THE EFFECT OF QUERCETIN-3-β-RUTINOSIDE ON BLOOD COAGULATION AND THE DEVELOPMENT OF ATHEROSCLEROTIC LESIONS IN SWINE

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ABSTRACT

The effect of quercetin-3-β-rutinoside (rutin) was studied in domestic swine for changes in blood clotting times, fibrinolysis times, and platelet counts. The swine were divided into four experimental groups: (1) Control group (2) Swine which received daily rutin injections, (3) Swine which received daily rutin injections and underwent denudation of the abdominal aorta and (4) Swine which received only aortic denudation. Histological sections of the subjects were made 45 days after the denudation process. The sections were examined for the development of atherosclerotic lesions. Weekly blood samples were measured for partial thromboplastin times (PTT), euglobulin lysis times and platelet counts.

It was found that rutin administration showed a decreased mean partial thromboplastin time and a prolonged euglobulin lysis time. Platelet counts remained unchanged from that of the control group. Histological examinations revealed no early lesion development in the group which only received rutin. Slight removal of the endothelium was observed in subjects which had received the denudation procedures. However, no smooth muscle cell proliferation or lesion formation was detected in these animals.

In conclusion, this study indicated a statistical significance between the normal controls and those which received rutin. This suggests that rutin may cause an accelerated clotting time and a prolonged fibrinolytic process, thus increasing the risk of formation of atherosclerotic lesions.

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INTRODUCTION

Atherosclerosis is a disease of the large and medium arteries and is characterized by the thickening of the inner portion of the artery. It is believed to be the number one cause of death in humans, with the possible exception of cancer (National Institute of Health, 1971). Atherosclerosis is the major cause of myocardial infarction, cerebral thrombosis, and other serious illnesses which involves the blood vessels. The major concern with atherosclerosis is that it is allowed to progress to an advanced stage before developing any noticeable symptoms.

The inner most wall of the artery is called the intima. It consists of the narrow region bounded on the lumenal side by a single, continuous layer of endothelial cells, and externally by a sheet of elastic fibers. The middle layer of the artery consists of smooth muscle cells surrounded by collagen, elastic fibers, and mucopolysaccharides. The outer-most layer, the adventitia, consists principally of fibroblasts, intermixed with smooth muscle cells loosely arranged between bundles of collagen, and surrounded by mucopolysaccharides.

In arteries with initial symptoms of atherogenesis, the intimal cell layer is principally involved, although secondary changes are occasionally found in the media. Three different lesion types are found; the fibrous plaque, the fatty streak, and the complicated lesion (Geer, McGill, and Strong, 1968).

The fatty streak is commonly found in young persons and is characterized by focal accumulation of relatively small numbers of intimal smooth muscle cells, containing and surrounded by deposits of lipid-like material. Hollander and Kramsh (1967), and Scott and Hurley (1970) associated the yellow color of these lesions to the presence of the lipid deposits around them. Most of these lipids were found in the form of cholesterol and cholesterol esters.

The fibrous plaque is characteristic of the advanced atherosclerotic lesion. It is characterized by a whitish appearance caused by lipid accumulation and is elevated so that it protrudes into the lumen of the artery (Geer and McGill, 1968). It consists of an accumulation of lipids around the intimal, smooth muscle cells. These cells are also surrounded by collagen, elastic fibers, and mucopolysaccharides.

The complicated lesion appears as a fibrous plaque that has become altered as a result of hemorrhage, calcification, cell necrosis, and thrombosis (Geer and

McGill, 1968). This type of lesion becomes associated with occulsive arterial disease.

One theory of atherogenesis is the response to injury hypothesis, which was formulated by Ross and Glomset (1976). Ross and Glomset noticed that after injury to the artery, platelets aggregated at the injured site, possibly allowing an unknown factor to stimulate the proliferation of the smooth muscle cells. Lorenzen, Garbarsch, and Mathessen (1971) found that within two weeks after a short lasting dilation of an artery the animal developed gross atherosclerotic lesions.

Jorgensen and Mustard (1974) histochemically determined that at the site of injury there was an increase in the space between the endothelial cells which allowed for the proliferation of the smooth muscle cells. Platelet aggregation was also observed in the region of lesion formation. This is supported by the findings of Nam, et. al. (1968,1976).

Atherogenic lesions are thought to result from injury which may be caused by hemodynamic force, smoking (Doyle, 1964), hyperlipidemia (Ross and Glomset, 1976), and hypertension (Baker, 1969). Tobacco smoking was associated with the incidence of myocardial infarction by Doyle (1964). He found that men who smoked more than twenty cigarettes per day experienced a risk of myocardial infarction and death three times higher than that of non-smokers. Myocardial infarction is caused by an

occulsion of the blood vessels leading to the heart.

Engelberg (1965) found that thrombosis formation was accelerated in smoking subjects as compared to non-smoking subjects. This platelet aggregation produced some type of injury to the blood vessel wall as shown by (Jorgesen, et. al. 1974). If the lesion is of sufficient size, and enough platelets aggregate to form a thrombosis, then the section of the coronary artery is blocked and those areas of the heart will receive no nutrients, and therefore die.

Engleberg (1975) also noted that the thrombus formation time of the blood was decreased soon after cigarette smoking. He suggested that thrombotic tendency is mediated by a substance found in the tobacco smoke which causes the release of clotting factors. Engelberg felt that the release of the substance was mediated by nicotine, which stimulated the release of epinephrine, which caused the accelerated response of the coagulation process.

Experiments by Ogston, et. al. (1969), Pozner, et. al. (1974), and Logan and Gerinn (1967) indicated that subjects who smoked showed increased plasma fibrinogen levels . Increased fibrinogen levels increased the acceleration of the clotting time. This is also supported by Nam, et. al. (1968). In animal subjects, Moscho, et. al. (1975), found that animals exposed to cigarette smoke had a significantly enhanced coagulation mechanism and an

increased platelet count.

Hasker and Slicter (1972), and Levine (1973), found that platelet aggregation is increased in heavy smokers, and aggregation also varies, depending on emotional stress, exercise, and elevated blood lipid levels. They felt that the normal hemostatic mechanism is designed to be activated by vascular injury. This response involves the interaction of vascular endothelium and platelets to form a hemostatic plug by fibrin stabilizations. This pathway is summarized in Figure 1.

Holeman (1963), studies the effect of blood vessel occulsion and the fibrolytic activity of the coagulation system. He found that the fibrolytic activity was stinulated in response to the clot formation. He observed through experimentation that there was an increase in the euglobulin fraction of the blood. Holeman also noted that plasminogen is converted to plasmin when a thrombus is produced which induces eventual fibrinolysis on the clot. The conversion scheme is illustrated in Figure 2.

Nicotine is thought to produce most of the biological reactions in tobacco condensate. Other compounds in tobacco smoke condensate are also biologically reactive in cigarette smoke. Becker and Dubin (1977), found a tobacco glycoprotein containing rutin (quercetin-3-β-rutinoside), which has a biological effect on coagulation.

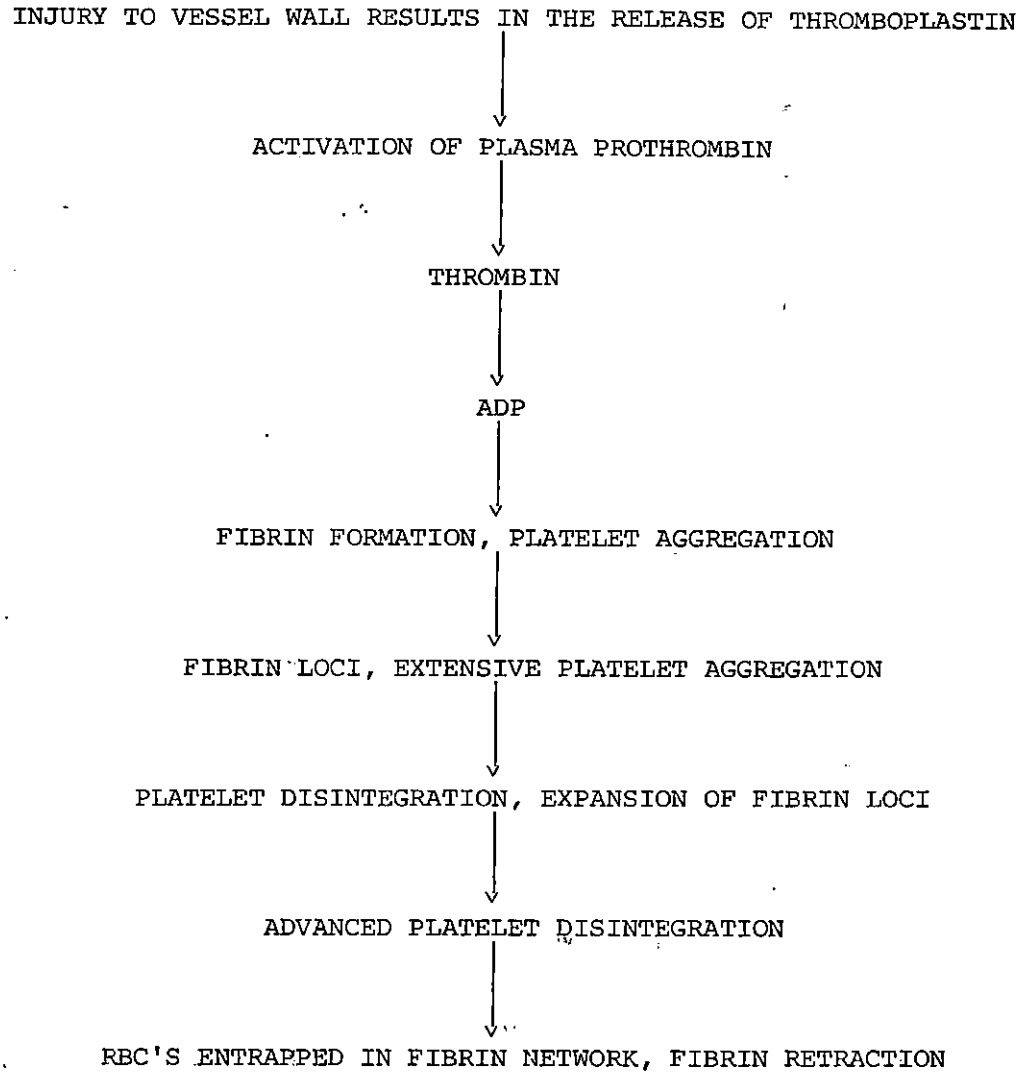

Figure 1. Fibrin clot formation with respect to platelet aggregation (Seeyers, 1968).

The experiments by Becker and Dubin indicated that rutin activates the Hagman Factor (Factor XII) of blood plasma which stimulates coagulation activity and kinin generation. Partial thromboplastin time (PTT) and euglobulin determination are measurements of coagulation and fibrolytic activities. A decrease in the partial thromboplastin time and a decrease of the euglobulin determination in the blood by rutin may result in the formation of the thrombosis, and possibly a change in the endothelial permeability which may produce an atherogeneic lesion.

This investigation proposed to study the role of rutin, and the coagulation process using swine as an experimental model, in the production and development of atherosclerotic lesions. The swine were divided into four groups:

Group 1. Controls which receive sham operative procedures.

Group 2. Swine which received daily injections of rutin, but did not undergo the denudation procedure.

Group 3. Swine which received surgical denudation of the abdominal aorta and daily rutin injections.

Group 4. Swine which received the denudation procedure, but were not administered rutin injections.

The denudation procedure was used to activate the response of the tissue to injury as proposed by Ross and Glomset (1976).

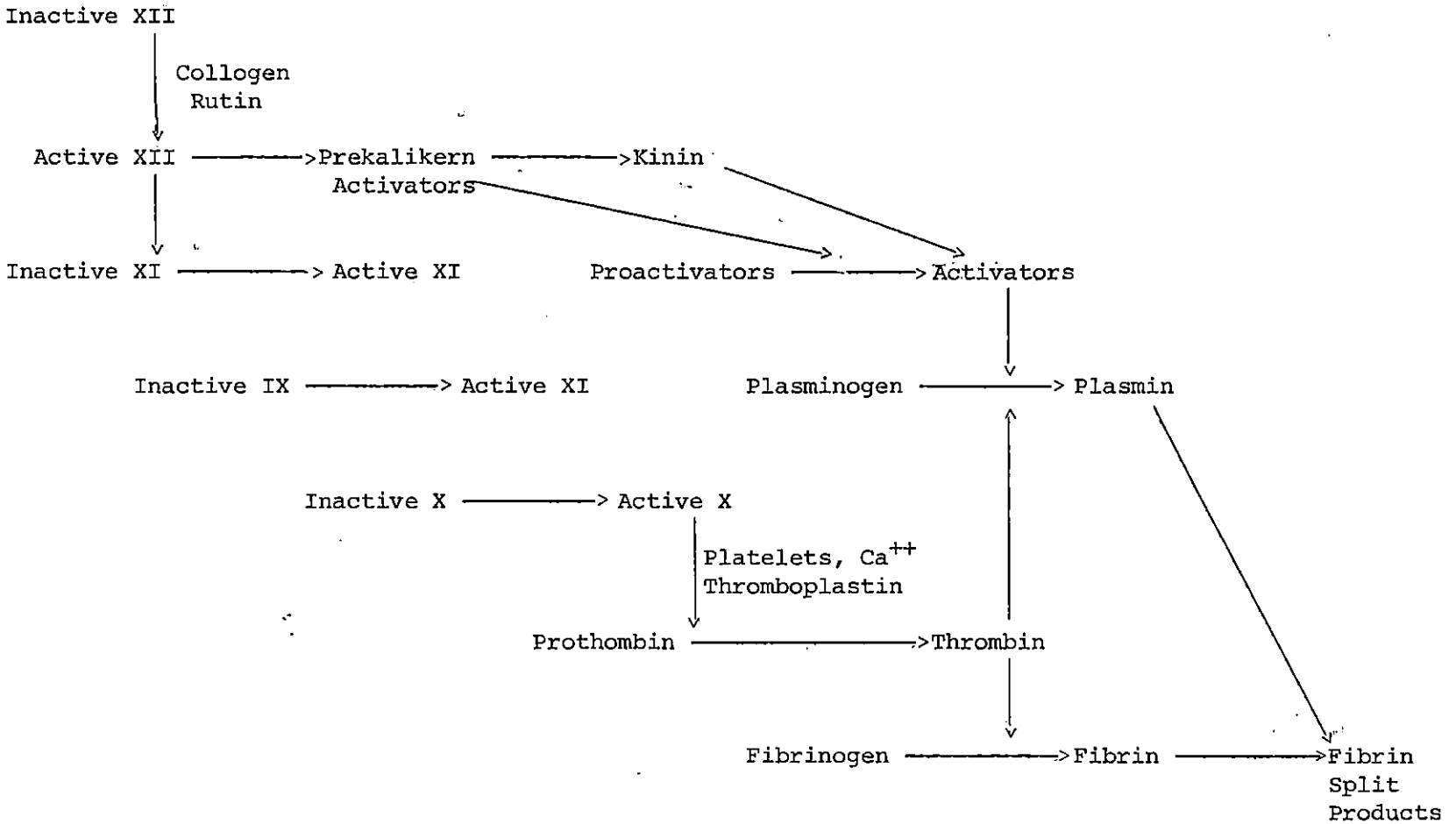

Figure 2. Routes of coagulation and fibrinolysis.

Blood samples were taken each week in order to study the effect of rutin on the blood coagulation system and the fibrinolytic system. The factors measured were the partial thromboplastin times (PTT), the euglobulin lysis times, and platelet counts.

After a forty five day period the swine were sacrificed and the abdominal aortas were extracted for histological studies. The histological sections were examined, by staining them with hematoxylin and eosin stain to delineate smooth muscle cells and arterial layer changes indicative of the development of early atherosclerotic lesions.

MATERIALS AND METHODS

Animal Care

In this study male, 6 to 12 weeks old, Yorkshire swine, fed a normal diet were used. Swine were selected as a model for this study because according to Ratclife and Lugiguhl (1971) the domestic swine are an excellent model for research of this type. Swine are anatomically similar to humans in the origin and distribution of blood vessels. Physiologically, domestic swine are also much like humans in their metabolic activities. The swine may also be subjected to stress in much the same way as humans.

Twelve swine were used for this study. They were divided into four study groups, with each group containing three swine. Group 1 contained swine which were sham controls. Group contained swine which received surgical denudation of the abdominal aorta and received daily injections of rutin for forty five days. Group 3 contained swine which had undergone the denudation procedures, but had not received rutin injections. Group 4 did not receive arterial denudation, but received daily injections of a rutin solution.

Surgical Procedures

All animals were subjected to routine surgical procedures and precautions. Groups 1 and 2, received sham procedures in which the femoral artery was isolated and ligated. Groups 3 and 4 received the same surgical procedure as Group 1 and 2 except that was was accompanied by arterial denudation.

The swine were anesthetized by the inhalation of chloroform. The surgical areas were shaved, and cleansed with laboratory green soap solution. The operative procedure involved making an incision through the skin and exposing the femoral artery. Once the femoral artery was well exposed, and isolated from the surrounding connective tissue a metal plate was placed under the artery. The artery was then clamped at two sites, and an incision was made in the artery between the clamped sites. A Forgarty 4 F embolectomy catheter (V. F. Muller Co.) was inserted and passed into the abdominal aorta, 20 centimeters from the point of incision, after the removal of the superior clamp. When the catheter was in the abdominal aorta it was inflated with 0.75 mL of saline to produce resistance to the movement of the catheter. The catheter was then moved superiorly and inferiorly within the artery for 10 seconds, to insure injury to the endothelial lining of the aorta. The catheter was removed and the femoral artery was

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ligated with surgical suture. The outer wound was sutured and clamped with animal wound clips to insure closure of the wound healed that suggested by Nam (1972).

The animals were allowed to recover in the laboratory before they were transported to the farm after the operative procedure. The swine were then injected with a penicillin solution, 12,500 units per mL to prevent infection.

Rutin Injections

One day after the surgical procedures, daily rutin injections were begun. A stock rutin solution was prepared in a concentration of 10 gm. per mL. of propylene glycol. The control groups received daily injections of propylene glycol. The amount of rutin which the swine received was based on the following data, (Saxon, 1978, Reese, 1978):

a. A 1 g cigarette contains 0.86 g of tobacco.

b. 55 mm of an 85 mm cigarette is smoked, equaling 6.56 g of tobacco.

c. 0.8% of tobacco is rutin, equaling 4.3 mg of rutin per smoked cigarette.

d. 15% of rutin is transferred from tobacco into tobacco condensate. This 15% transfer rate is based on the 5% to 25% transfer rate for scopoletin, a related chemical. 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.

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

g. 20 cigarettes per day is equal to 2.2 ug of rutin per mL of blood.

Swine in groups 2 and 3 were administered 2.2 ug of rutin per mL of blood. The volume of blood for each animal was determined by the body mass and 8.3% of the body mass is blood. The injections were given intramuscularlly each day for forty-five days.

Obtaining Blood for Testing

Weekly blood samples were taken from the swine for partial thromboplastin times (PTT), euglobulin lysis times, and platelet counts. The blood samples were obtained from the anterior vena cava with an 18 gauge, 4 inch needle inserted via the thoracic inlet. The blood, when obtained, was placed in vials containing a 3.8% sodium citrate solution. This anticoagulant binds with the calcium to prevent the blood from clotting. The ratio of sodium citrate used was one part to nine parts blood.

The samples were then placed in a cold environment (2-8°C) until tested. The samples were centrifuged for 15 minutes at 1500 rpm to separate the plasma and the cellular blood components. The plasma was then transferred to another tube.

Partial Thromboplastin Time

The partial thromboplastin time (PTT), is a test designed to measure the clotting factors in the intrinsic pathway of coagulation, with the exception of platelet factor III. The PTT test procedure used was the tilt tube technique. Plasma was incubated in a 37°C water bath for 5 minutes, then 0.1 mL of test plasma and p.1 mL of prewarmed Thrombofax reagent (Ortho Diagnostics Co.) was added and placed in a 37°C water bath for thirty seconds. While maintaining the tube in the water bath, 0.1 mL of $Cacl_2$, (0.02 M), (Ortho Diagnostics Co.) reagent was added to the plasma-Thrombofax mixture. A stopwatch was started simultaneously with the addition of the calcium chloride and this mixture was incubated for one minute. Then the tube was removed from the water bath, and gently tilted back and forth until a fibrin strand appears. The time elapsed was recorded as the PTT clotting time (Issacc, 1964).

Euglobulin Lysis Time

The euglobulin lysis test was utilized to evaluate the systemic fibrinolysis. The euglobulin fraction of the plasma, prepared by the dilution of the acidification of plasma, is relatively free of inhibitors of the fibrinolytic enzyme system, and therefore lysis of the clot formed from the fibrinogen in the euglobulin

precipitate occurs rapidly. The time for the clot to lyse is the euglobulin lysis time.

The Data-Fi kit (Dade Co.) was used for the euglobulin lysis test. A 0.5 mL sample of plasma was placed in a test tube and 6 mL of a 1% acetic acid solution was added to the test samples. The tubes were then placed in a 2-°C environment to precipitate the euglobulin fraction. After refrigeration for ten minutes the tubes were centrifuged at 3,000 rpm for 3 minutes. The supernatant was discarded and the clot fraction retained. A 0.35 mL volume of phosphate buffered saline, pH 7.35, was added to the sediment, then shaken gently until the sediment was dissolved. Using a separate pipet for each tube, 0.025 mL of buffered thrombin (Dade Co.) was added. A clot then formed and the tube was placed in a 37°C water bath. The time recorded, in minutes, for the clot to completely lyse was the euglobulin lysis time.

Platelet Counts

The platelet count was used to estimate the number of platelets in the perpherial circulation. A RBC pipet was filled to the 0.5 mark with blood and then the pipet was filled with diluting fluid, Platecount, (Scientific Products), to the 101 mark. The blood-Platecount mixture was maintained for ten minutes to insure complete hemolysis. After ten minutes a hemocytometer chamber was filled with the blood-Platecount solution. The

platelets were counted with a microscope at 400 x magnification. The small RBC portion of the grid was used to count the platelets.

Histological Study

At the end of the 45 day period the swine were sacrificed and the abdominal aortas were removed. The extracted arterial sections were then placed in a formalin fixitive. The sections were then dehydrated using a series of alcohol solutions and xylene. Next, the tissue was embedded in Histowax and sectioned with a microtome into 8 micron sections. The tissue was stained using a modified hematoxylin and eosin staining procedure used by Nam (1972). The modification was made by using a Harris stain. The modified Harris stain produced a sharp delineation of the smooth muscle cells, and the layers of the arterial section.

The staining method is indicated as follows:

1. Xylene - 1 minute
2. Xylene - 2 minutes
3. 100% alcohol - 2 munutes
4. 95% alcohol - 1 minute
5. 70% alcohol - 1 minute
6. 50% alcohol - 1 minute
7. Rinse in water
8. Hematoxylin - 45 seconds
9. Rinse in water

10. Eosin - 30 seconds
11. Rinse in water
12. 50% alcohol - 1 minute
13. 70% alcohol - 1 minute
14. 95% alcohol - 1 minute
15. 100% alcohol - 1 minute
16. Xylene - 1 minute

The sections were then examined under the microscope for evidence of arterial wall differences in each group of the animals.

RESULTS AND DISCUSSION

Weekly blood samples revealed a decrease in the mean PTT in the groups which received daily rutin injections as compared to the control group (Figure 3). Groups 2 and 3 showed a decrease in PTT clotting times, 53.6 $\pm$ 8.9 seconds, and 59.3 $\pm$ 1.0 seconds respectively, as compared to the control group of 66.3 $\pm$ 11.9 seconds. Group 4, which received the denudation procedure and was not administered daily rutin injections, had partial thromboplastin times of 64.3 $\pm$ 6.0 seconds. Thus, denudation procedures alone do not seem to play a role in the acceleration of the partial thromboplastin time.

Similar investigations by Becker and Dublin (1976) also found that rutin decreased the PTT. They thought that this decrease in the PTT was due to rutin's ability to stimulate the Hagman Factor. The Hagman Factor is the first step of the coagulation system which, when stimulated, causes a cascading effect to activate the entire system. Donaldson and Ratinoff (1965), showed that ellagic acid, a molecule which is structurally like rutin, is a known accelerator of the Hagman Factor. It is therefore thought that rutin may behave in such a manner as ellagic acid.

The Hagman Factor was found to be activated by the blood's exposure to collagen (Wildner, Nossel, and LeRoy 1968). This also supported the results of the study in

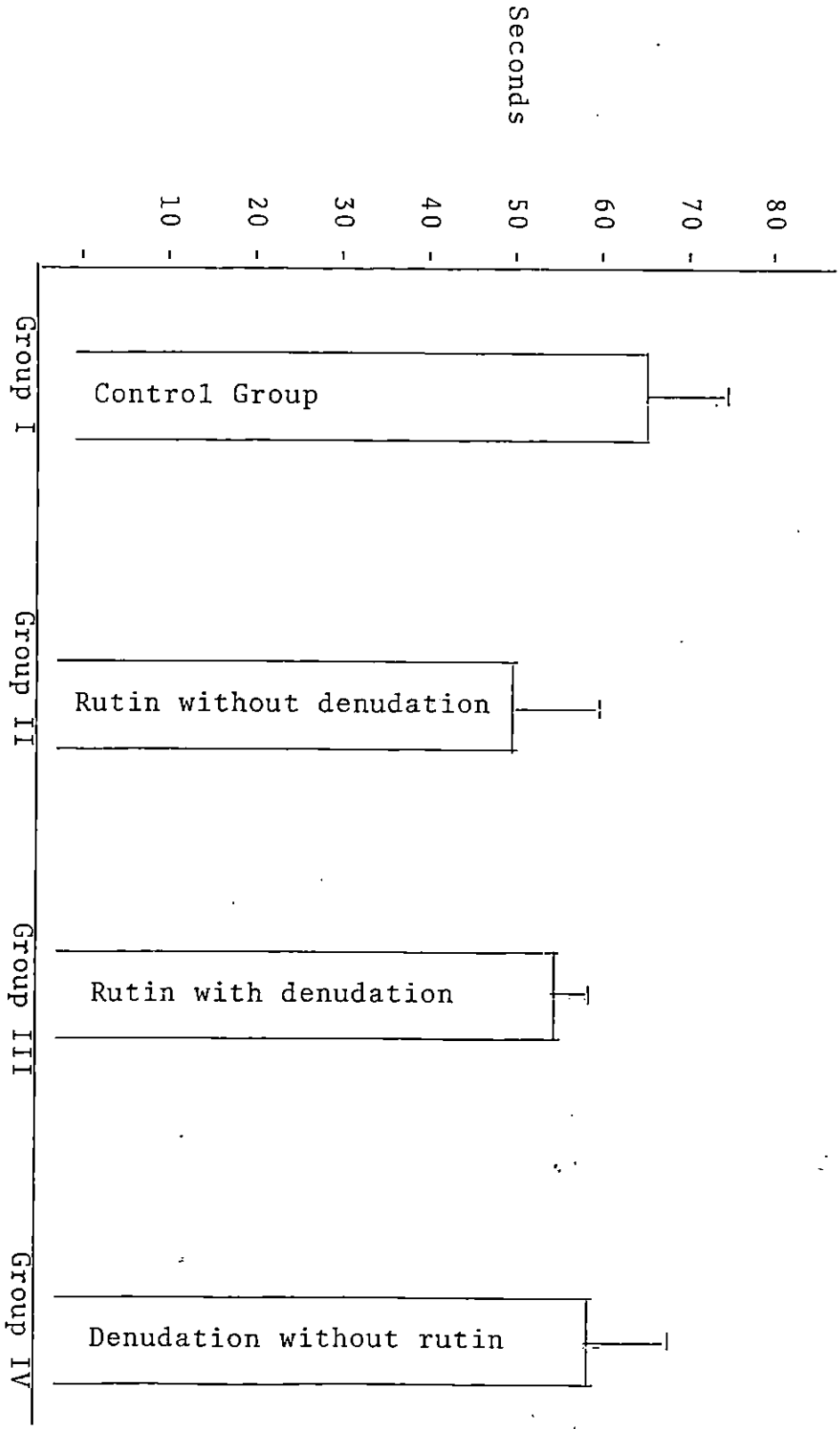

Figure 3. Mean Partial Thromboplastin Times in Swine.

that the mean PTT values of Group 4 were also decreased. The denudation procedure removed the endothelial tissue from a given area and exposed the collagen of the arterial wall to Factor XII present in the blood.

Euglobulin fraction determinations performed on the blood samples from test animals showed prolonged euglobulin lysis times as compared to 130 minutes ± 32 value for the control group (Figure 4). This data suggests that rutin alone caused a prolongation of the euglobulin fraction as is evidenced by the 278 minutes ± 21.3 of Group 2. Group 4, in which each test animal's abdominal aorta was denuded, also showed an increased euglobulin lysis time of 394.2 minutes ± 127 compared to the control values of 130 minutes ± 32. The results from Group 2 and 4 suggest that rutin does not exclusively cause such a prolongation, but the induced aortic injury may also cause the same results in fibrinolytic activity. Similar results were obtained by the experiments of Becker and Dubin (1976). They accredited the prolongation of fibrinolytic activity to rutin increasing the release of kinin from the arterial smooth muscle cells. The increase in kinin levels is currently thought to inactivate the enzymes of the fibrinolytic system.

Platelet counts were also performed on the test subjects. No changes were seen in peripherial platelet counts except immediately after surgery, however, this

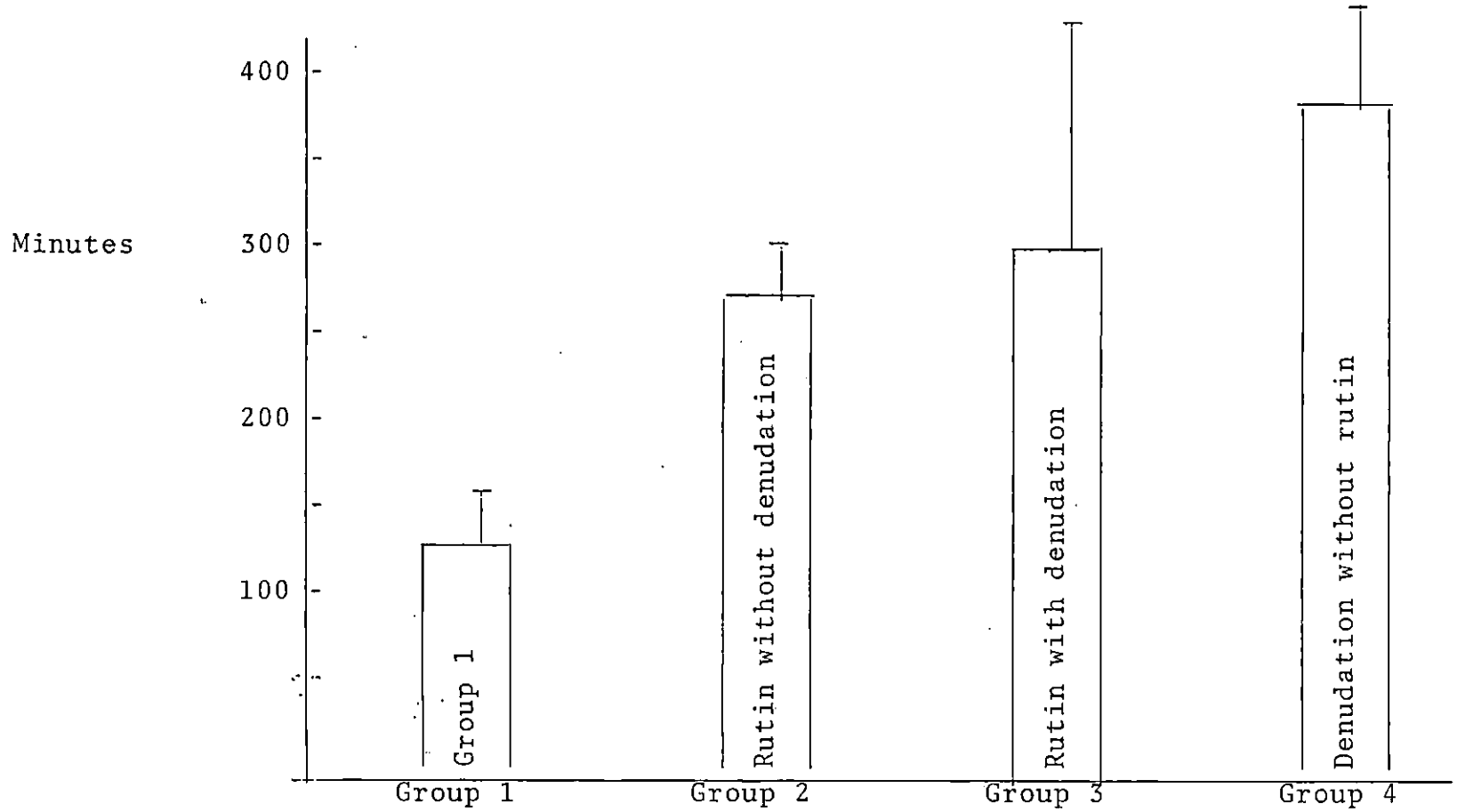

Figure 4. Mean Euglobulin Lysis Times in Swine.

is thought to be caused by the surgical procedure, since this was seen in all groups including the control subjects (Table I). Ross and Glomset (1976), theorized that when injury was induced upon the arterial tissue, platelets aggregated in the area of the injury. They believed that platelets carry an unknown platelet factor which is responsible for the growth and proliferation of the smooth muscle cells.

Gross examination of the histological sections showed no noticeable lesion formation of lipid accumulation (Figure 5 and 6). Upon staining the aortic tissue, the sections of Group 2 showed no endothelial damage or smooth muscle proliferation. Specimens of Group 3 and Group 4, which were obtained from denuded subjects, showed a removal of the endothelial layer, but failed to reveal any smooth muscle cell proliferation. No platelet aggregation was seen in the histological sections to indicate the formation of a thrombosis along the lumenal surface of the artery. Studies by Pillner (1976), showed that the administration of rutin to test subjects caused an injury response to endothelial tissue. Pillner theorized that the injurious result may be due to an increase in intravascular pressure and flow. This increase in vascular flow and pressure caused kinin release, which normally increases the activation of the enzymes responsible for the fibrinolytic system.

Table I. Platelet counts in swine.

Group	Mean platelets counts before surgical procedures ($/mm^3$)	Mean platelet count after surgical procedures ($/mm^3$)
Control 1	635,200	642,700
Rutin without denudation 2	622,500	625,000
Rutin with denudation 3	579,475	575,250
Denudation without rutin 4	632,250	629,500

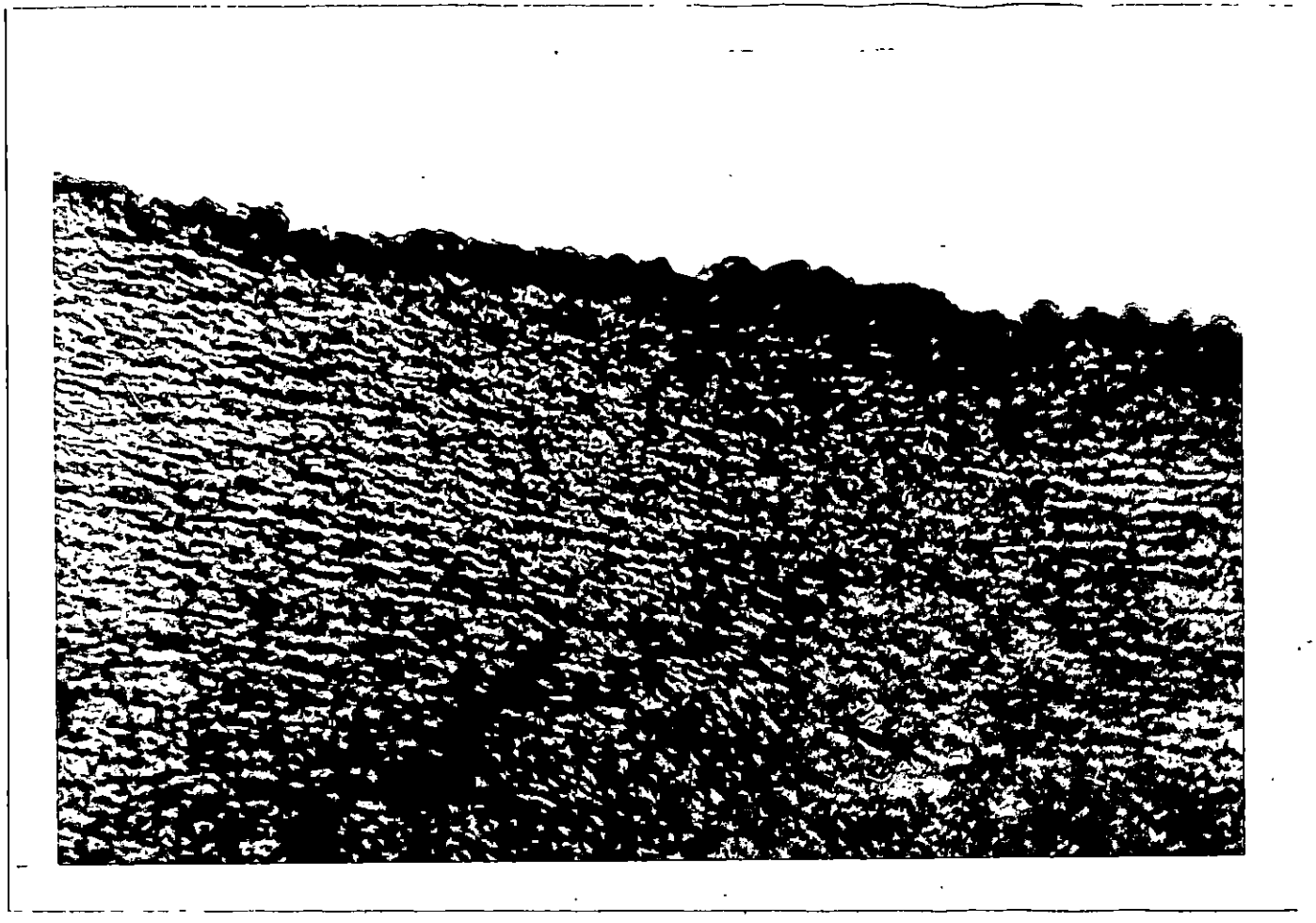

Figure 5. Photomicrograph of the abdominal aorta of the control group.

Figure 6. Photomicrograph of the abdominal aorta of the denuded artery.

Nam (1973), showed that an atherosclerotic lesion could be formed in test animals within 45 days after denudation with a high cholesterol diet. This study used animals on a normal diet, and no atherogenic lesions were seen after denudation.

Rutin is a polyphenolic glycoside which gives tobacco its yellow color. It has a molecular weight of 610 a.m.u. may freely pass into the blood system from the lungs, Barrows and Griffith (1978). Experiments by Barrows and Griffith (1967), showed that rutin was excreted from the body within four hours after it was introduced via smoke condensate. The molecule was excreted virtually unchanged. They believed that rutin may not remain in the body long enough to produce a morphological change to the arterial wall, but the four hour period may be long enough to produce a physiological change. This physiological change may cause kinin production, accelerated PTT's and prolonged euglobulin lysis times.

This investigation revealed an elevation in the mean PTT and a prolongation of the mean euglobulin lysis times (Table II). This was shown to be statistically significant at the .05 level. Platelet levels remained constant, with each of the four groups showing that in this study, platelets seemed to play no role in the atherogenic process. However, the change in PTT and

euglobulin lysis times may be prerequisital to the formation of atherosclerotic lesions. Rutin injections given several times daily for a more prolonged period could enhance lesion formation.

Table II. Partial thromboplastin times and euglobulin lysis times in test animals.

Groups	Mean PTT (sec.)	Std. Dev.	Mean Euglobulin Lysis Times (min.)	Std. Dev.
1 Control	66.3_a	±11.9	130_a	± 32
2 Rutin without denudation	53.6_b	± 8.9	278_b	± 21.3
3 Rutin with denudation	59.3_b	± 1.0	364.5_c	± 143
4 Denudation without rutin	64.3_a	± 6.0	394.2_c	± 127

Mean values with different subscripts are significantly different at $p < .05$.

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