THE EFFECT OF RUTIN ON SWINE SERUM CHOLESTEROL AND LIPOPROTEINS

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ABSTRACT

This study involves an investigation of the effects of rutin on serum cholesterol levels and high density lipoproteins (HDL) and the development of atherosclerotic lesions in the domestic swine. Swine were divided into four groups of three animals: (1) a control group receiving sham operative procedures, (2) a group receiving daily rutin injections, (3) a group receiving denudation of the abdominal aorta and daily injections of rutin, and (4) a group receiving only denudation of the abdominal aorta. Blood samples were taken weekly to analyze serum cholesterol and HDL levels. After 45 days, the abdominal aortae were removed and examined for gross and histological evidence of atherosclerotic lesion formation.

It was concluded that rutin significantly increases serum cholesterol levels. Significant decreases of HDL levels were observed in swine receiving daily rutin injections. Histological examination of those animals receiving denudation revealed a disruption of the endothelial layer, but failed to show thickening of the intimal and medial layers.

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INTRODUCTION

Atherosclerosis, an arterial disease recognized worldwide as a major cause of death, may progess unnoticed for many years before symptoms develop; therefore, making early diagnosis difficult (Ross and Glomset, 1976).

Atherosclerosis, as defined by the World Health Organization, is a variable combination of changes in the intimal layer of arteries consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits (Moon, 1972).

Normal arteries consist of three morphologically distinct layers, as described by Ross and Glomset (1976). The intima, or innermost layer, consists of a narrow region bounded on the lumenal side by a single continuous layer of endothelial cells, and bounded peripherally by a fenestrated sheet of elastic fibers, the internal elastic lamina. The media, or middle layer, consists entirely of diagonally oriented smooth muscle cells surrounded by variable amounts of collagen, small elastic fibers and mucopolysaccharides. The adventitia, or outermost layer of the artery, is comprised primarily of recognizable fibroblasts intermixed with smooth muscle cells loosely

arranged between bundles of collagen and surrounded by proteoglycans.

Normal intact arterial endothelium provides a protective barrier against injurious constituents of the blood. Factors such as hyperlipidemia, hypercholesteremia, smoking and increased sheer stress in hypertension may injure the endothelium and alter the nature of the endothelium as a barrier to the passage of blood constituents into the artery wall. This action alters endothelial cells and/or connective tissues associated with endothelial cells and permits hemodynamic forces to possibly detach endothelial cells from the arterial wall (Ross and Glomset, 1976).

The mechanical method of balloon denudation has recently been used for partially denuding an artery of its endothelium, with apparently only minimal damage to the underlying tissues (Stemerman and Ross, 1972). This method of study involves the movement of an inflated balloon catheter through a portion of the artery of an experimental animal. The balloon is inflated with saline until it produces resistance to movement in the artery. As the balloon is moved superiorly and inferiorly along the artery, patches of endothelial cells become detached and enter the circulating blood. Injury to the arterial endothelium causes an immediate cellular response. Within minutes after an injury is induced with a balloon catheter,

platelets aggregate and adhere to the subendothelial connective tissue at the site of injury (Ross and Glomset, 1976).

There are several ways in which enhanced platelet aggregation could contribute to vascular impairment. Increased aggregation of the platelets on a roughened surface could lead to thrombotic occlusion. Abnormal platelet aggregation could be injurious by direct occlusion of small peripheral vessels. Continuously excessive platelet encrustation on the intimal section of an injured artery may result in the incorporation of atheromatous plaques with progressive narrowing of the arterial lumen (Ward, et. al. 1978).

Thin mural thrombi form over the denuded areas within minutes. Interruption of the intact endothelium barrier is followed not only by a platelet response but also by a cellular response involving smooth muscle cells and endothelial cells. Five to seven days after balloon catheter injury, smooth muscle cells can be observed within fenestrae of the internal elastic laminae, apparently in the process of migrating into the intima (Ross and Glomset, 1976). After catheter induced arterial injury in normocholesterolemic animals proliferation of subendothelial smooth muscle cells occurs, and the endothelial cells multiply and eventually cover this thickened intima (Nam, et. al. 1973). Within one to three months,

the intima consists of 5-15 layers of newly formed proliferated smooth muscle cells, mainly surrounded by collagen, elastic fibers, and proteoglycans (Ross and Glomset, 1976). According to the hypothesis of Rosenthlum and El Sabban (1977), the release of pharmocologically active substances from platelets may result in smooth muscle proliferation, or damage to the arterial wall by release of factors that increase intimal permeability.

Restoration of the endothelial barrier normally occurs after injury and the lesion regresses if both the injury and tissue responses are limited. However, further proliferation of smooth muscle cells and accumulation of connective tissue and lipids occur if injury to the endothelium is continuous or repeated. Risk forces such as high serum cholesterol, abundant low density lipoprotein, or inadequate concentration of high density lipoprotein, may tip a critical balance between repair and further cellular response that eventually determines whether the lesion enlarges, remains relatively constant in size, or regresses. Cellular concentration of these factors may convert what might be a limited tissue response into an atheromatous lesion (Ross and Glomset, 1976).

Focal lesions of atherosclerosis are generally characterized by a proliferation of smooth muscle cells, deposition of intracellular and extracellular lipids (lipids are primarily cholesterol and cholesterol esters), and accumulation of an extracellular matrix components

including collagen, elastic fibers, and proteoglycans (Ross and Glomset, 1976). Therefore, one of the most striking biochemical abnormalities in human atherosclerosis is the accumulation of massive amounts of lipids in the lesions.

Lipids account for a great amount of energy expenditure. The body is confronted with the problem of transporting this large quantity of hydrophobic material in an aqueous environment. The problem is solved by associating the more insoluble lipids with more polar ones such as phospholipids, and the combining them with cholesterol and protein to form a hydrophilic lipoprotein complex (Brown and Goldstein, 1977).

Most nonhepatic body tissues do not synthesize cholesterol de novo, but rather obtain most of their cholesterol from plasma lipoproteins that are secreted by the liver or small intestine (Brown and Goldstein, 1977). Nonhepatic cells in the body may acquire cholesterol for such functions as membrane synthesis by cell surface receptors sites that bind the cholesterol-rich plasma lipoproteins. Binding of the low density lipoprotein, rich in cholesterol, is the first step by which cells acquire the lipoprotein by endocytosis, accumulate cholesterol, and suppress the endogenous cellular cholesterol synthesis (Brown and Goldstein, 1977).

One of the hallmarks of the atherosclerotic lesion is the accumulation of lipids within the arterial wall in

the form of cholesterol and cholesterol esters. In the severely diseased artery it is not unusual to find as much as a ten fold increase in the content of the unesterified cholesterol and more than a fifty fold increase in the cholesterol ester content (St. Clair, 1974). St. Clair (1974) also observed that an increase in fatty acid synthesis occurs in the area of the atherosclerotic plaque. Bjorkeurd and Bondjers (1974) discovered that the uptake of labeled cholesterol and cholesterol esters from the serum by the aorta is about four times larger in an area of injury than in an area of normal intact tissue. Thus, the condition of the arterial endothelium seems to have a determining influence on repair and accumulation of tissue lipids after injury to the artery.

Epidemiological studies of the evolution of cardiovascular disease in human populations have for many years emphasized the importance of the serum total cholesterol as a precursor of coronary heart disease. These population studies were stimulated by the observation that patients with clinical cases of coronary heart disease had higher cholesterol values than the non-coronary patients (Kannel, et. al. 1979).

Cigarette smoking and elevated serum cholesterol are well established risk factors for the development of coronary heart disease. Cholesterol levels and the number of cigarettes smoked per day prove to be powerful

predictors of coronary heart disease. Studies by Hjermann et. al. (1976) revealed a significant positive correlation between serum cholesterol and the exposure to cigarette smoke. Also in cigarette smokers, the serum cholesterol increased with an increase in the number of cigarettes smoked. Heavy cigarette smokers, persons who smoke more than 20 cigarettes per day, have higher levels of serum cholesterol than nonsmokers (Billimoria, et. al. 1975).

Devi, et. al. (1975) showed that there was an immediate and highly significant increase in cholesterol after a single cigarette. The overall mean cholesterol value was greater for heavy smokers, 236 ± 5 mg%, than the 215 ± 6 mg% for nonsmokers.

Sarma, et. al. (1975) reported that cigarette smokers have an abnormal increase in carboxyhemoglobin levels, which decreases the oxygen carrying capacity of the blood. The resulting hypoxia increases the permeability of the endothelial membrane, and may allow passage of macromolecules such as low density lipoprotein into the arterial wall. Pozner and Billimoria (1975) demonstrated a significant increase in low density lipoproteins (LDL) in cigarette smokers. The components of the arterial wall such as elastin, collagen, glycosaminoglycans could serve to entrap the LDL. The arterial wall, subjected to such an abnormal increase in LDL, could incorporate the excess lipid into an atherosclerotic lesion.

Lipoproteins may be ingested by smooth muscle cells of the intima and virtually all of the lipoprotein hydrolyzed within lysosomes to yield small, relatively soluble materials such as amino acids, fatty acids and monosaccharides which can leave the cell and be transported out of the artery wall. However, it there is a shortage of high density lipoprotein (HDL), whose function is to transport cholesterol from peripheral tissues to the liver for subsequent catabolism and excretion, the excess cholesterol accumulation could lead to atherosclerosis (Miller and Miller, 1975).

Recently, retrospective analysis of epidemological data has established an inverse relationship between decreased cholesterol in high density lipoprotein and premature heart disease (Miller and Miller, 1975).

Goldbourt and Medalue (1977) have demonstrated an inverse relationship between cigarette smoking and high density lipoprotein levels in 10,000 Israeli civil servants. Studies by Garrison, et. al. 1978 indicated that blood samples of men and women smokers had lower HDL levels than nonsmokers.

Experiments by Becker and Dubin (1977) indicated that a tobacco glycoprotein derived from cured tobacco leaves and from cigarette smoke condensate contained rutin. Rutin (quercetin-3-ß-rutinoside) was shown to activate factor XII in samples of human plasma resulting in the generation of clotting activity, fibrinolytic activity

and kinin activity. Activation of factor XII and initiation of thrombosis or kinin generation could result in increased endothelial permeability or focal vascular injury.

As a result of rutin causing focal vascular injury, or increasing endothelial permeability, the intima could be subjected to an overwhelming load of injurious plasma constituents such as LDL and cholesterol esters. Since cigarette smokers have a higher serum cholesterol level and a decrease in HDL, rutin could serve not only as a potential source of injury, but also affect the levels of cholesterol and lipoproteins.

Therefore, this study involved an investigation of the effect of rutin on serum cholesterol and lipoprotein levels and the development of atherosclerotic lesions. Swine were used as an experimental model for several reasons. Atherosclerosis in man and swine develop most frequently in aortas and coronary arteries. Anatomically, the origin and distribution of the aorta and coronary arteries of humans and swine correspond closely. The histological changes of growth and aging that apparently lead to atherosclerosis of aorta and coronary arteries are extremely similar in humans and swine (Ratcliff and Luginbuhl, 1971). Swine carry the majority of their cholesterol on LDL, as do humans, and the swine do develop atherosclerosis lesion formation spontaneously which also occurs in humans (Chase and Morris, 1976).

Swine were divided into four groups of three animals: (1) a control group receiving sham operative procedures, (2) a group receiving daily rutin injections, (3) a group receiving denudation of the abdominal aorta and daily injections of rutin, and (4) a group receiving only denudation of the abdominal aorta.

Blood samples were taken weekly to analyze serum cholesterol and HDL levels. After 45 days, the swine were sacrificed, and the abdominal aortae removed and examined for gross and histological evidence of atherosclerotic lesion formation. The histological specimens were stained using hematoxylin and eosin for analyses of thickening of the intimal and medial layers.

MATERIALS AND METHODS

Male, eight to twelve week old, Yorkshire swine were the animals chosen for the experimental procedure. Swine were housed at the Morehead State University Farm and given a normal diet of Purina Mash. Swine were divided into four groups of three animals: (1) a control group receiving sham operative procedures, (2) a group receiving daily rutin injections, (3) a group receiving denudation of the abdominal aorta and daily injections of rutin, and (4) a group receiving only denudation of the abdominal aorta.

Groups 1 and 2 were sham operated with the femoral artery being isolated and ligated. Groups 3 and 4 received denudation by the introduction of an embolectomy catheter.

Swine were anesthetized with choloroform and sterile surgical procedures were performed. An incision was made to expose either the right or left femoral artery. A small incision was made in the artery of those in the sham control group. For those animals receiving denudation a 4 F Fogarty arterial embolectomy catheter (V. M. Mueller, Chicago, Illinois) was introduced and passed into the abdominal aorta a distance of 20 centimeters. A volume of 0.75 mL of sterile saline was

injected into the balloon to distend the abdominal aorta moderately and produce considerable resistance to movement of the catheter. The balloon was then pulled superiorly and inferiorly in the abdominal aorta for 10 seconds. The saline was withdrawn and the catheter removed.

The artery was ligated with suture and the opening closed using animal wound clips and suture. Penicillin, in a concentration of 12,500 units, was given before and immediately after the operative procedure.

After recovering from surgery, the swine were returned to the enclosed swine facility at the farm. The day after surgery rutin injections were begun. A stock solution of rutin (Sigma Chemical Company) was prepared in a concentration of 10 mg per mL of proplyene glycol equal 2.2 micrograms of rutin per mL of blood. The dosage was determined as follows (Reese, 1978 and Saxon, 1978):

(a) a 1 g cigarette contains 0.86 g of tobacco.

(b) 55 mm of an 85 mm cigarette is smoked equaling 0.56 g of tobacco.

(c) 0.8% of tobacco is rutin which equals 4.3 mg of rutin per cigarette smoked.

(d) 15% of rutin is transferred from tobacco into tobacco condensate. This 15% transfer rate is based on the 5% to 25% transfer rate range for scopoletin. The transfer rate for rutin is not known; however, based on physical and chemical properties, the transfer rate of scopoletin would be very close to that of rutin. This yields 0.65 mg of rutin in the smoke of one cigarette.

(e) In humans (70 kg), 8.3% of the mass is blood which equals 5810 mL of blood. The volume of blood for each animal was determined by the body mass, since 8.3% of the body mass is blood.

(f) $\frac{0.65 \text{ mg rutin}}{5810 \text{ mL blood}}$ equals 0.11 ug rutin per mL of blood.

(g) 20 cigarettes per day equals 2.2 ug rutin per mL of blood.

The rutin was injected intramuscularly, where it would be slowly absorbed into the blood and bypass the liver, to prevent possible detoxification of the rutin before it reached the abdominal aorta.

Weekly blood samples were collected for analysis of serum cholesterol and HDL. Blood was drawn from the anterior vena cava in the region of the thoraic inlet using an 18 guage, 4 inch needle. Blood was collected in tubes containing sodium citrate, and, after collection, was placed in an ice bath. Blood samples were centrifuged at 1500 rpm for 15 minutes to obtain plasma.

Serum cholesterol levels were analyzed using Cholesterol Reagent purchased from Worthington Diagnostics, Freehold, New Jersey. (Cholestrol Reagent Set SM 526132, 1978)

CHOLESTEROL ASSAY

1. Cholesterol 16 Reagent containing the enzymes cholesterol oxidase, cholesterol esterase, and cholesterol peroxidase was resonstituted with 16 mL of cholesterol buffer.
2. Immediately after the addition of the buffer, the solution was inverted and swirled gently to dissolve the reagent.
3. A 1.0 mL disposal pipette was used to dispense the reconstituted reagent into a cuvette.

4. The initial absorbance (A_I) of the reagent at 500 nm was read and recorded.

5. A plasma sample of 10 microliters was added to the reagent and mixed gently by inverting.

6. Exactly 10 minutes after the addition of the sample to the reagent the final absorbance (A_f) was read and recorded at 500 nm.

7. The results were expressed in mg/dL using the following formula:

$$mg/dL = \Delta A \times 567$$

The principle of the cholesterol assay is outlined as follows:

$$\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol + fatty acid}$$

$$\text{Cholesterol + oxygen} \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{Cholesten-3-one + hydrogen peroxide}$$

$$\text{2 Hydrogen peroxides + 4-aminoantipyrene + phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine + water}$$

In the sequential reactions, cholesterol esters in plasma are enzymatically hydrolyzed to cholesterol and free fatty acids. The free cholesterol in plasma and the cholesterol produced are oxidized by cholesterol oxidase to cholesten-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide is oxidatively coupled with the chromagen 4-aminoantipyrene in the presence of phenol and peroxidase to yield a quinoneimine dye with an absorption maximum at 500 nm. The change in absorbance at 500 nm is directly proportional to the level

of total cholesterol in the sample.

High density lipoprotein (HDL) levels were determined with HDL Reagent purchased from Worthington Diagnostics, Freehold, New Jersey (HDL Reagent Set SM526121, 1978).

HIGH DENSITY LIPOPROTEIN ASSAY

1. A 0.1 mL solution of HDL Reagent containing manganese chloride and heparin was added to 1 mL of the plasma sample.
2. The test tube containing the reagent and the sample were vortexed for 3-5 seconds to insure complete mixing; it was then placed in an ice bath for 30 minutes.
3. The tube was centrifuged for 30 minutes at 2500 rpm.
4. The supernatant fluid was removed immediately after centrifugation, as this contains the HDL fraction.
5. The supernatant fluid was assayed for HDL cholesterol with Worthington Diagnostic's Cholesterol Reagent, with the following exception:

   the sample size was 0.75 microliter per 1.5 mL of Cholesterol Reagent.
6. The results were expressed in mg/dL using the formula:

$$mg/dL = \Delta A \times 567 \times 1.1$$

After 45 days of rutin injections, the swine were sacrificed and the abdominal aortae examined for gross morphological and histological examination. The aortae, measuring approximately three centimeters, in length, were placed in a 10% formalin fixative until they were embedded in Histowax. Sections measuring 8 microns in thickness were prepared. The specimens were stained with a modified Harris solution of Hematoxylin and Eosin for

analysis of possible differences in the thickening of the intima and media, since these layers are principally involved in atherosclerotic lesions.

The Hematoxylin and Eosin staining procedure utilized is outlined as follows:

1. Xylene - 1 minute
2. Xylene - 2 minutes
3. 100% alcohol - 1-2 minutes
4. 95% alcohol - 1 minute
5. 70% alcohol - 1 minute
6. 50% alcohol - 1 minute
7. Rinse in distilled water
8. Hematoxylin - 45-60 seconds
9. Counterstain with eosin for 30-45 seconds
10. Rinse in distilled water
11. 50% alcohol - 30 seconds
12. 70% alcohol - 30 seconds
13. 95% alcohol - 30 seconds
14. 95% alcohol - 30 seconds
15. 100% alcohol - 30 seconds
16. Xylene - 30 seconds

The sections were mounted on glass slides using Xylene Clearmount, and then covered with a glass cover slip.

RESULTS AND DISCUSSION

Serum cholesterol levels were determined for blood samples collected weekly from fasting animals. Swine receiving rutin injections had higher serum cholesterol levels than the control group, or the group receiving only denudation (Table 1). Rutin, may pass through the alveoli of the lung and gain access to the bloodstream. Burrows and Griffith (1977), suggested that rutin may exert a physiological effect while in the body. Such an effect may be instrumental in causing increased serum cholesterol levels. Kershbaum, et. al. (1963), indicated that cigarette smoking causes an immediate increase in free fatty acids as a result of catecholamine release from the adrenal medulla. Catecholamines may stimulate the following reactions:

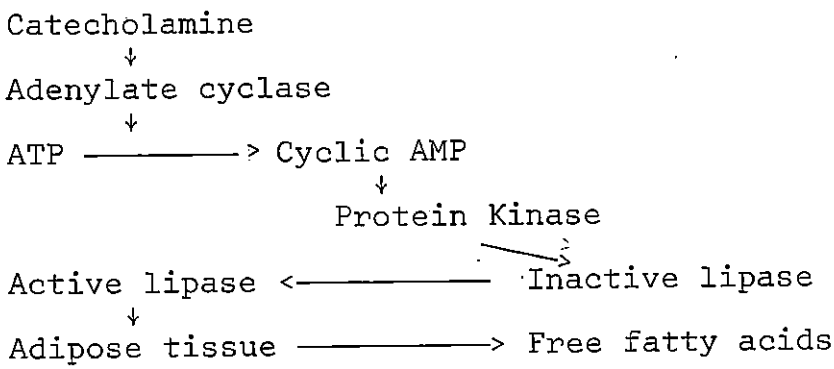

Figure 1. Mechanism by which catecholamines increase the activity of lipase in adipose tissue. Lundholm, et. al. (1978)

Table 1. Serum Cholesterol and HDL Levels.

Treatment	Serum Cholesterol Levels in mg %	Serum HDL Levels in mg %
Control (Group 1)	79.00 ± 9.57^{b}	22.33 ± 2.24^{b}
Rutin injections without abdominal denudation (Group 2)	104.00 ± 22.20^{a}	16.93 ± 3.50^{a}
Rutin injections with abdominal denudation (Group 3)	115.86 ± 22.90^{a}	13.66 ± 3.05^{a}
Abdominal denudation without rutin injections (Group 4)	77.80 ± 6.56^{b}	20.86 ± 2.35^{b}

Mean values with different superscripts are significantly different at $p < 01$.

It is possible that rutin may act in a similar manner in causing the release of free fatty acids. Increases in fatty acid levels may lead to excessive levels of serum cholesterol. Increases in serum cholesterol are in agreement with the findings of Devi, et. al. (1975), and Kannel, et. al. (1979), who both observed that cigarette smokers had significantly higher levels of serum cholesterol than nonsmokers.

It has been proposed that atherosclerosis has its source and origin in segregated areas of altered vacular permeability (Sarma, et. al. 1975). Becker and Dubin (1977), hypothesized that rutin could cause a localized alteration in vascular permeability associated with kinin release. With increased cholesterol levels due to the presence of rutin, there may be an increase in cholesterol permeating through a segment of the arterial wall. The infiltration of cholesterol into the arterial wall leads to the relentless growth of the atheromatous plague.

Blood samples were analyzed for HDL levels because recent epidemological studies have established an inverse relationship between decreased HDL and increased risk of coronary heart disease. Swine receiving rutin injections had decreased levels of HDL, (Table I). Garrison, et. al. (1978), indicated that smokers have lower HDL levels than nonsmokers. A decrease in HDL levels may interfere with cholesterol being removed from the arterial wall. This decrease in HDL levels, as a result of the action of rutin,

may be a critical factor in determining a lesion formation. A hypercholesteremic state may further enhance lesion formation.

Specimens of abdominal aortae from Groups 1, 2, 3, and 4 were cut longitudinally and examined, using a dissecting microscope, for evidence of gross morphological changes. The examinations failed to reveal any grossly visible atherosclerotic lesions in any of the groups.

After staining with hematoxylin and eosin, the aortae were microscopically observed for evidence of disruption in the endothelium and for proliferation of smooth muscle cells in the intimal and medial layers.

Swine receiving daily rutin injections without denudation failed to show evidence of endothelial damage or smooth muscle cell proliferation (Figure 2). Swine receiving daily rutin injections with abdominal denudation showed a removal of the endothelial layer, but no significant thickening of the intimal and medial layers were observed (Figure 3). Swine receiving denudation procedures without rutin injections showed evidence of the disruption of the endothelial barrier (Figure 3); small patches of endothelial cells were detached from the intimal layer. Two factors may be involved in the mechanical injury induced by balloon caterization: (a) An overstretching of the vascular connective tissue, particularly the smooth muscle cells, and the elastic fibers. (b) The intimal layer may be damaged by the

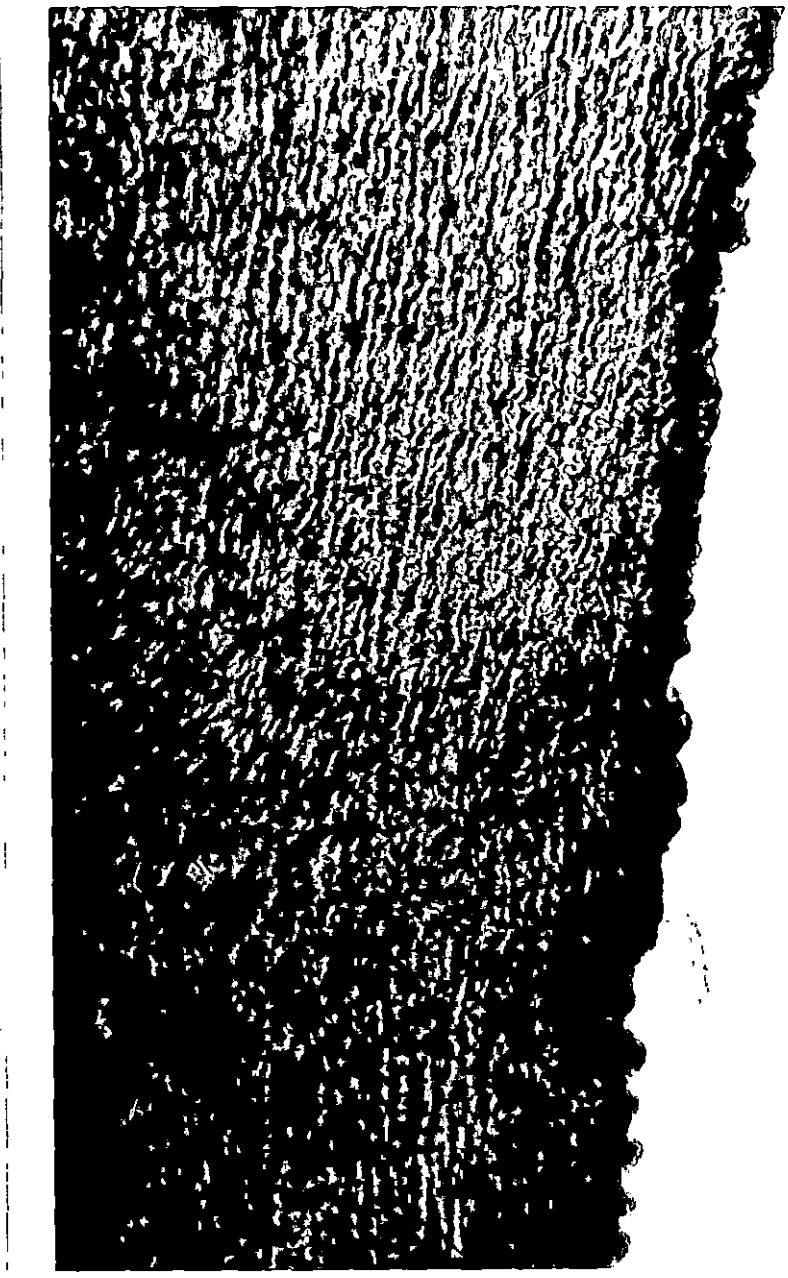

Figure 2. Photomicrograph of histological specimen from swine in which the aorta did not receive the denudation procedure.

Figure 3. Photomicrograph of histological specimen from swine that received the denudation procedure.

pressure and the friction of the balloon on the endothelial cells (Moon, 1976).

The failure of the rutin injections or the denudation to cause significant thickening of the intimal and medial layers may be explained in two ways. Rutin injections were only given for 45 days. If dosage levels were increased and given several times daily, a greater physiological effect possibly would occur. Studies by Burrows and Griffith (1977) revealed that rutin was excreted from the body with urine within four hours after it was introduced. Nam (1973) observed that the atherosclerotic lesion formation was greatly enhanced by the feeding of a hypercholesteremic diet in conjunction with denudation. Denudation procedures alone, failed to reveal a noticeable lesion until after the initiation of the procedure.

It is known that atherosclerosis is a disease of multiple etiology. This study indicates that rutin produced changes in swine that increase the risk for atherosclerosis. Those changes were an increase in serum cholesterol levels and a decrease in HDL levels.

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