To reinvestigate the relationship between diabetes and atherosclerosis in rabbits, we fed alloxan-diabetic, alloxan-nondiabetic, and control rabbits a low cholesterol atherogenic diet for up to 40 weeks. Concentrations of plasma total cholesterol, phospholipids, and triglycerides were higher, the percentage of very low density lipoproteins was higher, and the percentage of high density lipoproteins was lower in diabetic than in nondiabetic rabbits. Smooth muscle cell proliferation was prominent, atherosclerosis was more extensive, and a high incidence (29%) of large, sharply demarcated, ischemic myocardial lesions occurred in the diabetic rabbits. These results are in contrast to those of earlier studies where the diabetic state resulted in a partial protection against atherogenesis in alloxan-diabetic rabbits fed larger amounts of cholesterol. (Arteriosclerosis 4:586-591, November/December 1984)

Plasma cholesterol and triglyceride concentrations are higher in alloxan-diabetic rabbits fed cholesterol (0.5% to 2%) compared to cholesterol-fed nondiabetic rabbits. Serum phospholipid concentrations are also increased in cholesterol-fed diabetic rabbits but there is a reduction in the cholesterol/phospholipid ratio, and the Sf 12-30 lipoproteins. Although cholesterol-fed diabetic rabbits develop atherosclerotic lesions, there is a paradoxical decrease in the extent of disease unless insulin therapy is instituted, and then the degree of atherosclerosis in the diabetic rabbits is increased to the level found in the nondiabetic rabbits.

A number of undesirable toxic effects are present in rabbits fed 0.5% cholesterol or more. Development of low cholesterol atherogenic diets for rabbits permitted reinvestigation of the relationship between diabetes and atherosclerosis in rabbits under more physiologic conditions.

From the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington.
This work was supported by USPHS Grant HL-20441 and intramural funds of the Department of Microbiology and Pathology, Washington State University.


Received March 22, 1984; revision accepted June 6, 1984.

Methods

Animals

A group of 127 young (approximately 1.4 kg), male, Dutch Belted rabbits were housed individually in wire-bottomed cages, in air-conditioned rooms maintained at 18°C and 50% humidity, with 12 hours of light and 12 hours of dark each day. The housing and experimental procedures involving these animals were approved by the Washington State University Committee on Animal Care. The rabbits were fed commercial rabbit pellets during the 14-day quarantine period and the 4-week stabilization period after alloxan treatment. They were weighed at the beginning of the experiment and at 3-month intervals.

Approximately 3 ml of blood were obtained from the central artery of the ear and placed into ethylene-diamine tetracetic acid-treated tubes at the beginning of the experiment and at monthly intervals. A final 20-ml blood sample was collected from the posterior vena cava after the rabbits were killed by electrocution.

Alloxan Treatment

Alloxan monohydrate was dissolved in sterile physiological saline to make a 10% solution. The freshly prepared solution was administered via the marginal ear vein during a period of less than 10 seconds at a dose of 100 mg/kg per body weight. Alloxan solutions were discarded after 1 hour. Intra-
peritoneal injections of 5 ml of 50% glucose were given every 5 hours, for 24 hours, to counteract hypoglycemia caused by insulin release from necrotic beta cells.

Rabbits were classified as diabetic if their plasma glucose concentrations were 200 mg/100 ml or greater throughout the feeding trial. Alloxan-treated rabbits with plasma glucose concentrations of less than 200 mg/100 ml were considered nondiabetic. These alloxan-treated, nondiabetic rabbits were used as a second control group along with non-alloxan-treated rabbits.

**Diet**

The atherogenic diet (Table 1) was introduced 4 weeks after alloxan treatment. At this time the groups consisted of 34 diabetics, 32 alloxan-nondiabetics, and 27 controls. A free-choice diet was fed for 40 weeks. Fresh food was provided each day, and tap water was given freely.

**Urine Glucose and Ketones**

Qualitative methods were used to determine the presence of glucose and ketone bodies in the urine of alloxan-treated rabbits during the stabilization period.

**Plasma Glucose**

Plasma glucose concentrations were measured 4 weeks after alloxan treatment and at 4-week intervals thereafter by a glucose oxidase procedure.

**Plasma Lipids**

Plasma total cholesterol, phospholipid, and triglyceride concentrations were measured at the beginning of the feeding period and at 12-week intervals. Fresh plasma from the terminal bleedings was used for quantification of lipoproteins by a polyacrylamide-gel disc-electrophoresis procedure. The developed and stained gels were scanned at 600 nm in a linear transport mechanism attached to a Gilford spectrophotometer. The scans were recorded, and the percentages of very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) were determined by integrating relative surface areas under the respective curves.

**Necropsy and Histologic Procedures**

Tissues were preserved in 10% neutral-buffered formalin. The aorta was cut longitudinally and stapled to a cork board at its in situ length, with the intimal surface upward. The aortic intima was photographed, stained with Sudan IV, and rephotographed. Photographs were projected onto white paper and the lesions were traced. A grid with 3-mm squares was placed over each tracing, and the percentage of surface sudanophilia was determined by counting the squares covering the stained areas.

For quantitative evaluation, sections of aorta were taken immediately posterior to the branch point of the left subclavian artery, at the seventh intercostal, and immediately posterior to the celiac artery. The heart was sectioned 0.5, 1.0, and 1.5 cm from the apex. Each carotid artery was cut at 0.5-cm intervals. Each renal artery was sectioned 0.25 and 0.5 cm from its origin. Each basilar artery was sectioned 0.5 and 1.0 cm from the circle of Willis, and a section was taken through the circle of Willis. Paraffin-embedded sections of the above tissues were sectioned at 6 μm and stained by either the method of Masson or Verhoeff-Van Gieson. Select ed sections were stained with hematoxylin and eosin. One section of each aorta, just posterior to the left subclavian artery, was sectioned at 10 μm on a cryotome and stained with oil red 0 and light green stains.

The maximal intimal thickness of each section of aorta was measured with a micrometer eyepiece. The average intimal thickness was determined by summing the intimal thickness at the middle of every consecutive high power field (400 x ) across the total.

---

**Table 1. Atherogenic Diet**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/100 g dry mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.2</td>
</tr>
<tr>
<td>Powdered sucrose</td>
<td>36.25</td>
</tr>
<tr>
<td>Cellulose</td>
<td>12.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>4.0</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>0.4</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>2.5</td>
</tr>
<tr>
<td>Butter</td>
<td>19.0</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>2.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The dry ingredients were mixed except for the agar and sorbitol. The agar, sorbitol, and 100 ml of water per 100 g dry diet were mixed and heated to the point of gelling upon cooling; the hot agar solution was then mixed with the dry ingredients and allowed to set in the cold. By calculation, the diet contained 0.05% cholesterol.

*Each kg contained (g): CaCO3, 508.100; KH2PO4, 207.500; MgSO4, 12.5; MgCO3, 25; NaCl, 70; FePO4, 40; KIO3, 0.950; MnSO4. H2O, 20; ZnSO4, 7H2O, 0.625; CuSO4, 0.900; COCl2, 6H2O, 0.750; AIK (SO4)2, 12H2O, 0.175; NaF, 1.000; KCL, 112,500; MgCO3, 25; NcCl, 70; FePO4, 40; KIO3, 0.950; MnSO4. H2O, 20; ZnSO4, 7H2O, 0.625; CuSO4, 0.900; COCl2, 6H2O, 0.750; AIK (SO4)2, 12H2O, 0.175; NaF, 1.000; KCL, 112,500.*

†Each kg contained (g): inositol 50.00, thiamine 0.50, riboflavin 0.50, pyridoxine HCl 0.50, calcium pantothenate 1.50, niacin 2.50, folic acid 0.50, vitamin A acetate (500,000 IU/g) 2.90, vitamin D2 (50,000 IU/g) 2.90, DL-α-tocopherol 4.00, menadione 0.50, vitamin B12 (0.1% mannitol) 1.50, biotin 0.01, powdered sugar 932.19.
width of the aorta and dividing the total by the number of fields observed. The number of coronary arterioles and arteries greater than 20 μm in diameter that contained atheromatous lesions was counted and expressed as a percentage of the total number of vessels of that size observed. Likewise, the number of lesions found in the renal, carotid, and basilar arteries was expressed as a percentage of the number or cross sections observed.

Paraffin-embedded sections of both kidneys, the spleen, the adrenal glands, two lobes of lung, three levels of brain, and two lobes of liver were stained with hematoxylin and eosin.

Statistical Evaluation

Statistical evaluation of obtained data was facilitated by the Amdahl-V7 and SAS76 computer systems. Analysis of variance and correlation coefficients were computed to obtain evidence for significant differences and correlation of variables. The significance level established was $p < 0.05$. Only rabbits surviving at least 35 weeks were included in the statistical analysis.

Results

Clinical and General Findings

Twenty-four diabetic, 24 alloxan-nondiabetic, and 23 control rabbits completed 35 weeks or more of the feeding period. The principal cause of death in all groups were Pasteurella pneumonia, infectious enterocolitis, and fractured vertebrae (from handling). Hair chewing was seen in 20 rabbits and was equally distributed among the three groups.

Mean initial weights at the beginning of the feeding period were 1.4 kg for diabetic, 1.5 kg for alloxan-nondiabetic, and 1.6 kg for control rabbits. The average weight gains at the termination of the experiment were 1% for the diabetic rabbits, 10% for the alloxan-nondiabetic rabbits, and 22% for the control rabbits. All rabbits consumed approximately 100 g of food per day after the first 10 days.

Bilateral cataracts developed in 1 alloxan-nondiabetic and in 5 diabetic rabbits. The alloxan-nondiabetic rabbit with cataracts had been hyperglycemic for 1 month before reverting to a nondiabetic state after beginning the atherogenic diet.

Alloxan Treatment

Ten deaths from apparent hypoglycemia occurred within the first 24 hours after alloxan treatment despite glucose injections. The next critical period was 5 to 10 days after starting the alloxan treatment when severe and fatal ketoacidosis developed in 20 of the diabetic rabbits.

Plasma Glucose

Plasma glucose concentrations were expressed as a mean of the monthly results (Table 2). Differences in plasma glucose concentration between diabetic and either alloxan-nondiabetic or control rabbits were significant.

Plasma Lipids

Plasma cholesterol, triglyceride, and phospholipid concentrations were significantly higher in diabetic as compared to either alloxan-nondiabetic or control rabbits (Table 2). The cholesterol/phospholipid ratio was significantly elevated in diabetic rabbits compared to the other two groups.

The percentage of VLDL was increased and the percentage of HDL was decreased in diabetic rabbits killed at 40 weeks compared to either of the control groups (Table 3).

Gross and Microscopic Tissue Evaluation

Aortic surface area sudanophilia was significantly greater in diabetic than in either alloxan-nondiabetic or control rabbits (Table 4). In the less extensively involved aortas, lesions were restricted to the aortic arch and ostia of branch vessels. More extensive lesions were prominent in the aortic arch and ostia, but the atherosclerotic plaques were confluent and often included the abdominal portion of the aorta in diabetic rabbits. The area just anterior to the ostia of the mesenteric vessels was a frequent site for atherosclerosis in the more extensively involved aor-
Table 4. Aortic Atherosclerosis (35-40 Weeks)

<table>
<thead>
<tr>
<th></th>
<th>Diabetic (24)</th>
<th>Alloxan-nondiabetic (24)</th>
<th>Control (23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface sudanophilic (%)</td>
<td>Mean ± SD 48 ± 24*</td>
<td>20 ± 10</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>Intimal thickness (µM)</td>
<td>Mean ± SD 40 ± 35*</td>
<td>12 ± 10</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>Maximal ± SD 112 ± 80*</td>
<td>45 ± 22</td>
<td>26 ± 10</td>
</tr>
</tbody>
</table>

Number of rabbits is given in parentheses. *Significantly different from other groups (p < 0.05).

Table 5. Percentage of Atheromatous Lesions (at 35 to 40 Weeks) in Vessels Observed with Lesions

<table>
<thead>
<tr>
<th></th>
<th>Diabetic (24)</th>
<th>Alloxan-nondiabetic (24)</th>
<th>Control (23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary (%)</td>
<td>12*</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Carotid (%)</td>
<td>22*</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Renal (%)</td>
<td>29*</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Basilar (%)</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Number of rabbits in parentheses. *Significantly different from other groups (p < 0.05).

Most atherosclerotic lesions in the aorta were nonraised fatty streaks, but 12 diabetic and 3 alloxan-nondiabetic rabbits had grossly raised, white plaques (Figure 1).

Microscopically, the majority of aortic lesions were small, raised plaques of collagen and smooth muscle, with intracellular and extracellular lipid. These lesions were highly proliferative with little foam cell formation or cholesterol clefts except in the thickest plaques. Lipid was observed 4 to 6 elastic lamina deep in oil-red-0-stained sections of aorta. Fragmentation and reduplication of the internal elastic membrane were common. Lesions were more numerous, thicker, and more advanced in diabetic rabbits than in the other two groups. Proliferation of smooth muscle cells was particularly striking in the diabetic rabbits compared to the controls. The mean aortic intimal thickness in diabetic rabbits was more than three times the mean thickness of nondiabetic controls (Table 4).

The total number of coronary, carotid, and renal arterial atheromatous lesions was significantly greater in diabetics than in either alloxan-nondiabetic or control rabbits. The absolute number of atheromatous lesions in the basilar arteries was greater in diabetic than nondiabetic rabbits, but differences were not statistically significant (Table 5). Proximal coronary arterial plaques were highly proliferative, particularly in diabetic rabbits, while those in distal arterioles were more foamy and amorphous (Figure 2).

Large, sharply demarcated, scarred areas typical of either ischemic injury or infarction were seen in 12 rabbits — 10 diabetic and 2 alloxan-nondiabetic (Table 6). These large scarred areas were found entirely in the left ventricular myocardium and intraventricular septa. Three morphologic variations were noted. Some scars were transmural with a few viable myocardial fibers remaining in the subendocardial and subepicardial areas. In others, long, linear scars involving about one-quarter of the ventricular wall were found in the subendocardial area. In a few hearts, one or more papillary muscles contained significant and discrete scars. The ischemic lesions were associated with stenotic lesions of proximal and distal coronary arteries, but thrombi were not found.

Degranulation, vacuolation, and absence of beta cells and small foci of residual or regenerative beta cells were seen in the pancreas of alloxanized rabbits. These changes were more severe in diabetic than in nondiabetic rabbits.

Hepatic lipidosis of varying degrees was noted in 22 rabbits (16 diabetic, 5 alloxan-nondiabetic, and 1 control). Of 12 severely fatty livers, 11 were from diabetic rabbits. Centrilobular hepatic fibrosis was found in eight diabetic rabbits. Vacuolated tubular epithelial cells (Armanni-Ebstein lesions) were seen in the kidneys of eight diabetic rabbits.

Table 6. Myocardial Ischemic Lesions (All Rabbits)

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Alloxan-nondiabetic</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial ischemic lesions</td>
<td>10/34 (29%)</td>
<td>2/32 (6%)</td>
<td>0/27 (0%)</td>
</tr>
</tbody>
</table>
Discussion

Atherosclerosis was more extensive and severe in diabetic than in nondiabetic rabbits fed a low cholesterol diet. This finding is in contrast to earlier observations, and in the present study the expected positive correlation was present between mean serum cholesterol concentrations and degree of atherosclerosis among groups.

The design of the present experiment did not permit precise identification of the factors responsible for differences in atherogenesis among rabbits fed various amounts of cholesterol. Both serum lipid and blood glucose concentrations were positively correlated with the extent and severity of cardiovascular lesions. But multiple regression analysis failed to demonstrate a significant difference between the relative importance of serum lipid or glucose concentrations on the various cardiovascular lesions. Additional experiments are needed to answer the question of whether the more severe cardiovascular lesions in diabetic, compared to nondiabetic, rabbits result from a higher serum-lipid concentration or from some other effect of the diabetic state, or both.

There are profound alterations in lipid metabolism in rabbits fed large amounts of cholesterol as reflected by exceedingly high concentrations of plasma cholesterol (≈ 2000 mg/100 ml), the presence of circulating lipophages, and a generalized lipid storage disease. With low cholesterol diets, the atherogenesis is milder and slower in development, and smooth muscle cell proliferation is more prominent. The cholesterol/phospholipid ratio was elevated in diabetic rabbits in this experiment, but the ratio was lower in diabetic rabbits fed much larger amounts of cholesterol. Higher cholesterol/phospholipid ratios are usually associated with more extensive atherosclerosis.

The alloxan-nondiabetic rabbits provided a control group for the possible toxic effects of alloxan, aside from its effect as a diabetogenic agent. In general, the degree of atherosclerosis in alloxan-nondiabetic rabbits was similar to that of the nonalloxanized controls. However, a few nonalloxan-diabetic rabbits did have rather severe atherosclerotic disease. This was not unexpected since some of the alloxan-nondiabetic rabbits were probably hyperglycemic, or at least glucose-intolerant, at times.
Atherosclerosis in Diabetic Rabbits

Miller and Wilson 591

Although there was considerable variation in the degree of atherosclerosis in diabetic rabbits, the disease was more extensive and severe in this group than in either of the control groups. The marked prolife-
ration of smooth muscle cells in the diabetic rabbits was striking. From earlier studies, it seemed that in addition to hypercholesterolemia, normal concentrations of insulin were essential for maximal athero-
genesis in rabbits. Insulin promotes proliferation of cultured primate arterial smooth muscle cells. On the other hand, in serum from alloxan-diabetic rabbits, some other factor — not glucose, insulin, or lipid — promotes growth of rabbit smooth muscle cells in culture. Likewise, a factor in the serum of diabetic people, probably growth hormone, stimulates cell proliferation of rabbit aortic medial cell cultures. Alloxan-diabetic rabbits are only relatively insulin-defi-
cient since they live for months under relatively sta-
ble degrees of diabetes.

The high incidence (29%) of large myocardial ischemic lesions in diabetic rabbits is significant. In a study using the same diet as this experiment, only three myocardial infarcts were found in about 70 normal rabbits fed the diet for as long as 5 years. Epidemiologic studies have indicated that ischemic myoc-
dardial disease and myocardial infarction are two to three times more frequent in diabetic than in nondia-
betic people and that primary mortality is two to three times higher in diabetic patients.

The results of the present study show that a combina-
tion of time, low dietary cholesterol intake, and moderate diabetes results in arterial and myocardial lesions in rabbits similar to those seen all too fre-
quently in diabetic people.

References

6. Duff GL, Brechin JH, Finkelstein WE. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. IV. The effect of insulin therapy on inhibition of athero-
7. Wilson RB, Newberne PM, Conner MC. An improved semi-
9. Alpert, NL. Instrument series: Report #50, Model 23A Glu-
cose Analyzer. Yellow Springs, Ohio: Yellow Springs Instru-
ment Company, 1976
12. Carlson LA. Determination of serum triglycerides. J Ather-
13. Nakao HK, Wada M, Ehrhart LA, Lewis LA. Polyacrilamide-
15. Morgan RS, Adams CMM. A graticule for measuring athero-
17. Wilson RB, Miller RA, Middleton CC, Kinden D. Athero-
sclerosis in rabbits fed a low cholesterol diet for five years. Atherosclerosis 1982;2:228–241
19. Ledet T, Fischer-Dzoga K, Wissler RW. Growth of rabbit aortic smooth-muscle cells cultured in media containing dia-
21. Sievers J, Blomquist G, Brink G. Studies on myocardial infarction in Malmo 1935 to 1954. VI. Some clinical data with particular reference to diabetes, menopause and heart rup-

Index Terms: atherosclerosis • diabetes mellitus • myocardial infarcts • rabbits
Atherosclerosis and myocardial ischemic lesions in alloxan-diabetic rabbits fed a low cholesterol diet.
R A Miller and R B Wilson

Arterioscler Thromb Vasc Biol. 1984;4:586-591
doi: 10.1161/01.ATV.4.6.586

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/4/6/586