lipid

Table 1. Histological study

	P.L.			E.O.L.		
	3M	5M	7M	3M	5M	7M
B+D	3± 2+ (5)	5+ (5)	5+ (5)	3e 2em (5)	2e 3em (5)	5a (5)
B+P+D	1− 3± (4)	3± 1+ (4)	1± 1+ 1++ (3)	4− (4)	4e (4)	2e 1a* (3)
D	4− (4)	4− (4)	4− (4)	4− (4)	1− 3e (4)	1− 3e (4)
P+D	5− (5)	5− (5)	5− (5)	5− (5)	2− 3e (5)	2− 3e (5)

M = months; () Number of animals examined, the control rat was not included
P.L. = Parietal lesions: − No lesion; ± Enlargement of interlamellar space; + Thinning, elastolysis of internal lamellae; + + Disorganization of the media
E.L.O. = Endothelial overload lipid: − No lipid; e Endothelial overload; em Endothelial overload with lipidic penetration in the media; a Atheroma over half the circumference; a* 1 plaque only

overload was seen in conjunction with an increase in the quantity and density of collagen.

Group II (B+P+D). Alteration of the internal media, to a varying extent in this group reveals the action BAPN treatment. Lipid overload was constant but the lipids did not penetrate deep into the media and there were no widespread atheromatous plaques (Fig. 3). After 7 months treatment, only one animal presented a localized atherosclerotic plaque.

The histological data were confirmed by ultrastructural examination. Lipid overload was less intense than in the B+D series. After 3 months lipids were observed with a frequency maximum in the intimal cells, restricted zones of interstitial lipid however were also noted in the media. At 5 and 7 months intercellular lipids were also found in the intima and the first two spaces of the media but never reached the outer layers.

Group III (D). The diet alone for 7 months caused an endothelial lipid overload from the 5th month, it was constantly found but isolated, penetration of the media was not observed. In spite of the lenght of the treatment, the deposit remained endothelial but spread along the surface of the vessel.

Electron microscopy showed the presence of lipid in the intima (intra and inter-cellular) and in a few myocytes of the first space of the media.

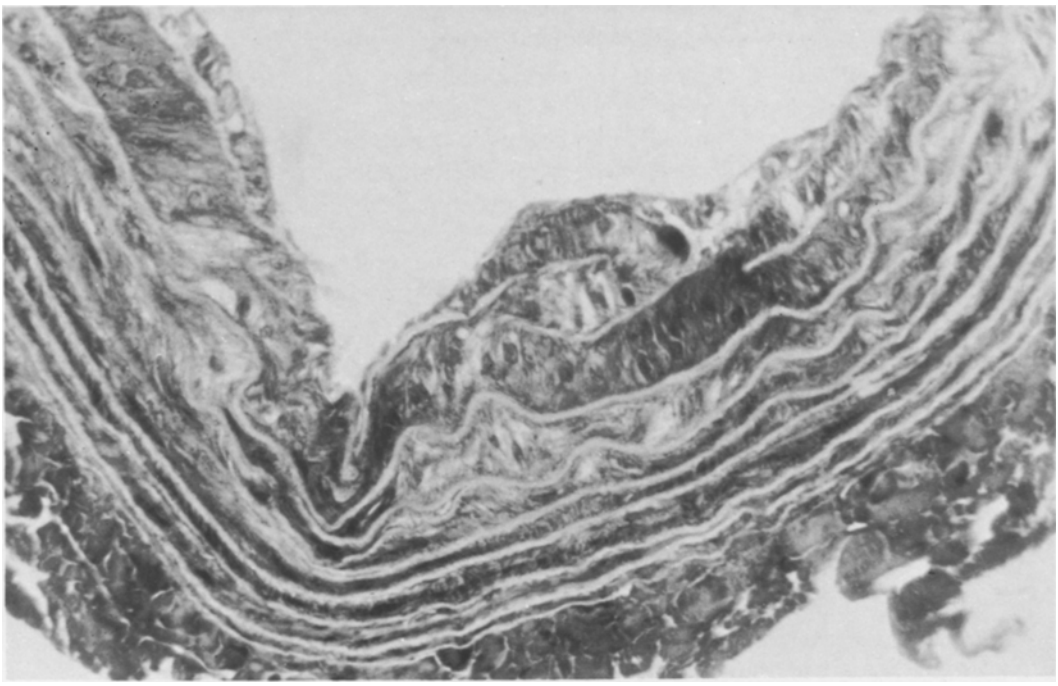

Fig. 2. Group I (B+D). After 7 months treatment: lipid penetration into the internal $^{2}/_{3}$ of the media and angiolathyrism lesions. Atheromatic plaque affected whole vascular wall. Masson's blue trichrome stain ×25

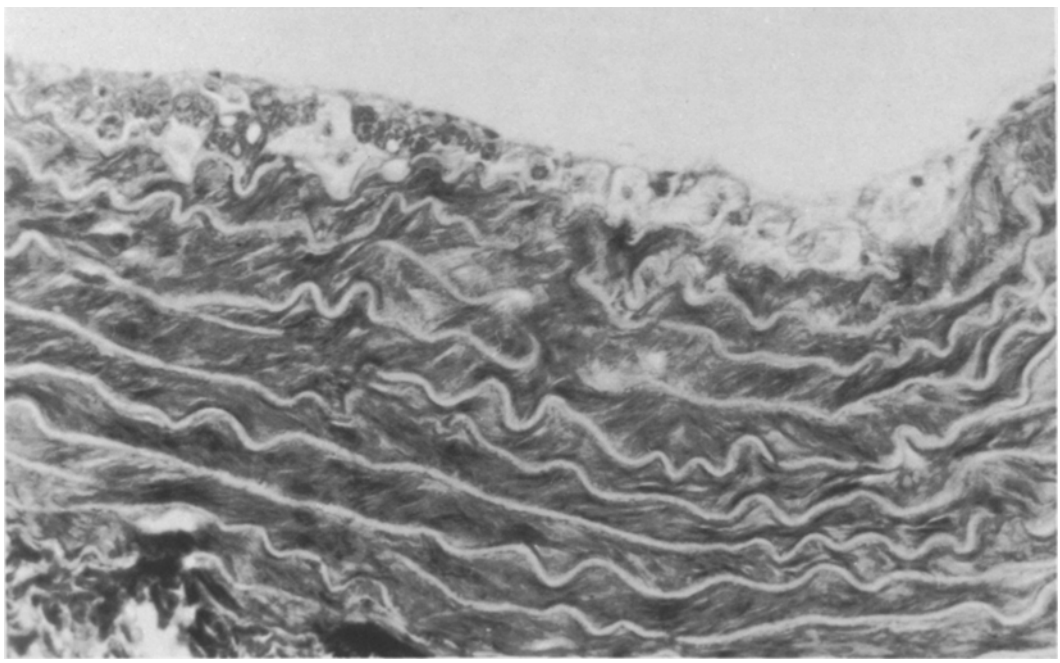

Fig. 3. Group II (B+P+D). After 7 months treatment: only constant lipid overload. PDC prevent atheromatous lesions. Masson's blue trichrome stain ×25

Group IV (P+D). PDC modified neither the intensity nor the frequency of the lipid deposit observed with the diet alone.

Under the electron microscope the lesions were seen to be similar to those of series III. The lipid deposits were essentially intracellular and mainly localized in the intima. A few interstitial lipid pools were seen in the proximity of the first elastic lamella.

2. Biochemical Investigations

The values of the average total and esterified aorta cholesterol contents and the statistical analyses are grouped in Tables 2, 3 and 4.

With respect to the controls, the hyperlipidic diet induced a very highly significant increase in the total cholesterol content from the third month. It was still present at the other dates with an even lower dispersion of the values. All groups receiving the diet showed this increase in aorta cholesterol. Maxima were reached at the third month followed by a decrease except in the B+D and B+P+D series.

When compared to the D group, the P+D series showed no significant difference at 3 months; at 5 months, the levels of total cholesterol were the same but the level of esterified cholesterol in the P+D group was lower implying an increase in free cholesterol; at 7 months, there was no significant difference between the levels of total and esterified cholesterol.

In the B+D series the level of cholesterol was always very highly significantly greater than in the control group. Comparison of the values of the B+D series and those of the B+P+D series at 3 months showed no significant difference in the levels of cholesterol. At 5 months a significant difference did appear corresponding to a decrease in the level of total cholesterol in the B+P+D group. At 7 months the levels of total cholesterol were not significantly different in the two groups; however the level of esterified cholesterol was significantly lower in the B+P+D group showing an increase in free cholesterol.

Table 2. Comparison of aortic cholesterol by student's *t* test (3 months)

Groups		C	D	B+D	P+D	B+P+D
C	0.400 ±0.023	0				
	0.0194 ±0.0012	*0*				
D	0.885 ±0.173	5.68****	0			
	0.261 ±0.077	*6.24****	*0*			
B+D	0.960 ±0.075	14.35******	0.66 ns	0		
	0.315 ±0.036	*16.27******	*1.26* ns	*0*		
P+D	0.885 ±0.080	11.71*****	−0.13 ns	−1.38 ns	0	
	0.281 ±0.027	*19.55*******	*0.49* ns	*−1.50* ns	*0*	
B+P+D	0.931 ±0.054	17.92*******	0.39 ns	−0.59 ns	0.99 ns	0
	0.317 ±0.021	*28.24*******	*1.39* ns	*0.08* ns	*2.08* ns	*0*

At 3 months, levels of total cholesterol and esterified cholesterol (italicized number)
Each value in mmol/100 g
NS=non significant. $*P<0.05$; $**P<0.01$; $***P<0.001$; $****P<0.0001$; $*****P<0.00001$; $******P<0.000001$; $*******P<0.0000001$

Table 3. Comparison of aortic cholesterol by student's *t* test (5 months)

Groups		C	D	B+D	P+D	B+P+D
C	0.390±0.026	0				
	0.023±0.004	*0*				
D	0.769±0.046	13.98******	0			
	0.225±0.045	*8.21****	*0*			
B+D	1.582±0.175	13.33******	8.87****	0		
	0.468±0.086	*10.37******	*4.92****	*0*		
P+D	0.751±0.139	5.04***	−0.24 ns	−7.33****	0	
	0.086±0.020	*6.29****	*−5.26****	*−8.69****	*0*	
B+P+D	1.050±0.181	6.95****	2.89*	−4.13**	2.53*	0
	0.315±0.092	*6.35****	*1.72* ns	*−2.44**	*4.87****	*0*

At 5 months, levels of total cholesterol and esterified cholesterol (italicized numbers)
Each value in mmol/100 g

Table 4. Comparison of aortic cholesterol by student's *t* test (7 months)

Groups		C	D	B+D	P+D	B+P+D
C	0.410±0.024	0				
	0.035±0.005	*0*				
D	0.848±0.067	12.30*****	0			
	0.204±0.045	*7.49****	*0*			
B+D	1.709±0.216	11.97*****	7.62****	0		
	0.681±0.057	*22.49*******	*13.19******	*0*		
P+D	0.798±0.079	9.40*****	−0.98 ns	− 7.93****	0	
	0.243±0.025	*15.92******	*1.5* ns	*−14.05******	*0*	
B+P+D	1.686±0.201	12.58******	7.89****	− 0.16 ns	8.21****	0
	0.436±0.116	*6.91****	*3.74***	*− 3.80***	*3.26***	*0*

At 7 months, levels of total cholesterol (large sized numbers) and esterified cholesterol (small sized numbers)
Each value in mmol/100 g

Comparison of the B+D and the D groups showed no significant difference at 3 months. At 5 months however there was a very highly significant increase in the level of total and esterified cholesterol in the B+D series. The same was seen at 7 months with a higher increase in the esterified cholesterol.

Interpretation of the Results

The diet alone did not cause atheromatous lesions. After 7 months treatment there was simply an endothelial overload without parietal overload. These histological observations were confirmed by biochemical analysis and are in agreement with results obtained previously (Bouissou et al., 1978).

The simultaneous administration of BAPN and D causes formation of atheromatous lesions. These results are in agreement with those of an earlier experiment (BAPN administered for 9 weeks) but long term BAPN did not favour

the appearance of premature atheroma. This experiment confirms the important role of the aorta wall in atherogenesis, an undamaged wall prevents lipid penetration. Alteration of the elastic framework favourizes lipid deposit.

PDC protects the wall, minimizes BAPN lesions and slows their appearance. Histologically this action can be seen through the lesser intensity of the elastic modifications observed at 3 and 5 months and by the clear decrease of the atheromatic lesions at 7 months. These results are reinforced by the lower aorta total cholesterol contents in the series receiving PDC representing weaker penetration.

PDC favourizes parietal cholesterol elimination. This hypothesis was confirmed by the results of the B+P+D and P+D series. In effect the level of esterified cholesterol was lowered corresponding to an increase in free cholesterol, free cholesterol being a form suitable for cellular cholesterol elimination.

Conclusions

Pyridinol Carbamate, in association with a long term lathyrism inducing treatment and hyperlipidic diet (7 months) minimized and slowed the appearance of aortic lesions thus preventing the formation of atheroma. It also favourized the parietal alimination of cholesterol by increasing the free cholesterol content of the aorta.

It would seem therefore that Pyridinol Carbamate acts both on the structure of the aorta and on parietal cholesterol metabolism.

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References

Bouissou, H., Julian, M., Pieraggi, M.Th.: Effet protecteur du Pyridinol Carbamate sur l'athérome expérimental du rat. Path. Biol. **22**, 713–720 (1974)

Bouissou, H., Thiers, J.C., Douste-Blazy, L., Pieraggi, M.Th., Julian, M.: Chronic lathyrism and atheromatosis in the rat. Study of the VLDL and plasma and arterial wall. Virchows Arch. A Path. Anat. and Histol. **380**, 107–117 (1978)

Bouissou, H., Thiers, J.C., Julian, M., Pieraggi, M.Th.: Lathyrisme chronique. Plasma, aorte et peau du rat traité par 5 mois de Beta-Aminopropionitrile avec ou sans régime hyperlipidique. Path. Biol. (Submitted)

Julian, M., Fabre, M.Th., Bouissou, H.: Lathyrisme chronique. Modifications simultanées des tissus conjonctifs cutané et aortique. Path. Biol. **19**, 389–399 (1971 a)

Julian, M., Pieraggi-Fabre, M.Th., Bouissou, H.: Lathyrisme expérimental. La paroi aortique au cours de l'intoxication chronique avec régime athérogène. Path. Biol. **19**, 1037–1044 (1971 b)

Julian, M., Pieraggi, M.Th., Bouissou, H.: Lathyrisme et régime athérogène chez le rat. Path. Biol. **20**, 271–278 (1972)

Julian, M., Pieraggi, M.Th., Bouissou, H.: Lathyrisme chronique. Modèle expérimental du vieillissement du tissu conjonctif humain. Gerontologia **19**, 220–239 (1973)

Pieraggi, M.Th., Julian, M., Bouissou, H.: Aortic lathyrism. Ultrastructural study of lesions in the aortic wall and the protective effect of Pyridinol Carbamate. Gerontologia **20**, 193–211 (1974)

Rose, H.G., Ocklander, M.J.: Improved procedure for the extraction of lipids from human erythrocytes. J. Lipid Res. **6**, 428–436 (1965)

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