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Preventive Effect of Gliclazide on Experimental Atherosclerosis in Rabbits

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Summary. Administration of a hypoglycaemic sulphonamide, gliclazide, at 10 mg/kg/day p.o. to rabbits for 60 days did not affect the development of plasma lipid disturbances induced by a high cholesterol diet. The accumulation of cholesterol in the liver was significantly reduced by up to 34% when compared with animals on the high cholesterol diet. The high concentrations of glycerides and fatty acids in the aorta were significantly decreased towards normal values and histology showed that the gliclazide strongly inhibited the development of aortic and particularly coronary lesions induced by the atherogenic diet. A normal appearance of coronary arteries was noted in more than 50% of cases.

Key words: Plasma, fatty liver, atherosclerosis, coronary cholesterol, sulphonylureas, gliclazide, rabbit.

The longer survival of diabetic patients has revealed a high incidence of vascular complications involving either arteries or capillary bed or both. Sulphonylureas have been used to control blood glucose.

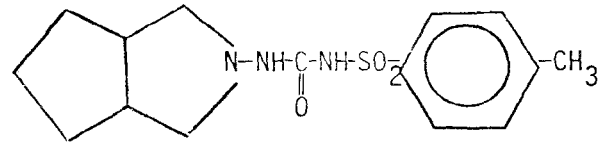
Concomitant treatment of arterial disease with the earlier hypoglycaemic sulphonamides produced controversial results at both the experimental and clinical levels. Punsar and Hartel [29] and Hartel et al. [16] showed that carbutamide can have a hypercholesterolaemic effect and can aggravate experimental and spontaneous atherosclerosis in chickens, the frequency of coronary lesions in particular being greatly increased. These results were also confirmed by the use of high doses of chlorpropamide in rabbits subjected to an atherogenic diet [23]. Chlorpropamide appeared to inhibit the elimination of the excess fat in atherosclerotic lesions, following termination of the atherogenic diet [24]. At the clinical level, the enquiry by the U.G.D.P. [1 and 2] concluded that long term therapy with tolbutamide had adverse effects on cardiovascular complications. On the other hand, Keen and Jarrett [19] recently gave a report on the beneficial effect of using tolbutamide in hyperglycaemic patients. Paasikivi and Wahlberg [28] came to the same conclusions. Under these circumstances, we thought it appropriate to investigate experimentally whether gliclazide could affect the development of arterial lesions induced in the rabbit by a diet enriched with cholesterol.

Materials and Methods

Experiments were conducted over a period of two months on 52 fawn Bourgogne rabbits each weighing approximately 3 kg. Eighteen rabbits received no treatment, 18 rabbits were given 400 mg of cholesterol (with no oily excipient) per body wt daily, and the remaining 16 rabbits received, in addition to the cholesterol, 10 mg per kg body wt per day of gliclazide orally in two doses. The food allowance was 150 g per day in the form of commercial granules for rabbits, and water was given ad libitum. The weight curve for rabbits treated with gliclazide was comparable to that for the control rabbits and the animals to which only cholesterol was administered.

All sacrifices were performed simultanéously to escape bias resulting from seasonal effects.

The chemical structure of gliclazide is:



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For plasma lipid determination, venous blood samples were collected from the rabbit's ear after the 15th, 30th, 45th and 60th days of treatment between 9 and 11 a. m.

Slices of aorta were homogenised with a mixture of chloroform methanol (2:1 by volume) within a virtis apparatus.

Extraction of the plasma or tissue lipids was carried out in accordance with the method of Folch et al. [12]. The quantity of total fatty acids was determined by a micro-method developed from the works of Sperry and Brand [32], Folch et al. [12] and Albrink [3]; total cholesterol by the colorimetric method of Lieberman-Burchard. Phospholipids were determined by a method developed from Delsal and Manhouri [4], followed by quantitative colorimetric determination [10]. Free fatty acids were measured by the method of Laurell and Tibbling [21] and plasma glucose by the method of Somogyi [30]. The quantitative composition of the plasma and liver in terms of triglycerides, cholesterol esters and free cholesterol was determined by chromatography separation on a column packed with a mixture of silic acid and supercell [17]. Lipid separation by thin layer chromatography according to Landiscrina et al. [20] followed by densitometer quantification gave similar figures.

Macrocospic assessment of the degree of aortic atherosclerosis was made for each animal over the entire arterial trunk in accordance with the usual criteria which enable a distinction to be drawn between 5 stages – 0, 1, 2, 3 and 4 [27] : 0 = normal artery; 1 = fatty streaks and small plaques up to 10% of the aortic area; 2 = plaques covering 10–25% of the aorta; 3 = plaques extending over 25–50% of the area; 4 = from 50% to complete involvement. Samples were taken of the arch of the aorta, the thoracic aorta, the abdominal aorta and the myocardium for the purpose of histopathological examination. The tissues were stained with Masson's trichrome and Verhoeff's iodised haematoxylin in order to confirm the degree of modification of the elastic fibres. The presence and extent of the arterial lesions was evaluated against an atherosclerosis scale ranging from 0 to 4 [13] : 0 = normal artery; 1 = disorganization in connective tissue (collageneous increase and superficial elastic degeneration); 2 = lipid infiltration (thickening of sub-endothelial space with appearance of foam cells and numerous fibres of collagen); 3 = extensive lipid plaques (the internal elastic limiting membrane was fragmented, the internal zone of the tunica media consisted of smooth muscle cells invaded by lipids scattered elastic elements and disordered collagen fibres, the external zone of the tunica media and the adventitious tunica were not affected); 4 = converging lipid plaques.

Samples of the thyroid gland were taken and stained with Masson's trichrome and thyroid activity assessed on Kampelmann's scale [18]. The degree of thyroid activity was classified from 1 to 5 : Stage 1, the vesicles were of small diameter, the colloid very sparse, the nuclei rounded and located halfway up the cell, the gland strongly vascularized; stage 2, the vesicles were rather small, the colloid sparse, the epithelium with a central nucleus, the gland was well vascularized; stage 3, the thyroid vesicles were of medium size, the colloid became abundant, the epithelium cubic, the vascularization remained important; stages 4 and 5, the vesicles were of great diameter, the colloid abundant or very abundant and acidophilic, the cells of the thyroid epithelium flat and their nuclei oval and attached to the base, vascularization was poor.

The data were analysed statistically using Student's t test. The mean values and standard error of the mean are given in tables. The criterion for statistical significance is $p \leq 0.05$.

Results

Effect of Cholesterol Rich Diet Alone

Plasma and Liver Lipids: The rabbits to which only cholesterol was administered showed marked hyperlipaemia especially of cholesterol esters. The content of esterified cholesterol reached 910 ± 70 mg/100 ml on the 60th day of the diet as compared to 21 ± 2 mg/100 ml for the same animals before treatment. There was a slow, but progressive increase in plasma free fatty acids. The plasma glucose values were unchanged (Table 1). An increase in liver mass was observed, the lipid surplus being considerable at the end of the experiment; the contents of free and esterified cholesterol were particularly high, with those of phospholipids and glycerides increasing to a lesser degree (Table 2)

Arterial Lipids: In cholesterol-fed rabbits, there were large lipid accumulations involving cholesterol more than phospholipids or triglycerides. Cholesterol content was approximately ten times higher than in control animals (Table 3).

Arterial Pathology: Macroscopic lesions were generalised over the entire arterial trunk and characterised by extensive lipid deposits, frequently converging at the level of the arch of the aorta (Table 4).

Histopathological examination of these 'lipid pla-

Table 1. Evolution of plasma lipids in cholesterol fed rabbits – effects of gliclazide

Biochemical analysis	Treatment period (days)	Group: Normal control [18]	Cholesterol fed rabbits [18]		Cholesterol fed rabbits + Gliclazide [16]	
Total fatty acids (mg/100 ml)	0	118 ± 6	115 ± 6		118 ± 6	
	15	121 ± 7	410 ± 35[c]		603 ± 55[b]	
	30	120 ± 5	777 ± 53[c]		710 ± 64	
	45	127 ± 7	1030 ± 85[c]		946 ± 69	
	60	132 ± 11	1140 ± 75[c]		1045 ± 58	
Free fatty acids (mg/100 ml)	0	11 ± 0.7	12 ± 0.7	NS	12 ± 1.1	NS
	15	11 ± 1	18 ± 1.4		21 ± 2	
	30	13 ± 2	22 ± 1.2		20 ± 1	
	45	16 ± 3	22 ± 1.5		21 ± 1.6	
	60	15 ± 2	25 ± 1.8		21 ± 1.2.	
Phospholipids (mg/100 ml)	0	76 ± 5	80 ± 5		71 ± 3	
	15	81 ± 6	200 ± 17[c]		266 ± 23[a]	
	30	82 ± 7	340 ± 25[c]		323 ± 27	
	45	93 ± 11	400 ± 27[c]		374 ± 29	
	60	95 ± 13	425 ± 32[c]		466 ± 26	
Total cholesterol (mg/100 ml)	0	33 ± 2	31 ± 2		32 ± 1	
	15	31 ± 3	400 ± 45[c]		555 ± 43[c]	
	30	35 ± 6	925 ± 75[c]		767 ± 70	
	45	41 ± 7	1330 ± 75[c]		1014 ± 72	
	60	47 ± 11	1375 ± 95[c]		1147 ± 63[a]	
Glucose (mg/100 ml)	0	135 ± 3	132 ± 2	NS	141 ± 3	NS
	15	131 ± 4	138 ± 2		138 ± 3	
	30	137 ± 3	133 ± 4		138 ± 3	
	45	129 ± 2	128 ± 3		132 ± 4	
	60	133 ± 4	138 ± 3		134 ± 4	

The number of animals in each group is indicated in brackets. The values for the plasma lipids and glucose are expressed as mean ± SEM
The degree of significance is calculated for cholesterol group vs control and cholesterol + gliclazide group vs cholesterol group
[a] $p \leq 0.05$ [b] $p \leq 0.01$ [c] $p \leq 0.001$

Table 2. Effects of gliclazide on the lipid content of the liver of rabbits given cholesterol

Biochemical analysis	Group: Normal control [18]		Cholesterol fed rabbits [18]		Cholesterol fed rabbits + gliclazide [16]	
	mg/100 g wet wt	mg/kg body wt	mg/100 g wet wt	mg/kg body wt	mg/100 g wet wt	mg/kg body wt
Total fatty acids	3550 ± 300	1050 ± 30	6300 ± 300[c]	2010 ± 100[c]	5850 ± 300	1800 ± 100
Phospholipids	4015 ± 70	1010 ± 50	4400 ± 500[c]	1400 ± 50	4420 ± 130	1360 ± 40
Total cholesterol	315 ± 10	80 ± 3	3810 ± 160[c]	1215 ± 60[c]	2780 ± 205[c]	865 ± 72[c]
Cholesterol esters.	55 ± 5	20 ± 3	2630 ± 150[c]	835 ± 50[c]	1726 ± 99[c]	537 ± 38[c]
Free cholesterol	260 ± 25	60 ± 2	1180 ± 70[c]	380 ± 25[c]	1029 ± 96	320 ± 34
Glycerides	1330 ± 100	340 ± 20	2950 ± 200[c]	950 ± 70[c]	2810 ± 320	860 ± 100
Liver wt as % body wt	2.50 ± 0.05		3.20 ± 0.10[c]		3.05 ± 0.10	

The number of animals in each group is indicated in brackets. The values for the hepatic lipids are expressed as mean ± SEM
The degree of significance is calculated for cholesterol group vs controls and cholesterol + gliclazide group vs cholesterol group
[c] $p \leq 0.001$

ques' permitted assessment of the extent of such lesions. The endothelial cells were little affected, resting on a sub-endothelial space consisting of cells packed with lipids, the 'foam cells' or 'xanthoma cells', and numerous fibres of collagen; the internal elastic limiting membrane was fragmented; the internal zone of the tunica media consisted of smooth muscle cells invaded by lipids, scattered elastic elements and disordered collagen fibres. The external zone of the tunica media and the adventitious tunica

Table 3. Effects of gliclazide on the aortic lipids of rabbits given cholesterol

Biochemical analysis		Group: normal control [18]	Cholesterol fed rabbits [18]	Cholesterol fed rabbits + Gliclazide [16]
Total fatty acids mg/100 g wet wt		1870±10	3030±140[c]	2100±100[c]
Phospholipids mg/100 g wet wt		565±20	1051±43[c]	1080±43
Cholesterol mg/100 g wet wt	Total	151±5	1225±130[c]	1085±138
	Esterified	9±1.5	885±115[c]	695±135
	free	142±4.5	340±31[c]	390±55
Glycerides mg/100 g wet wt		1650±70	2260±140[b]	1280±90[c]

The number of animals in each group is indicated in brackets. The values for the aortic lipids are expressed as mean ± SEM
The degree of significance is calculated for cholesterol group vs controls and cholesterol + gliclazide group vs cholesterol group
[b] $p \leq 0.01$ [c] $p \leq 0.001$

Table 5. Effect of gliclazide on the histological appearance of the thyroid gland of rabbits given cholesterol

Group	Normal control [18]	Cholesterol fed rabbits [18]	Cholesterol fed rabbits + gliclazide [16]
Index of thyroid activity	2 ± 0.019	3.67 ± 0.039[c]	2.4 ± 0.033[c]

All animals were sacrificed at 60 days
Thyroid activity has been assessed according to Kampelmann's scale
The number of animals in each group is shown in brackets. The values for the index of thyroid activity are expressed as mean ± SEM
The degree of significance is calculated for cholesterol group vs controls and cholesterol + Gliclazide group vs cholesterol group
[c] $p < 0.001$

Table 4. The degree of atherosclerosis in the aorta of rabbits treated with cholesterol alone − effects of gliclazide

Arterial pathology (Degree of atherosclerosis)		Group: Cholesterol fed rabbits [18]	Cholesterol fed rabbits + Gliclazide [16]
Macroscopic examination	Aortic arch	3.7 ± 0.1	3.0 ± 0.3
	Thoracic aorta	3.1 ± 0.2	1.9 ± 0.3[b]
	Abdominal aorta	1.8 ± 0.2	1.0 ± 0.2[b]
Microscopic examination	Aortic arch	3.1 ± 0.2	2.2 ± 0.2[b]
	Thoracic aorta	2.4 ± 0.2	1.1 ± 0.2[c]
	Abdominal aorta	1.2 ± 0.3	0.6 ± 0.2
	Coronary arteries	1.8 ± 0.3	0.6 ± 0.2[b]

All animals were sacrificed at 60 days. There were no lesions of aorta in normal controls, except at the level of the arch of the aorta where there were sometimes lipidic patches
The number of animals in each group is indicated in brackets. The values for the degree of atherosclerosis are expressed as mean ± SEM
The degree of significance is calculated for cholesterol + gliclazide group vs cholesterol group
[b] $p < 0.01$ [c] $p < 0.001$

presented a normal appearance. Moréover, examinations involving the coronary artery showed evidence of local sub-endothelial accumulation of foam cells, the underlying tunica media being frequently modified. The lesions were particularly clear around the small coronary vessels; these minor ramifications were partially or completely occluded by the hyperplasia of the tunica intima.

Thyroid Histology: In the control rabbits, sustained thyroid activity was observed; the vesicles were of small diameter, colloid very sparse, the epithelium prismatic, the nuclei rounded and located half way up the cell and acidophilia was weak. This histological appearance of the gland corresponds to the stage 2 of Kamplemann's scale. In the animals treated with cholesterol for 60 days, the thyroid vesicles were of medium size, sometimes large, the colloid abundant and acidophilic, the cells of the thyroid epithelium flat and their nuclei oval and attached to the base. The greatly diminished thyroid activity of these hypercholesterolaemic animals. although there were fairly marked individual differences, corresponded to stages 3 and 4 of Kamplemann's scale (Table 5).

Effect of Cholesterol Rich Diet + Gliclazide

After treatment with gliclazide at the low-doses used (10 mg/kg/day) plasma glucose values were unchanged in the non-fasted animals (Table 1).

Plasma and Liver Lipids: At the 15th day, gliclazide-treated animals had a significant increase in plasma lipids.

Thereafter there were no significant modification of lipid components except for total cholesterol which was decreased on the 60th day of treatment (Table 1). With regard to the liver, no changes were observed in the content of triglycerides or phospholipids. However, it was noted that the addition of gliclazide to the diet rich in cholesterol greatly reduced the hepatic accumulation of cholesterol esters (Table 2). All these values, although lower than the high cholesterol values were still vastly greater than in the animals on a normal diet.

Arterial Lipids: Gliclazide had a most noticeable prophylactic effect on the entire arterial trunk. Biochemical analysis revealed that the aortic lipid content was reduced significantly (Table 3); for the entire group of animals treated with gliclazide, the mean content of total fatty acids was only slightly higher that the normal values. The glycerides were likewise affected; but the cholesterol fractions were only slightly decreased.

Arterial Pathology: Macroscopic examination showed that the arterial lesions were also greatly diminished in relation to those in cholesterol fed animals. The presence was noted of papules, isolated or rosary like but these were rarely in the form of patches (e. g. an index of 1.9 ± 0.3 as compared to 3.1 ± 0.2 at the level of the thoracic aorta); in some animals no change could be detected over a large part of the aortic trunk.

The favourable results were largely confirmed at the histopathological level. In most cases, degenerative changes in the superficial elastic fibres were observed with sometimes a few conjunctival hyperplastic reactions which are the initial stages of development of atherosclerosis (Table 4). The lipophages were likewise very small in number in the sub-endothelial space. Gliclazide had a particular prophylactic effect on the coronary arteries, which had a normal appearance in more than 50% of cases. In the other animals, the hyperplastic intimal reactions were always limited and the absence of foam cells was more or less general while in the cholesterol-treated rabbits most of the secondary ramifications were partially or completely occluded.

Thyroid Histology: Finally, with regard to the thyroid gland, it was noted that in those animals in which activity was partly maintained (stages 2 and 3 of Kampelmann's scale; Table 5), the supranuclear cytoplasm remained large, the vesicles were relatively reduced and the colloid was somewhat sparse.

Discussion

Gliclazide administration to rabbits fed a high cholesterol diet induced a marked decrease of atheromatous lesions without noticeable effects on plasma lipids. Interpretation of the transient rise in plasma lipids observed on the 15th day is difficult. Thereafter, gliclazide did not produce any consistent modification of the high-cholesterol-diet-induced lipid disturbances.

This is an agreement with the work of Smit-Sibinga and Wieringa [31] who observed no effect on serum cholesterol in rabbits at a dose level of 15 mg/kg. According to these authors, hypercholesterolaemia is only seen at hypoglycaemic doses e. e. 25 mg/kg.

However, treatment with gliclazide in the present experiments did significantly reduce the hepatic accumulation of cholesterol by up to 34% in relation to control values. To our knowledge, this effect on hepatic cholesterol metabolism has not been reported for other hypoglycaemic sulphonamides. Although further research is being undertaken into the mechanism of this action, it may be worthwhile at this stage to speculate on the possible involvement of thyroid gland function.

Earlier sulphonamides slow-down thyroid activity; on the contrary, gliclazide maintained noticeable histological thyroid activity, but measurements of levels of circulating thyroid hormones are necessary to confirm histology data. Since thyroxine is known to promote greatly the biliary excretion of cholesterol metabolites [9] and increase synthesis [11]; it is possible that gliclazide counteracted hepatic cholesteatosis by promoting thyroid activity. This hypothesis must be studied.

The patterns of arterial lesions in cholesterol-fed rabbits are altogether in agreement with the many descriptions in the literature. In previous studies we showed that gliclazide could forestall the atherosclerotic syndrome [22]. The present results confirm this, demonstrating that in the aorta accumulation of glycerides and fatty acids was significantly reduced and the severity of atherosclerotic lesions throughout the arterial trunk considerably diminished (both at the macroscopic and microscopic levels) following administration of the drug. These results are sup-

ported by work on another experimental model of atheroma in which gliclazide prevented arterial infiltration of lipids following local irradiation and treatment with cholesterol [31]. The present experiments also showed that in more than 50% of the animals treated with gliclazide the histological appearance of the coronary arteries was completely normal in contrast to the cholesterol treated controls where most of the coronary ramifications were partially or totally occluded. These antiatheromatous properties were not related to the level of plasma lipids, suggesting a vascular metabolic effect of the drug. It must be stressed that earlier reports of studies performed with old sulphonamides revealed both an increase of lipid disorders and of vascular lesions, especially of the coronary arteries in the chicken [16, 29] and the rabbit [23, 24].

The experimental model uses a normoglycaemic animal. Abnormal glucose tolerance test appears after longer cholesterol administration [25].

From our results, low gliclazide dose does not induce significant modification in plasma glucose in fed animals. A dynamic study of plasma immunoreactive insulin may be helpful, but the relationship between insulin levels, diabetes, hyperlipaemia and atheroma are unclear. Data from studies performed with gliclazide do not indicate a direct relationship between hypoglycaemic and anti-atheromatous properties.

Results of studies in progress with diabetic rabbits and psammomys obesus could help to elucidate the mechanism of action of gliclazide.

Several other pharmacological effects could be involved in these antiatherogenic properties. This sulphonamide has been shown to reduce markedly platelet adhesiveness [6] to decrease strongly platelet aggregation (by an inhibition of platelet ADP release [33], to enhance fibrinolysis [5] and to reduce the exaggerated vasoconstrictor responses to adrenaline in experimental diabetes [7, 8]. All these actions could influence to varying degrees the preventive effect of the drug on aortic and coronary atherosclerosis.

In conclusion, our study indicates that gliclazide, at the low-doses used, reduced the accumulation of cholesterol in the liver and strongly inhibited the development of aortic and particularly coronary lesions induced by the cholesterol rich diet. But the mechanism of action of gliclazide is unclear.

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