THE EFFECT OF QUERCETIN RUTINOSIDE ON EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN RABBITS

by

DUNCAN SCHWARZ

August 1988

Department of Pathology
McGill University,
Montreal

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

EFFECT OF QUERCETIN RUTINOSIDE ON EXPERIMENTAL ATHEROSCLEROSIS

TABLE OF CONTENTS

ABSTRACT

Quercetin Rutinoside, commonly known as rutin, is a polyphenol molecule that is present in tobacco, cigarette smoke, cigarette smoke condensate and many edible plants consumed in man's daily diet (asparagus, buckwheat, apples, etc.). It has been reported to stimulate smooth muscle cell proliferation *in vitro*. Quercetin rutinoside has been suggested as one possible risk factor for increased atherosclerosis and coronary artery disease in smokers. This study tests the ability of quercetin rutinoside to modify experimental cholesterol atherosclerosis in rabbits. Fifty five white male New Zealand rabbits were fed a Purina rabbit chow diet rich in cholesterol, peanut oil and BHA (butylated hydroxyanisole) to produce a myogenic type cholesterol atherosclerosis. Fifteen rabbits were fed this diet and acted as controls while forty were fed the control diet plus 2 g of quercetin rutinoside per day for 14 weeks. Aortic atherosclerosis containing a high proportion of myogenic type, lipid laden foam cells was produced in both groups. The extent of aortic atherosclerosis expressed as a percent of the total aortic intimal area covered with visible gross lesion was assessed in both groups. Cell proliferation (DNA synthesis) was assessed by determining the incorporation of tritiated thymidine into newly synthesized DNA of proliferating cells. There were no significant differences between groups with respect to tritiated DNA synthesis as an index of cell proliferation and aortic or pulmonary cholesterol atherosclerosis. It is concluded that quercetin rutinoside does not modify this type of atherosclerosis, but this does not preclude the possibility that quercetin rutinoside may modify other types of atherosclerosis [82].

RESUME

Le "Quercetin Rutinoside", communément appelé "rutin" est une molécule polyphénique qui est présente dans le tabac, la fumée de cigarette et la fumée condensée de celle-ci. Il est rapporté que le "quercetin rutinoside" stimule la prolifération cellulaire des muscles lisses *in vitro*. On suggère donc que le "quercetin rutinoside" pourrait être un facteur responsable de la sévérité plus élevée de l'athérosclérose et des maladies de l'artère coronaire chez les fumeurs. La présente étude vérifie l'habileté du "quercetin rutinoside" à modifier l'athérosclérose cholestérolique expérimentale ches les lapins. Cinquante-cinq lapins blancs, mâles, de la Nouvelle Zélande ont été nourris une diète au cholestérol et à l'huile d'arachide afin de produire une athérosclérose cholestérolique de type myogène. Quinze de ces lapins, qui ont été nourris cette diète, représentaient un groupe contrôle, tandis que quarante ont été nourris la diète contrôle et 2 g de "quercetin rutinoside" par jour, pendant 14 semaines. L'athérosclérose aortique produite contenait une proportion élevée de cellules spumeuses lipides, de type myogène. Le degré de l'athérosclérose aortique exprimée selon le pourcentage de la surface totale de l'intima de l'aorte recouverte par les lésions macroscopiques a été évalué chez les deux groupes. La prolifération cellulaire (synthèse de l'ADN) a été évaluée en déterminant l'incorporation de la thymidine tritiée dans l'ADN, nouvellement synthétisée des cellules proliférées. Il n'y avait aucune différence entre les 2 groupes quant au pourcentage de la surface recouverte par les lésions et la valeur de synthèse de l'ADN tritié (prolifération cellulaire). On en conclut donc que le "quercetin rutinoside" ne modifie pas ce type d'athérosclérose mais ceci n'empêche pas la possibilité que le "quercetin rutinoside" puisse modifier d'autres types d'athérosclérose [82].

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor, Dr. B.I. Weigensberg for giving me the opportunity to work in his laboratory and for his continued guidance, encouragement and penetrating criticism. Special thanks to Dr. Sean Moore (thesis advisor) and Dr. Edith Zorychta (chairperson of graduate studies) for giving invaluable advice concerning thesis preparation. I would also like to express my sincere thanks to the following for their technical assistance: Mrs. M. Giannetti (cholesterol determinations), Mr. W. Kingsley (preparation of the diets), Mrs. L. Renaud (DNA determination) and Mr. M. Lopez (feeding animals and performing autopsies). I am also grateful to Dr. M.Z. Alavi, Mr. S. Berezowski, Dr. W. Weiss and Dr. L. Vitou for translating articles in German, Polish, Italian and French. Dr. H. Robson and Dr. D. Briedis from the Department of Microbiology together with Dr. J. Fitzgerald of the Village Veterinary Clinic gave invaluable information concerning the possible treatment of the experimental animals with antibiotics. Dr. Mike Vassyadi and Dr. Louise Vitou were kind enough to read drafts and suggested improvements to the thesis. Many thanks to Mrs. L. Marshall and Ms. J. Gauthier-Whitham for typing the manuscript.

LIST OF ABBREVIATIONS

TGP	tobacco glycoprotein
PDGF	platelet derived growth factor
TAR	water soluble, nondialyzable, delipidated extract of cigarette smoke condensate
BSA	bovine serum albumin
LDL	low density lipoprotein
DNA	deoxyribonucleic acid
BHA	butylated hydroxyanisole
PTAH	phosphotungstic acid haematoxylin stain
MT	Masson trichrome stain
RPM	revolutions per minute
TCA	trichloroacetic acid
Ci	Curie
SMC	smooth muscle cell
Pulm	pulmonary artery
delip	delipidated
wt	weight
U.S.P.	United States Pharmacopoeia
mg	milligram
ml	millilitre
g	gram
SD	standard deviation
SE	standard error
N	number
kg	kilogram

LIST OF TABLES

LIST OF FIGURES

REVIEW OF THE LITERATURE

The Role of Smooth Muscle Cells in Atherosclerosis

Most major theories of atherogenesis encompass the belief that the lesion is characterized by extensive cellular proliferation in the arterial intima [1,2]. Although atherosclerosis is a disease of the arterial intima, any hypothesis must encompass three crucial features of the advanced lesion:

1. focal cellular proliferation, 2. synthesis of large amounts of connective tissue matrix components, particularly collagen and elastin, but also glycosaminoglycans and proteoglycans, and 3. deposition of intra and extracellular lipid [3,4].

Many but not all major theories of atherogenesis place an overwhelming emphasis on the role of smooth muscle cell accumulation within the arterial intima. Evidence from many sources confer that smooth muscle proliferation determines the extent of fibrous plaque formation, which in turn leads to progressive clinically significant occlusive disease [5]. Without fibrocellular proliferation, clinical sequelae would probably not occur. Since atherosclerosis is a disease involving focal proliferation of cells in the arterial intima, it is of importance to know why smooth muscle cells modulate their phenotype, proliferate and elaborate matrix components.

Smoking and Atherosclerosis: The Role of Quercetin Rutinoside

The National Heart, Lung, and Blood Institute [6] has identified three primary risk factors for atherosclerosis: elevated serum choles-

terol, hypertension and cigarette smoking. Because smoking is an important risk factor in atherosclerosis, there is considerable interest in increasing the understanding of the tobacco constitutents that may be involved and in elucidating their mechanisms of action.

It is still not fully known whether inhaled smoke contains mitogens that could induce medial smooth muscle proliferation or hapten moieties that stimulate specific antibody production resulting in "injury" via immune complexes or release of vasoactive amines. Tobacco constituents capable of inducing somatic cell mitogenesis are potentially fundamental to the pathogenesis of the fibroatheromatous plaque.

Research to elucidate specific compounds acting at the cellular level was carried out by Becker et al. [7]. Tobacco glycoprotein (TGP), a polyphenol rich antigen (purified from flue cured tobacco leaves) was tested *in vitro* for possible mitogenic effects against bovine smooth muscle cells of the arterial wall. TGP is present in cigarette smoke and cigarette smoke condensate [7]. The carbohydrate moiety (glucose and rhamnose), the molecular weight (18000), the iron content, the amino acid sequence and the colour of the TGP correspond to that of an iron-protein-chlorogenic acid - quercetin rutinoside complex present in tobacco as a dark brown pigment [8-10]. This tobacco glycoprotein (pigment complex) can comprise as much as 4% of cigarette smoke condensate [11]. When inhaled, the relatively small size (MW 18000) of the TGP may permit it to cross the thin capillary-alveolar membrane (0.5 microns) and gain access to the circulation. The concentration of such substances in the pulmonary venous blood of cigarette smokers is at present unknown. However, it has been demonstrated that the inhalation

of tar-derived material (TAR) is associated with the activation of Hageman factor (XII) dependent pathways in dogs, indicating that TAR can enter the circulation [12]. Furthermore, quercetin rutinoside alone or coupled to albumin, activated factor XII *in vitro* and *in vivo* [13,14].

Becker et al. [7] reported that concentrations ranging from 25 to 100 ug/ml of tobacco glycoprotein (TGP), TAR (the water soluble, non-dialyzable, delipidated extract of cigarette smoke condensate), quercetin rutinoside - bovine serum albumin conjugates (20 epitopes/mol BSA), quercetin, the aglycone of quercetin rutinoside (Figure 1) and chlorogenic acid are all mitogenic for bovine aortic smooth muscle cells *in vitro* (expressed as percent increase over control) (Table 1). The capacity to be mitogenic together with the ability of TGP to activate factor XII and shorten the partial thromboplastin time [13] is derived from polyphenol epitopes on carrier molecules. The ability of TGP to stimulate smooth muscle cell proliferation indicates that the polyphenol moiety (i.e. quercetin rutinoside) is not only present in molecules of TGP but is arranged in such a way that it is immunologically reactive [13]. Also, the extent of cell proliferation was inversely proportional to the epitope density with lower numbers of epitopes being more mitogenic than higher numbers. From these results, it was speculated by Becker et al. [7] that quercetin rutinoside and epitopes like it, i.e. quercetin, can bind smooth muscle surface receptors inducing them to become cross-linked, resulting in the initiation of mitosis.

Critical Commentary on Becker's Hypothesis and *In Vitro* Work

Since each pack of cigarettes potentially exposes the smoker to approximately 1 mg of tobacco glycoprotein [15] Becker et al. [7] have put

forward the hypothesis that the mitogenic effects of quercetin rutinoside *in vitro* may affect smooth muscle cell proliferation in the arterial intima *in vivo*, thus compounding the growth of pre-existing atherosclerotic lesions. Therefore it becomes of interest to examine the hypothesis carefully and determine if it is realistic. The reported evidence that a tobacco glycoprotein (containing quercetin rutinoside) from smoke can stimulate smooth muscle cell proliferation *in vitro* does not prove that it can stimulate cellular proliferation *in vivo*.

It is not possible to explain the aetiology of increased atherosclerosis associated with smoking simply in terms of *in vitro* cell proliferation. Cell proliferation is only one parameter in the growth of atherosclerotic lesions, and therefore it alone cannot be regarded as the only indicator of atherosclerosis as a whole. Many other factors serve to promote/modify atherogenesis *in vivo*, these include: LDL, PDGF, age, hypertension, diabetes mellitus to name just a few. Yet many questions are raised concerning Becker's hypothesis. Firstly does TGP survive in the systemic circulation long enough without being altered/inactivated by the binding of serum proteins and secondly does it circulate in the blood in sufficient concentration to penetrate or diffuse across the endothelial barrier to modify cell proliferation? It has been shown that TAR can enter the blood stream [12], but is the TGP sufficiently separate from the TAR to be a distinct component, thereby potentially eliciting specific cellular responses on its own?

In view of the fact that Becker and colleagues have put forward the hypothesis that quercetin rutinoside (as part of TGP) is a tobacco constituent which renders smoking a risk factor for atherosclerosis, it be-

comes important to review the evidence carefully. Becker et al. [7] reported that concentrations ranging from 25 to 100 ug/ml of tobacco glycoprotein (TGP), tar derived material (TAR), quercetin rutinoside conjugated with bovine serum albumin (20 epitopes/mol BSA), chlorogenic acid and quercetin (the aglycone of quercetin rutinoside) are mitogenic for bovine aortic smooth muscle cells but not mitogenic for adventitial fibroblasts. A study by Gotzos et al [16] however, reported that quercetin rutinoside in doses of 6.25 ug/ml and 100 ug/ml could induce fibroblasts to proliferate *in vitro* as well as to increase protein synthesis, although the fibroblasts were not bovine in origin. The results indicating that TGP may be a selective mitogen were supported by evidence that this pigment complex is also mitogenic for B lymphocytes, but not T lymphocytes [17]. Furthermore, it was demonstrated by Capelli et al. [18] that smooth muscle cells contained within embryonic arteries of chicks explanted *in vitro* in standard Wolff-Haffen medium in the presence of unconjugated quercetin rutinoside were induced to proliferate. This proliferation over control values resulted in increased muscular and elastic tissue. The observation that quercetin rutinoside induces arterial smooth muscle cells to proliferate was quantified histologically, yet no attempt was made to definitively characterize the degree of cell proliferation or to define the actual number of smooth muscle cells [18]. It is interesting to note that smaller doses of quercetin rutinoside were more effective than larger doses. The fact that smaller doses exerted a stronger proliferative effect [18] seems to parallel the data presented by Becker et al. [7] whereby lower numbers of quercetin rutinoside epitopes were more mitogenic than higher

numbers.

Becker et al. [7] reported that when pre-incubated with semi-confluent aortic smooth muscle cells for 30 minutes the capacity for mitogenicity was highest for quercetin (expressed as peak percent of proliferation over control at 50 ug/ml) followed by chlorogenic acid, PDGF type one, TGP, quercetin rutinoside pre-incubated with tissue culture medium, quercetin rutinoside - BSA, TAR and lastly unconjugated quercetin rutinoside. This is in contrast to tobacco constituents that were incubated for 84 hours (Table 2). TGP exercised roughly a ten fold increase over control values when incubated for 30 minutes (expressed as percent of cell proliferation over control at 50 ug/ml) versus roughly a two and a half times greater increase over control when incubated for 84 hours (expressed as number of cells at 50 ug/ml). This is significant as TGP did not require more time to exert full mitogenicity. Clinically, this may be relevant in terms of chronic or chain smokers who may be continually exposed to TGP, although the kinetics of TGP in blood are as yet not known. It is important to note that quercetin rutinoside conjugated with bovine serum albumin influenced smooth muscle proliferation (expressed as number of cells x 10,000 with ~ 2 x 10,000 cells per well or as percent increase over control with 2 x 100,000 cells per well) in a dose dependent fashion. This is in contrast to both TGP and TAR (expressed as percent increase over control)which influenced smooth muscle proliferation up to 50 ug/ml, then leveled off. Quercetin Rutinoside conjugated with bovine serum albumin (50 ug/ml) also influenced smooth muscle proliferation up to 84 hours while both TGP and TAR leveled off or decreased slightly at 60 hours (expressed as the

number of cells x 10,000)[7].

The high degree of mitogenicity of quercetin at 50 ug/ml (Table 1), is not clinically relevant as only polyphenol epitopes within TAR/TGP are inhaled/absorbed *in vivo*. However, the measured incorporation of tritiated thymidine into newly synthesized DNA of smooth muscle cells was significantly less for all tobacco constituents (excluding chlorogenic acid) compared to commercially available platelet derived growth factor type 1 (PDGF) which acted as a positive control [7]. It is important to note that tobacco glycoprotein (TGP) is composed of both quercetin rutinoside and chlorogenic acid and may account for its increased capacity (although not significant), over quercetin rutinoside bovine serum albumin conjugates to stimulate smooth muscle proliferation. This increased mitogenic capacity presumably acts via the combined or synergistic effects of both these molecules , although it was reported [7] that quercetin rutinoside conjugates with a lower epitope density (~ 6) were more mitogenic than those with larger numbers (~ 20-49). It should also be noted that tobacco glycoprotein reached a peak percent of smooth muscle proliferation at a concentration of 50 ug/ml, while at a concentration of 100 ug/ml the percent of smooth muscle proliferation decreased slightly. This differential effect according to epitope density may relate to the reported [19] biphasic action of quercetin rutinoside since it has been shown that it exhibits a cytostimulatory/cytoinhibitory effect at different doses.

Crucial to this thesis is the observation that free quercetin rutinoside (unconjugated) was not as mitogenic as pre-incubated quercetin rutinoside to bovine aortic smooth muscle cells *in vitro* (Table 1)

[7]. Preincubation of quercetin rutinoside with tissue culture medium for 30 minutes before addition to the cell cultures enhanced its mitogenic capacity. It was concluded that absorption of quercetin rutinoside to media proteins enhanced its mitogenicity to equal that of quercetin rutinoside bovine serum albumin conjugates. It is important to note that chlorogenic acid did not need to be conjugated to bovine serum albumin to be mitogenic. This is significant as chlorogenic acid is a component of TGP, and therefore cannot preclude the possibility that chlorogenic acid is an active epitope within TGP.

Recent evidence suggests a differential reaction to potential atherogenic stimuli of smooth muscle cells according to their respective phenotype [20]. Fowler et al. [21] demonstrated that bovine aortic smooth muscle cells grown *in vitro* under different conditions show dramatic differences in enzyme content, physical properties and morphological appearance: dilation of endoplastic reticulum, increase golgi apparatus, etc. This becomes extremely important since smooth muscle cells must modulate their phenotype from the contractile to the synthetic in order to proliferate [22]. The existence of synthetic state smooth muscle cells due to culturing *in vitro* could potentially bias the *in vitro* response of cells to suspected mitogens. The results of Fowler et al. [21] demonstrate that differences in specific cellular characteristics/functions of aortic smooth muscle cells exist when comparing *in vitro* versus *in situ* data. It has been reported [20] that the proportion of synthetic versus contractile state smooth muscle cells is exaggerated in tissue culture because synthetic state smooth muscle cells have a higher plating efficiency (80%) compared to freshly dis-

sociated aorta (65%). Becker et al. [7] when conducting electron-microscopic analysis to confirm cells to be either smooth muscle or fibroblast cells did not definitively characterize the degree of phenotypic expression as being either contractile or synthetic smooth muscle cells.

Nature of Quercetin Rutinoside

Flavonoids, to which quercetin rutinoside belongs, are a group of plant polyphenols that consist of a 2-phenyl-benzo- pyrane or flavane nucleus consisting of two benzene rings (A and B) attached to an oxygen containing pyrane ring C [23]. Quercetin Rutinoside (quercetin-3-O-B D-rutinoside), commonly known as rutin, was discovered by Weiss [24] in 1842 in the leaves of the Ruta graveolens. Quercetin Rutinoside is a flavonol glycoside which can be prepared in a highly purified state. Quercetin Rutinoside is an ubiquitous, naturally occurring bioflavonoid distributed throughout the plant kingdom, being present in at least 34 plant families and 77 plant species [25]. With 800 different flavonoids known to exist, they are the most widely occurring group of secondary plant metabolites that are consumed in man's daily diet [23]. In addition, many flavonoids including quercetin rutinoside are found in the leaves of the tobacco plant (nicotiana tabacum) in a concentration of 0.5% [26].

Effect of Quercetin Rutinoside on Experimental Atherosclerosis and Hypercholesterolemia

A number of experimental studies [27-32] have reported that quer-

cetin rutinoside lowers serum cholesterol levels and reduces experimental atherosclerotic lesions. However, it cannot be overemphasized that the literature on these compounds is highly ambiguous and very often contradictory.

Effect on Serum Cholesterol

The contradictory nature of the literature is illustrated by several studies that do not report a reduction in serum cholesterol levels [33,34]. One study reported that quercetin rutinoside lowered total and esterified cholesterol in serum but showed that free cholesterol was significantly increased [28].

More importantly, several studies [30,31] have introduced confounding variables within the experimental protocols in the form of specific vitamin deficient states as well as exposing the experimental animals to high degrees of physical stress (extreme lack of motion), etc. These non-essential variables cannot be accounted for in other respective studies nor in the present one. Therefore it renders it exceedingly difficult to extrapolate the data from the literature to the present investigation. Numerous studies [29-31] were conducted under experimental conditions whereby serious deficiencies in methodology were noted. In particular, one study [29] examined the possible hypocholesterolemic action of quercetin rutinoside, however the measurements for cholesterol were made three weeks after discontinuation of cholesterol feeding, hence the lower levels cannot be definitively attributed to the influence of quercetin rutinoside.

Effect on Experimental Atherosclerosis

Research on seventy-three rabbits [32] tried to confirm what pre-

vious studies had investigated in other species. It was concluded that quercetin rutinoside inhibits the formation of aortic lesions in experimental peroxide injury atherosclerosis under conditions of hypercholesterolemia. To clearly evaluate the role of quercetin rutinoside in this type of atherosclerosis, it is fundamental to differentiate between cholesterol atherosclerosis (induced by hypercholesterolemia) and injury atherosclerosis (induced by peroxides). In the study put forward by Voskresensky [32], these two different forms of experimental atherosclerosis are superimposed one on another. Friedman [35] and later Weigensberg et al. [36] described and produced three distinct types of atherosclerosis (cholesterol, injury and thrombo-atherosclerosis) in terms of morphological constituents, evolution and fate of aortic lesions, as well as different biochemical compositions. Therefore to properly evaluate the role of quercetin rutinoside it is necessary to study these lesions as distinct and separate entities. Comparison of quercetin rutinoside on other forms of atherosclerosis [27,32] other than cholesterol induced lesions is therefore not relevant to the present investigation.

In summary, the conflicting reports taken with the different experimental lesions employed and varying deficiencies in methodology render it extremely difficult to extrapolate or adequately assess the influence of quercetin rutinoside in the treatment of hyperlipidemia or atherosclerosis. To date, there exists not one study that adequately measures the influence of quercetin rutinoside on cholesterol content or smooth muscle proliferation in aortic tissue. It must remain strict conjecture at present, as to the influence of quercetin rutinoside in

experimental cholesterol atherosclerosis and hypercholesterolemia.

Effect of Quercetin Rutinoside on Capillary Filtration and Perfusion

The vast majority of literature on quercetin rutinoside and related compounds consists of clinical reports that have emphasized the use of flavonol glycosides in conditions characterized by spontaneous capillary bleeding believed to be the consequence of increased capillary fragility and filtration [26,37]. The exact mechanism of action of these flavonoids on the microcirculation is not known.

As early as 1938, Winternitz et al. [38] and later Barger et al. [39] demonstrated evidence for neovascularization in the regions of atherosclerotic plaques. These reports [38,39] raise fundamental questions concerning the role of neovascularization in atherosclerosis. Namely, the existence of capillary vessels within the arterial wall (plaque and tunica media) may mean that mitogenic agents from the blood stream could be brought into intimate contact with smooth muscle cells, perhaps for longer periods of time than the uninvolved arterial wall. However, neovascularization is a characteristic of advanced complicated lesions and not of the type of lesion employed within the present investigation. Hence the role of quercetin rutinoside in microcirculatory changes is not expected to be a major factor in the present investigation.

Absorption, Metabolism and Excretion of Quercetin Rutinoside

The publications on the absorption, distribution, biotransformation and excretion of quercetin rutinoside have yielded conflicting data.

Early research on the metabolic fate of orally administered quercetin rutinoside demonstrated that its metabolites were composed of ring fission products [23]. Studies have since shown that several of these flavonoid metabolites [40] are derived from the lumen of the lower intestinal tract by gut microfloral catabolism [41].

Kinetics

Quercetin Rutinoside administered orally to rabbits is absorbed and excreted within twenty-four hours [42,43]. The flavonoids that are not catabolized by microflora cross the mucosa and are bound as glucuronides or sulphate conjugates [23,26]. The conjugation of natural flavonoids with glucuronic acid and with sulfate is the most common final step in the metabolism of these intact molecules. Varying amounts of yellow fluorescing metabolites of quercetin rutinoside have been demonstrated in bile and urine after oral administration, indicating that tissue metabolism plays a role in elimination of this bioflavonoid [44].

In conclusion, quercetin rutinoside metabolism has been studied in rats, rabbits, guinea pigs and man [23]; in all cases the metabolic fate between the species was demonstrated to be a duplicate one. Because the metabolism to phenyl acyl fragments is mediated by micro-organisms of the GI tract, it became necessary then to administer large amounts (within the range of 445 mg/kg - 1000 mg/kg) to ensure that bacterial catabolism did not significantly interfere with the absorption of quercetin rutinoside.

Toxicology of Quercetin Rutinoside

Quercetin Rutinoside as an integral part of the human diet has long

been considered to be non-toxic and is purported to be beneficial in the treatment of numerous vascular diseases. Recent reports have illustrated widespread biochemical effects of quercetin rutinoside on mammalian cells.

In contrast to the beneficial clinical reports, a direct tissue toxicity study was conducted on isolated heart and lung preparations of rabbits and guinea pigs exposed to tobacco glycoprotein isolated from cigarette smoke condensate [45]. The heart preparations developed cardiac anaphylaxis characterized by sinus tachycardia, idioventricular tachyarrhythmias and decreased coronary perfusion and contractility. In addition, the isolated pulmonary preparations developed acute dysfunction characterized by spasms of the arterial bed causing significant reduction or complete arrest of the pulmonary arterial flow. It must be emphasized that 1 mg of tobacco glycoprotein or roughly the same amount present in one pack of cigarettes was injected into the arterial flow during *one* circulation through the isolated organs [45]. This clearly represents a very small amount of antigen that is capable of binding cell surface receptors during a single circulatory pass.

Intravenous injection of quercetin rutinoside in dogs and monkeys induces acute cholecystitis together with margination of neutrophils in the lung which is sometimes accompanied by pulmonary edema and focal necrosis of alveolar capillaries [14]. If inhaled, quercetin rutinoside or cigarette smoke condensate induce focal myocardial necrosis in rabbits which is characterized by nuclear pyknosis, contraction bands and hypereosinophilia [46]. Apart from cardiac manifestations, the inhalation of quercetin rutinoside induces clumping, margination and emigra-

tion of leukocytes in the pulmonary vasculature of rabbits [46]. Alveolar extravasation of erythrocytes and microthrombi, together with the activation of factor XII dependent pathways and the shortening of the partial thromboplastin time also occur [46]. In another study it was reported that inhalation of the non-dialyzable constituents of cigarette smoke condensate, or "tar", activated factor XII dependent pathways and induced acute acalculous cholecystitis and pneumonitis in dogs [12]. The formation of thrombi in the pulmonary vasculature was also observed. The capacity of tobacco glycoprotein in cigarette smoke condensate to activate factor XII of the intrinsic pathway was shown to depend on the presence of quercetin rutinoside.

In summary, when administering quercetin rutinoside *in vivo*, the various toxicity studies [12,14,45,46] must be assessed in deciding the ultimate route of ingestion. It is concluded that enteral administration of quercetin rutinoside would result in the lowest degree of toxicity.

Experimental Cholesterol Atherosclerosis in Rabbits

Historical Perspective

Cholesterol feeding as a procedure for generating fatty streak lesions in rabbits originated in the work of Ignatowski in 1908. The strong resemblance between the experimental lesions produced by Ignatowski and the typical lesions of human atherosclerosis were quickly noted. Ever since Anitschkow and Chalatow first described the production of hypercholesterolemia and atherosclerosis in cholesterol-fed rabbits in 1913, the rabbit has been the main animal species used in ex-

perimental atherosclerosis research between 1915 and 1950, with more than 90% of the published experimental studies involving this animal. The formation of fatty streak lesions in experimental models of atherosclerosis has also been studied in many other species such as pigeons, guinea pigs, baboons and chickens when fed a diet containing added amounts of cholesterol. However, with the success of developing arterial lesions from cholesterol feeding in animals other than rabbits, there arose a continuous decline in the use of the rabbit for experimental atherosclerosis research. This decline was hastened by negative comments (which were later found to be incorrect) concerning the type of atherosclerotic lesions that could be induced and their dissimilarity to human disease. Yet various animal species show different susceptibilities to varying degrees of cholesterolemia, and thus to experimental atherosclerosis, resulting in different stages of lesion progression [47,48]. However among the animals which have been studied experimentally the rabbit is unique in that no other animal responds to dietary cholesterol to such a high degree that elevation of plasma cholesterol and intimal lesions result with ease, regularity and consistency.

It is both logical and intuitive that in the empirical investigation of any disease it is highly important to study a reasonable facsimile in an animal model and to be able to modify the disease under different experimental conditions. Although there are definite morphological similarities between the lesions of experimental atherosclerosis in rabbits and those in man, definite differences are also easily demonstrable.

<u>The Rabbit Model: Different degrees of Hypercholesterolemia</u>

The use of the rabbit in atherosclerosis research has remained controversial over the years because of certain proposed limitations to this model. It is evident from the beginning that the rabbits' tissues encounter the large quantities of dietary cholesterol as something foreign. Rabbits are hyperresponders when fed a cholesterol-rich diet and become extremely hypercholesterolemic developing arterial lesions after a matter of a few months. The mechanism for handling exogenous cholesterol in rabbits is highly deficient with respect to its metabolism and excretion. Physiologically unprepared to dispose of ingested cholesterol, rabbits develop "cholesterol storage disease" with large amounts first accumulating within the reticuloendothelial system. The deposition of cholesterol in the wall of the aorta is only part of a systemic infiltration of the tissues which include the liver, heart, lungs and kidneys and has no counterpart in the "typical" non-familial homozygotic atherosclerotic lesions in man.

Various investigators have stressed the point that rabbits exhibit exceedingly high levels of serum cholesterol when placed on atherogenic diets (values of 2000 mg% and higher are commonplace) and have objected to such levels as being far higher than those observed in human sera. The development of intimal lesions is produced far more quickly when very large daily doses of cholesterol are used. This discrepancy between serum cholesterol values may result from the relatively slow formation of atherosclerotic lesions in man, as compared with the rapid induction and forced accumulation (systemic infiltration) of cholesterol in rabbits. However, several studies have demonstrated that cholesterol atherosclerosis will develop in rabbits and other laboratory animals

when their normal (50± 10 mg%) cholesterol levels are raised to the normal human range (155-230 mg%) [49,50].

The Rabbit Model: Lesion Morphology

It is now recognized by many that the lesion in 12 to 14 week cholesterol-fed rabbits is a model of the typical fatty streak lesion that occurs at predictable sites in the thoracic aorta. Because the morphology of this lesion has been described by many previous investigators [51-55] only a very brief review will be presented about their microscopic appearance and only insofar as they are relevant to the comparison at hand. The components of the intercellular matrix material together with the two cell types, the polyhydrol non-myogenic foam cell and the PTAH positive myogenic lipid laden foam cell, represent the same constituents of both human and rabbit intimal lesions. In contrast to the human intimal atherosclerotic lesions which are generally quite abundant in myogenic lipid laden foam cells and with fewer numbers of non-myogenic foam cells, the early atherosclerotic lesion of the cholesterol-fed rabbit usually contains a predominance of polyhydric non-myogenic lipid laden foam cells that have the immunological characteristics of macrophages. The ongoing deposition of cholesterol in the rabbit aorta promotes the continued hyperplasia of the original subendothelial foam cells along with an increased proliferation of smooth muscle cells into the base of the plaque [56]. However, many investigators have criticized the lesions produced in rabbits as being unlike the human lesion because it consists almost exclusively of cushions of foam cells, and does not show the typical atheroma with gruel, dead cells, necrosis and the fibrous cap. In addition, the complications of

human atherosclerosis such as hemorrhage, ulceration, thrombosis and calcification rarely occur. It is now known that these features will form if more time is allowed on the diet together with alternate periods when cholesterol is withheld [57]. If used under well defined conditions, it is possible in the rabbit model to produce sub-types of cholesterol atherosclerosis containing predominantly one cell type or the other. It is widely recognized that alterations of the fat used in atherogenic diets can influence the gross and microscopic character of the lesion. Therefore, it is possible to produce a severe intimal proliferation with moderate amounts of fibrosis. This predominantly myogenic proliferation together with the deposition of collagen and elastin produces a fibrocellular lesion that radically differs from foam cell lesions and is remarkably human-like in comparison. As well, human-like lesions can be produced at an accelerated rate if the artery is subject to injury and the same risk factors that occur in man (i.e. hypertension, etc.) in association with the cholesterol feeding.

Analysis of the experimental cholesterol atherosclerotic lesion in the rabbit demonstrates that it does compare to many stages that occur in the lesions of man. On the most basic level in both humans and rabbits, the pathogenesis of atherosclerosis is derived from the direct response of smooth muscle cells in the intima and media to excess lipids. Differences in response seem to be a matter of degree.

Although not anatomically identical with human atherosclerosis, it is in many instances very striking that the experimental lesion in the rabbit frequently involves areas around the mouths of branching arteries much the same way as in man [51,52]. One major difference concerning

the anatomical distribution is the strong tendency of the lesion in the rabbit aorta to become most advanced in the arch. On the contrary, in man it is more common to observe the greatest severity in the abdominal aorta. As well, the pulmonary artery is severely affected in the rabbit while in man it is usually spared.

The comparison between the rabbit and man is not complicated by the existence of spontaneous arterial lesions in the rabbit. Descriptions of spontaneous disease in the arteries of rabbits are confined exclusively to a single type of lesion in which changes are found chiefly in the media of the aorta [52]. The lesions exist in localized areas in the middle of the tunica media with smooth muscle cells dying and becoming necrotic, calcification being a common end result. Thus, the spontaneous arterial lesion in the rabbit is easily differentiated from the arterial lesions produced by cholesterol diets and human atherosclerosis.

The interpretation of significant experimental results in relation to human atherosclerosis can be made in relation to lipoprotein metabolism. The normal rabbit plasma lipoprotein classes are comparable to those of man, although at lower concentrations [58]. Several studies indicate as well that rabbit apolipoproteins are also comparable to those of man [59,60]. The similarity of rabbit apolipoproteins and lipoproteins to their human counterparts imparts an even greater value of the rabbit as a tool for the study of hyperlipoproteinemia in the elucidation of atherosclerosis.

It is clear therefore that there are many striking similarities as well as many differences in anatomical distribution, lipid deposition,

morphology (grossly and histologically) and lipoprotein metabolism in arterial lesions of experimental disease and that of the disease which naturally occurs in man. In conclusion, cholesterol atherosclerosis in the rabbit provides a high degree of equivalence to human atherosclerosis and thus justifies the applicability of experimental observations to many aspects of human disease.

EXPERIMENTAL DESIGN

Object of this Study

In a previous study [7] it was established that tobacco glycoprotein (containing quercetin rutinoside) and quercetin rutinoside coupled to bovine serum albumin alone are mitogenic to bovine aortic smooth muscle cells *in vitro*. The design of this research was undertaken to investigate the effect of quercetin rutinoside on experimental cholesterol atherosclerosis composed predominantly of the smooth muscle cell type. This was carried out within the rabbit model. The experiments reported are preliminary in nature and were undertaken to answer some simple questions regarding the role of quercetin rutinoside in atherosclerosis.

Two groups of white male New Zealand rabbits were used. The number of animals allocated to the control and treated groups were determined by considerations of cost and of statistical requirements for groups large enough to provide significant results. In addition, the size of the treated group was taken into account on the basis of purported toxic effects of quercetin rutinoside that could possibly result in a decreased survival rate of the treated animals in reaching the termination of the experiment (14 weeks). The rabbit model was selected on the basis of its high susceptibility to cholesterol feeding resulting in a rapid lesion induction. The rabbit is easily manipulated, inexpensive and biologically well-characterized. If used under well defined conditions the rabbit model is unique and is very useful in that several biochemically and morphologically distinct types of atherosclerotic le-

sions can be produced [36]. As well, it is also possible to produce cholesterol lesions containing predominantly one cell type or the other. Because it is possible to produce sub-types of cholesterol atherosclerosis it becomes important to study the effects of quercetin rutinoside on these "pure lesions". The atherosclerotic lesion of the cholesterol fed rabbit is thus an excellent *in vivo* model on which to base this preliminary study. Apart from the ability to produce specific cholesterol lesions, the rabbit is suitable to study the effects of quercetin rutinoside because its metabolism of this flavonol is essentially the same as in man [23].

Production of a Myogenic Type of Atherosclerosis

Alterations of the fat used in atherogenic diets has long been a widely recognized method for influencing the gross and microscopic character of experimental atherosclerotic lesions. Vesselinovitch et al. [61] reported that feeding a diet containing 25% peanut oil produces a more cellular, proliferative lesion containing more collagen than does feeding butter or corn oil to monkeys on a 2% cholesterol diet. In accordance with Vesselinovitch et al., it has been reported in monkeys as well as rabbits that peanut oil fed along with cholesterol produces a more prominent myogenic proliferation, together with more collagen and fibrous tissue than would be present in lesions if cholesterol were fed alone or accompanied by other fats or oils [62-67]. A cholesterol peanut oil diet is inordinately atherogenic in rabbits mainly because of the severe intimal proliferation and fibrosis that occurs. This fibrocellular type of lesion is characterized by less lipid deposition, and shows a mixture of smooth muscle cells, collagen and some slight

degenerative changes. Peanut oil is a good example of a dietary fat that may speed up the progression of a fatty streak lesion to a more fibrous type lesion.

The rationale for feeding a diet containing peanut oil and butylated hydroxy anisol (BHA) to produce the myogenic type lesions is based on reports that each of these materials by themselves could produce myogenic type atherosclerosis with spindle shaped foam cells [68]. Therefore the rationale for feeding all three of these materials was to provide a more reliable regimen than feeding higher doses of any one of these alone. Wilson et al. [68] writes, in describing the lesions produced by feeding cholesterol combined with the antioxidant butylated hydroxy anisole, that "the atherosclerotic lesions consisted of a proliferation of smooth muscle cells in the intima with intracellular and extracellular lipid". Similar descriptions to the one above exist for the effects of feeding peanut oil. Peanut oil was chosen specifically since it has been shown to cause increases in myogenic foam cells.

We feel it is fundamental to this project to study this myogenic sub-type of cholesterol atherosclerosis in a "pure" state in order to fully test the hypothesis put forward by Becker et al. [7].

The amount of quercetin rutinoside present within the daily diet of the treated group was determined from numerous metabolic studies. Limited solubility together with relative microfloral catabolism required the administration of a large dose. The oral route of administration was chosen over an inhalation method (nebulizer) because of the factors of time and convenience and of inhalation toxicity. To subject roughly forty rabbits to a nebulizer each day for fourteen weeks

would add considerable expense to the project in addition to the difficulty of acquiring such hardware previously not found in this laboratory. The nebulizer method is not without advantages though. To bypass the gastrointestinal tract would be ideal in order to avoid microfloral catabolism. The possibility of treating the animals with antibiotics to reduce the microfloral catabolism of quercetin rutinoside was investigated. The narrow spectrum antibiotics necessary to effectively reduce the microflora were found to be toxic to rabbits [69]. If however, the antibiotics were well tolerated during the 14 weeks of the experiment, then selection of resistent strains and supra-infections would result. As well, antimicrobial therapy could only reduce the microflora a few orders of magnitude, clearly not sufficient to eliminate or effectively reduce flavanol catabolism.

A potential problem associated with the intravenous use of quercetin rutinoside is its low water solubility. Pfeifer et al. [70] showed that quercetin rutinoside precipitated out of solution and formed concretions with associated suppurative inflammation in the renal channels and liver bile ducts of newborn hamsters, rats, mice and newborn or premature babies following parenteral dosage. The absolute ease of the feeding method assured that all rabbits consumed equal amounts of this flavonoid.

Hypothesis to be Tested

The specific objectives of this study were to determine the quantitative effects of quercetin rutinoside on cell proliferation (tritiated DNA synthesis), serum, aortic and pulmonary artery choles-

terol levels, as well as the extent of atherosclerosis estimated by the percent of intimal area covered by lesion.

From a study conducted on atherosclerotic plaques of rabbits using tritium labelled nuclei, it was found that the initiation and sequential evolution of the atherosclerotic plaque was closely associated with the proliferative reaction of the arterial wall involving medial smooth cells [71]. Because myointimal proliferation is the critical event in the formation of the fibrous lesion and because this proliferation forms the basis of Becker's hypothesis that quercetin rutinoside may be one factor in smoke responsible for making it a risk factor, it becomes important to obtain quantitative data concerning the degree of cell proliferation (DNA synthesis). One parameter of this study was to determine the level of DNA synthesis measured by the incorporation of tritiated thymidine into newly synthesized DNA of atherosclerotic lesions.

Although the use of tritiated thymidine provides a good index of cellular proliferation, it is still necessary to evaluate the aorta and pulmonary artery in terms of percent surface area covered with lesions. This procedure gives a clearer index of the involvement, i.e. extent in terms of distribution of the lesions within the vessels. Moreover, when combined with the incorporation of tritiated thymidine into newly synthesized DNA (as an index of cell proliferation) and total cholesterol content of serum and aortic and pulmonary tissue, this approach provides a basis on which to judge the effect of quercetin rutinoside on this type of atherosclerosis.

Because several studies have reported that quercetin rutinoside ex-

hibits a possible cholesterol lowering action on serum and other tissues thereby potentially slowing the formation of atherosclerosis, it becomes important to measure serum cholesterol in order to evaluate the role of quercetin rutinoside in cholesterol atherosclerosis. Although measuring the total cholesterol content of serum, aortic and pulmonary tissue does not test Becker's hypothesis directly, it does impart a greater understanding of this flavonoid in an important paramater that is necessary to assess its role in atherosclerosis [72]. As well, it sheds light on quercetin rutinoside's potentially beneficial function in vascular disease and may lend some credence to previous reports of its therapeutic application.

The time allocated for the animals to be on the diets was based on previous empirical work conducted within this laboratory demonstrating that fourteen weeks was sufficient time to develop atherosclerosis.

MATERIALS AND METHODS

Diet

White male New Zealand rabbits weighing approximately 2 to 4.5 kg and about 3 months of age were fed a cholesterol-peanut oil diet with BHA and ethanol to produce aortic atherosclerosis containing predominantly myogenic type, lipid laden foam cells. The daily food ration contained within the 52 grams of pellets (Purina Rabbit Chow) consisted of: 500 mg USP-grade crystalline cholesterol along with 1 g of ethanol, 6 g of peanut oil and 0.06 g butylated hydroxy anisole (referred to hereafter as the control group). The rabbits were divided into control and experimental groups at random, 15 rabbits acted as controls while 40 were fed 2 g of quercetin rutinoside in the diet per day. Quercetin rutinoside was obtained as a yellow crystalline powder (Sigma Chemical Company) and was certified over 95% pure with the remainder made up of water of crystallization. The quercetin rutinoside was incorporated into the diet by dissolving it in ethanol and sprinkling it on the food. The ethanol was then allowed to evaporate under vacuum.

All rabbits were sacrificed after 14 weeks on the diet with sodium pentobarbital (100 mg/kg body weight). Blood obtained just prior to autopsy (14 weeks) was analyzed for total serum cholesterol. The spleen and liver from each rabbit were removed, cleaned free of fat and weighed. The aortae were carefully dissected out and flushed through with saline, opened longitudinally and incubated in tissue culture Medium 199^{R} (Grand Island Biological Co., N.Y.) for one hour at 37°C under 95% O_2, 5% CO_2 to measure DNA synthesis in terms of tritiated

thymidine incorporation into DNA. This medium contains Earle's Salts, L-glutamine and 5 microcuries tritiated thymidine per ml incubation medium. The tritiated thymidine (New England Nuclear) had a specific activity of 6.7 Ci per millimole and radiopurity in excess of 99% as determined by chromatography. Following incubation, the aortae were rinsed in stirring ice-cold 0.01 molar non-labelled thymidine in 5% trichloroacetic acid (TCA) for 5 minutes, then in 3 changes of thymidine in 0.9% saline, 2 minutes each time. The rate of DNA synthesis was measured in terms of tritiated thymidine incorporation into DNA expressed as disintegrations per minute (DPM) per mg delipidated tissue.

It was necessary to immediately incubate the aortae once removed from the rabbit to ensure cell death did not occur. After incubation, the extent of cholesterol atherosclerosis in the whole aorta, its arch, the thoracic segment and the abdominal segment were estimated visually in terms of percent surface area covered with lesions as previously described by McMillan et al. [72]. A small section from one random lesion from the aortic arch and one random lesion from the thoracic aorta of each rabbit was fixed in 10% formaldehyde and was used for light microscopy with sections stained with phosphotungstic acid haemotoxylin (PTAH) and Massons Trichrome (MT).

After removing small segments of the arch and thoracic area for histology, the remainder of the aorta not used for histology was cut up into lesion and non-lesion areas. This procedure was performed to differentiate the respective DNA synthesis of lesion and non-lesion segments. In addition, mixed tissue was obtained (lesion and non-lesion areas that could not be definitively separated) in order to render a

ratio of lesion and non-lesion areas.

Determination of Total Cholesterol in Serum

Under anesthesia, blood was obtained during sacrifice by inserting a large bore syringe into the left ventricle and drawing blood while the heart was still beating. Blood obtained from each rabbit during the sacrifice was left overnight in a fridge, then centrifuged at 4000 RPM for 20 minutes. A 1 ml aliquot of serum was obtained, placed in 150 ml of ethanol for 20 minutes and extracted in a Soxlet apparatus by boiling ethanol for 24 hours and then extracted in diethyl ether (Fischer Scientific Co.) at room temperature for 8 hours. The ethanol extracts were combined with the diethyl ether extract, reduced in volume, and evaporated to dryness under vacuum at room temperature. The lipid residue was then dissolved in 100 ml of analytical grade chloroform (Mallinckrodt Chemical Works). It was essential that all traces of water, alcohol, and diethyl ether be absent from the chloroform solution. 2 ml of a reagent that consisted of 1 ml of concentrated sulfuric acid (Fischer Scientific Co.) and 40 ml of acetic anhydride (Mallinckrodt Chemical Works) was added to 5 ml of the sample in chloroform. Each vial containing reagent and sample was mixed well and kept at 25°C in a water bath in darkness for 20 minutes. After 10 minutes, the solutions were checked for the intensity of the color. A dilution cocktail was added if the color of the solution was too dark for accurate reading. The amount of dilution cocktail was recorded so that the proper dilution factor was used during the calculation of the concentration. After 20 minutes, the samples were transfered into colorimeter tubes and covered tightly with plastic stoppers and were

read immediately at 660 milli-microns on a Bausch and Lomb Spectronic 21 spectrophotometer with the room lights off. All cholesterol determinations were calibrated and standardized with intra-assays performed on a regular basis. Cholesterol standard determinations were carried out at concentrations of 160 ug/3.5 ml (N=95), 400 ug/3.5 ml (N=31) and 320 ug/3.5 ml (N=16). The combined standard errors were 0.25, 0.34 and 0.23 for the respective concentrations. The combined standard deviations were 2.29, 0.92 and 0.90 for the respective concentrations. No significant intra-assay variations were detected.

Determination of Total Cholesterol in Aortic and Pulmonary Arteries

After removing the adventitia, each cleaned aorta and pulmonary artery was individually weighed (fresh wet weight), frozen, freeze dried for 72 hours to constant weight, again individually weighed and delipidated in a Soxlet apparatus with boiling alcohol for 24 hours and further rinsed in diethyl ether at room temperature for 8 hours. The diethyl ether was then allowed to evaporate under vacuum. Methods for cholesterol determinations in aortic and pulmonary arteries paralleled the serum cholesterol determination as previously described for serum. No further lipid could be extracted from the residues by more prolonged extraction with these or other solvents. Cholesterol concentrations were expressed as mg lipid per 100 mg freeze-dried tissue.

Scintillation Counting

The tissues were delipidated, reweighed and dissolved in 2 ml of 0.5 molar solution of Protosol^{R} tissue solubilizer (Dupont) overnight at 55°C. To the liquified tissue residue in Protosol^{R} was added 20 ml of scintillation cocktail. The scintillation cocktail contained 4 g of

2.5-diphenyloxazole and 50 mg dimethyl POPOP and was made up to one litre in toluene. The amount of scintillation solution together with concentration of primary and secondary fluor molecules provided for efficient/effective solvent-solute interactions thereby negating the possibility of any dilution or concentration quenching. It was absolutely essential that all traces of water, polar compounds, inorganic acids or colored substances (hemoglobin) be absent from the tissue residue and scintillation cocktail. This ensured that impurity (chemical) quenching did not interfere in energy transfer or color quenching did not prevent the photons from being detected by the photomultiplier tubes. In all procedures glass borosilicate scintillation vials were free from dirt, condensation or fingerprints and in all cases light trapping was not significant enough to produce photon quenching.

All precautions were taken in sample preparation (handling and techniques) and monitoring to avoid non-beta coincidence pulses. Non-specific scintillation arising from chemiluminescence as a result of peroxides and/or alkalinity or from photoluminescence (sunlight, etc.) did not give any false positives in either nonradioactive controls or sample vials. The use of borosilicate glass vials significantly reduced the possibility of static electricity discharge which could potentially result in single photon event. As well, the borosilicate glass vials did not alter the concentration of fluor molecules or sample, thereby eliminating the possibility of quench.

Tritium was counted by scintillation counting using a Rack Beta LKB liquid scintillation system with a counting efficiency of 35%. Prior to

liquid scintillation counting the decay correction based on the half-life of the tritium was determined. The Rack Beta system automatically counted all samples to the same statistical error using 2 sigma standard deviations (percent error). However, samples were not counted to a percent error smaller than the maximum technical error arising during preparation (pipetting, preparation error, weighing, etc.).

The significance of the differences between means was calculated using the student t-test [73].

RESULTS

Mortality, Body and Organ Weights

Table 4 shows the mortality rate expressed as a percentage of the number of rabbit deaths over the 14 week duration of the experiment compared with the number of rabbits started on each diet. Also shown are the initial and terminal body weights and terminal organ weights (liver, spleen, delipidated aorta). As seen in Table 4, the mortality rate of the experimental group (35%) was five times the rate of the control group (7%).

The administration of the control diet did not impair the ability of the rabbits to gain weight. The percent change in body weight from initial to final weight was +18.3%. However, the administration of quercetin rutinoside did impair the ability of the rabbits to gain weight. The percent change in body weight from initial to final weight was -1.26%. However, the initial weight of the quercetin rutinoside fed group was 413 g heavier than that of the control, meaning a higher starting weight was observed.

Extent of Aortic and Pulmonary Atherosclerosis

Table 5 shows the extent of gross visible cholesterol atherosclerosis in terms of the percent intimal area involved with lesions. At the termination of the experiment at 14 weeks, the extent of atherosclerosis was estimated by visually examining the aortic arch, thoracic aorta, abdominal aorta, pulmonary artery and the entire aorta. Under the conditions of this study, the results do not show a sig-

nificant elevation in the extent of atherosclerosis of the quercetin rutinoside fed group in comparison to the control.

In terms of percent area involved with lesions in the aortic arch (mean value 56.2±33.1%) and thoracic aorta (mean value 15.0±17.4%) of the experimental group fed 2.0 g of quercetin rutinoside per day, it was shown not to be significantly different ($P>0.80$ and $P>0.90$, respectively) than the extent of atherosclerosis in the arch (mean value 57.9±24.0%) and thoracic aorta (mean value 14.4±21.4%) of the control group not fed quercetin rutinoside. The extent of atherosclerosis as a percent of the total aortic area covered with gross atherosclerosis in the quercetin rutinoside fed group, consisted of a mean value of 20.7±16.8% and this was not significantly different ($P>0.95$) from the mean value of 20.9±18.4% area covered with lesions in the control group. In addition, the extent of atherosclerosis in the abdominal aorta (mean value 7.6±11.7%) and pulmonary artery (mean value 30.6±25.6%) of the quercetin rutinoside fed group was not significantly different ($P>0.60$ and $P>0.10$, respectively) than the corresponding extent of atherosclerosis (mean value 9.7±12.1%) and (mean value 20.5±16.4%) of the control group.

Total Cholesterol in Serum, Aortic and Pulmonary Arteries

Table 6 shows the mean serum concentration of total cholesterol and the total cholesterol accumulation per whole aorta and pulmonary artery of both groups at the termination of the experiment at 14 weeks. Cholesterol concentrations were expressed as mg of cholesterol per 100 ml serum or as mg per 100 mg freeze-dried weight. The terminal serum

cholesterol concentration of the quercetin rutinoside fed group was not significantly changed (P>0.80) from the serum cholesterol concentrations found in the control group. The hypercholesterolemic effect of the control diet was apparently not affected by the addition of quercetin rutinoside.

Similar lack of significant differences between the quercetin rutinoside and control group were found in cholesterol concentrations in freeze-dried tissue of the aorta and pulmonary artery (P>0.60 and P>0.70, respectively). Large amounts of lipid accumulated within the aortae producing predominantly myogenic lipid laden foam cells in both groups. There was no evidence that quercetin rutinoside had any modifying influence on the cholesterol accumulation or cholesterol concentration in these tissues or in this type of lesion. The aortic and pulmonary artery cholesterol concentrations, for each group, ran parallel to the extent of aortic and pulmonary atherosclerosis expressed as a percent of the area covered with lesions.

Tritiated Thymidine Incorporation into Newly Synthesized DNA by Aortic and Pulmonary Arteries

Table 6 shows tritiated DNA synthesis (cell proliferation) in both the control and quercetin rutinoside fed group as measured by tritiated thymidine incorporated into newly synthesized DNA. Values of tritiated thymidine incorporated into DNA are expressed as DPM/mg delipidated tissue. Under the conditions used in this study the control group incorporated a mean value of 4474 DPM/mg delipidated tissue into sections of aortic wall (excluding adventitia) containing lesions. Aortic le-

sions from the experimental group fed quercetin rutinoside did not show an elevation in tritiated DNA concentration compared with the control group. The results show that there was a slight difference ($P>0.20$) indicated by a marginal depression in tritiated DNA synthesis (which was not considered statistically significant) of the experimental group corresponding to a mean value of 3481 DPM/mg delipidated tissue. This decrease in DNA synthesis of the experimental group fed quercetin rutinoside as far as labelling of DNA with tritiated thymidine does not correspond to a decrease in extent of atherosclerosis as expressed in mean percent surface area of the entire aorta (Table 6).

In comparing the DPM tritiated thymidine incorporated into DNA of mixed lesions (lesion and non-lesion areas that could not be definitively separated) tissue from aortae from both groups, there was no significant changes ($P>0.90$) in tritiated DNA "concentration" of the quercetin rutinoside fed group, mean value 1173 DPM/mg delipidated tissue compared to 1128 DPM/mg delipidated tissue of the control group. Mixed lesions (lesion and non-lesion areas) from the pulmonary artery from both groups showed similar amounts of tritiated thymidine incorporation into DNA. The mean value of the quercetin rutinoside fed group (pulmonary artery mixed tissue) was 3779 DPM/mg delipidated tissue which was not different ($P>0.50$) from the control group, 3256 DPM/mg delipidated tissue. The normal aortic wall without lesions of the control group incorporated 518 DPM/mg delipidated tissue. Statistical analysis showed that this was not significantly different ($P>0.40$) with the quercetin rutinoside fed group, 453 DPM/mg delipidated tissue.

DISCUSSION

In a previous study it was established by Becker et al. [7] that tobacco glycoprotein isolated from cigarette smoke condensate (containing quercetin rutinoside) and quercetin rutinoside conjugated with bovine serum albumin are mitogenic to bovine aortic smooth muscle cells *in vitro*. Since each pack of cigarettes potentially exposes the smoker to the ingestion of approximately 1 mg of tobacco glycoprotein (containing quercetin rutinoside) [15], Becker et al. have put forward the hypothesis that the mitogenic effects of quercetin rutinoside directed against vascular smooth muscle cells *in vitro* may affect smooth muscle cell proliferation *in vivo*, thus compounding the growth of pre-existing atherosclerotic lesions.

The design of this present research was undertaken to investigate the effect of quercetin rutinoside on experimental cholesterol atherosclerosis carried out within the rabbit model. The specific objectives of this study were to determine the quantitative effects of quercetin rutinoside on: cell proliferation measured by the incorporation of tritiated thymidine into newly synthesized DNA of aortic lesions, mixed aortic tissue (lesion and non-lesion areas) and mixed pulmonary artery tissue (lesion and non-lesion areas); total cholesterol within serum, aortic and pulmonary arteries and on the extent of gross visible aortic and pulmonary atherosclerosis estimated by the percent surface area covered by lesion.

In the experiments outlined above, no significant differences were found between experimental and control groups with respect to the extent

of gross aortic or pulmonary atherosclerosis, cellular proliferation (tritiated DNA synthesis) or total cholesterol of the aorta, the serum or the pulmonary artery. However, there was a five-fold increase in mortality in the group fed quercetin rutinoside versus the control group.

Mortality, Body and Organ Weights

The addition of significant amounts of cholesterol to a normal diet (purina rabbit chow) can increase the mortality rate compared to that of the normal diet alone. This higher mortality rate in rabbits fed cholesterol has been recognized by many researchers and has been discussed in detail by Constantinides [74]. The mortality from cholesterol toxicity is mainly due to liver failure with the rabbits developing various symptoms of cholesterol overdose, i.e. refusing to eat, weight loss, and jaundice. This significantly higher percentage rate found within the quercetin rutinoside fed group cannot be attributed to cholesterol toxicity. The existence of a mortality rate within the experimental group (35%) that is some five times higher than the control group (7%) evidently resulted from toxic levels of quercetin rutinoside being absorbed from the gastro-intestinal tract into the blood stream. The basis for this conclusion is drawn from the fact that the same cholesterol peanut oil diet was administered to both experimental and control groups. Since only a single rabbit died while on the control diet, then toxic levels of quercetin rutinoside must have been achieved, therefore resulting in a significant increase in mortality.

In light of the reports in the literature concerning microfloral catabolism of this bioflavonoid within the gastrointestinal tracts of

experimental animals, it was found necessary to administer large amounts of quercetin rutinoside (2 g/day) to ensure adequate amounts were absorbed as intact molecules (i.e. not catabolized) from the gut into the blood stream. However, serum levels of quercetin rutinoside could not be determined in this study using rabbits due to the interference of very high serum cholesterol levels on the sensitive aluminum-chloride colorometric method used to determine serum values. Due to this technical problem, the significantly higher mortality rate of the experimental group (fed quercetin rutinoside) was taken as indirect evidence that this component of the diet was absorbed in sufficient concentration during the experimental period. It is felt that if more quercetin rutinoside were administered (in excess of two grams) then the mortality rate would approach fifty percent.

The percent change in body weight over the duration of the experiment (from the initial to final weight) for the control group was +18.3%. This is in contrast to the group fed 2 g/day of quercetin rutinoside which had a percent change in body weight of -1.26%. Although a random separation of rabbits for either experimental or control group was conducted prior to the start of the experiment, a chance differential weight distribution of 413 g occurred between experimental and control groups. This was only discovered at the end of the experiment.

No significant differences were reported between organ weights of the experimental group and that of the control group. However, the experimental group fed quercetin rutinoside had consistently larger organ weights than that of the control group. These differences cannot be explained at the present time, although they may be related to toxic

levels of quercetin rutinoside, particularly relevant to liver and spleen weights. The initial body weight found in the experimental group may mean that these organ weights were larger from the start.

The results substantiate previously reported data within the literature [12,14,45,46] concerning toxic effects of quercetin rutinoside in laboratory animals. However, due to the considerable and highly controversial biochemical responses of quercetin rutinoside on living systems, no definitive conclusions can be offered concerning the overall effects on mortality in the absence of hypercholesterolemia.

Extent of Aortic and Pulmonary Atherosclerosis

The object of this study was to function as an *in vivo* extension of previous *in vitro* work that showed that quercetin rutinoside could stimulate smooth muscle cells to proliferate [7]. The quintessential objective of this investigation was to study the influence of quercetin rutinoside on developing cholesterol atherosclerosis within the aortic and pulmonary arteries. This was accomplished grossly by measuring the percent aortic or pulmonary intimal area covered with lesions. It was concluded that quercetin rutinoside under the conditions of this study, did not modify the extent of myogenic cholesterol atherosclerotic lesions in the aortic arch, the thoracic, abdominal or in the whole aorta. The percent surface area covered with lesions in the quercetin rutinoside fed group was nearly identical to that of the control group, with the exception of the pulmonary artery. The pulmonary artery in the experimental group displayed a slight but not statistically significant increase in surface area covered with lesion: 30.6% versus a 20.5% for the control group. This slight difference cannot be explained at the

present time.

Because atherosclerotic lesions enlarge by the accumulation of very low density lipoproteins (rabbits) within the arterial wall, by the production of collagen and proteoglycans, etc., and lastly but most importantly, by the proliferation of smooth muscle cells, it becomes fundamental to discuss the reasons for any change in the extent of atherosclerotic lesions (within the aorta or pulmonary artery) in terms of these parameters. It is particularly relevant to the discussion of the extent of aortic or pulmonary atherosclerotic lesions to address the issue of smooth muscle cell proliferation, as the latter determines the extent of fibrous plaque formation (synthesis of matrix tissue components and sequestering of intra and extracellular lipid). Therefore, any further discussion of the extent of atherosclerotic lesions will be addressed in subsequent sections.

Total Cholesterol in Serum, Aortic and Pulmonary Arteries

Expressed as mg per 100 mg freeze dried weight or mg per 100 ml serum, no significant differences existed in total cholesterol concentrations in the aorta, pulmonary artery or serum of the experimental group (fed quercetin rutinoside) compared with that of the control group. Because several studies reported in the literature a possible hypocholesterolemic effect of quercetin rutinoside [28-31], it was found necessary to measure total cholesterol in the respective tissues as a crude preliminary measure. No other measure of free or esterified cholesterol concentrations, phospholipid concentrations or lipoprotein cholesterol concentrations or ratios were undertaken. Because total cholesterol is only one lipid component of the atherosclerotic plaque,

any conclusion on the effect of quercetin rutinoside on total cholesterol cannot preclude the possibility of any effects on other lipid profiles within serum or the plaque (phospholipids, esterified cholesterol, triglycerides, etc.).

All rabbits within the experimental or control groups exhibited hypercholesterolemia with the deposition of lipid in both aortic and pulmonary arteries. The deposition of lipid in both experimental and control groups (aortic and pulmonary arteries) ran roughly parallel to the extent of atherosclerotic lesions in both experimental and control groups as measured by the percent surface area covered in lesion. However, the extent of area covered in lesion for the pulmonary artery (though not significantly different from the control) did not correspond to as large an increase in total cholesterol deposition within the pulmonary artery. This difference cannot be explained at the present time. In contrast to the body of purported clinical and experimental evidence in the literature concerning the therapeutic application of quercetin rutinoside in treating hypercholesterolemia and atherosclerosis [27-32], the results do not substantiate previous evidence.

Tritiated Thymidine Incorporation into Newly Synthesized DNA

Under the conditions used in this study the normal rabbit aortic wall (excluding adventitia) of the control group incorporated 518 DPM thymidine per mg delipidated tissue. This is in contrast to the normal aortic wall of the experimental group which is marginally lower (although not significantly), incorporating 453 DPM thymidine per mg delipidated tissue. Aortic lesions, mixed aortic lesions or pulmonary artery mixed lesions from the groups fed 2 g of quercetin rutinoside did

not result in an elevation in tritiated thymidine incorporated into newly synthesized DNA as compared with the control groups. The failure to find an increase in tritiated DNA synthesis in these groups could be due to many reasons. Firstly, does it remain in the blood in an "active" form long enough for it to penetrate or diffuse into the aortic wall to promote smooth muscle cell proliferation. Quercetin rutinoside could potentially have been diluted in the total blood volume, however this seems unlikely in the face of such a high mortality rate exerted by toxic levels of this component. Glucuronide or sulfate conjugates of quercetin rutinoside could have possibly altered its capacities to penetrate into cells or bind cell surface receptors. It has been reported [75] that there is increased endothelial permeability to horseradish peroxidase yet more importantly endothelial cell sloughing did not occur in hypercholesterolemic rabbits. Although there is increased endothelial permeability, a conformation change of quercetin rutinoside by conjugation may not allow it access past the endothelial barrier. It is important to note that in the *in vitro* work carried out by Becker et al. [7], the endothelium was absent, therefore allowing unhindered access to the smooth muscle cells.

What is more important than the <u>many</u> conflicting studies presented in the literature is the reported biphasic effects [19] of this flavonoid. It is reasonable to speculate that a certain level of quercetin rutinoside could have exerted a cytoinhibitory effect on the replicating potential of the aortic cells in this study. The data bears witness to a slight but not statistically significant decrease in tritiated thymidine incorporated into newly synthesized DNA of the ex-

perimental group (3481 DPM/mg delipidated tissue) compared to that of the control group (4474 DPM/mg delipidated tissue). It should be mentioned that there was no difference in the extent of atherosclerotic lesions between control and experimental groups, meaning that a possible cytoinhibitory effect did not express itself as a decrease in the extent of the atherosclerotic lesions.

The oral administration of quercetin rutinoside may have influenced its biological activity. Because different segments of the gastrointestinal tract have different pH values, it is possible that upon reaching the blood stream it may have significantly diminished biological properties, if in fact pH values alter its activity. Quercetin Rutinoside present within tobacco glycoprotein would not be subject to such a wide range of pH values when inhaled.

Because quercetin rutinoside is an anti-oxidant [23], it is possible that this component in the experimental group could have potentially influenced the lesions to be more myogenic in nature. The results do not show any increase in percent area involved with lesions (aortic or pulmonary), therefore, the degree to which quercetin rutinoside could have potentially exerted an anti-oxidant effect is at present not known. The possibility of quercetin rutinoside being hydrolyzed within the gastrointestinal tract into quercetin (the aglycone of quercetin rutinoside) is at present not known. *In vitro*, quercetin exerted a very high mitogenic effect against bovine smooth muscle cells. Yet no increase in tritiated thymidine incorporated into newly synthesized DNA was observed for any lesion or mixed lesions, whether it was aortic or pulmonary artery in origin.

There have been no quantitative studies reported in the literature of DNA synthesis using definitive methods to assess the role of quercetin rutinoside on cell proliferation in atherosclerosis. The selection of the *in vitro* method for quantifying cell proliferation in terms of the incorporation of tritiated thymidine into newly synthesized DNA was based on the following advantages. Firstly, this method avoids the many inherent problems that arise in using methods such as the *in vivo* administration of tritiated thymidine or in counting mitotic figures. An accurate measure of the potential influence of quercetin rutinoside on vascular smooth muscle requires an accurate measurement of cell proliferation. It has been reported [76] that in the normal arterial wall of the rabbit the rate of cell renewal is extremely low, with the tunica media being virtually stable. Although hypercholesterolemia increases the proliferative activity of the aortic wall, identifiable mitotic figures are still observed infrequently [77]. Because mitotic figures are rare, it is necessary to use colchicine to arrest the cell cycle, accumulating cells in metaphase so that the characteristic mitotic figures can be counted. Since colchicine itself inhibits DNA synthesis, this method is self defeating since it interferes in the sensitivity to adequately assess the influence of quercetin rutinoside on proliferating smooth muscle cells.

With the possibility of underestimating cell proliferation when counting mitotic figures, quantitative measurement of cell proliferation must be based on tritiated thymidine being incorporated into newly synthesized DNA, either by radioautographic counting of labelled cells or by chemical determination of tritiated thymidine using liquid scintilla-

tion. In assessing availability of labelled precursor, fundamental differences exist between *in vitro* and *in vivo* techniques. One important consideration when assessing intravenous administration is the duration of the availability of tritium labelled thymidine to the cell population being studied. Comparative research previously undertaken within this laboratory reveal that the *in vitro* method of assessing DNA synthesis by incubating sections of aortic tissue and lesions for one hour at a concentration of 5uCi per ml of tissue culture medium gave twenty to fifty times greater DNA labelling than was obtained one hour after an *in vivo* injection of 1uCi per gram body weight. Possible reasons for this inequality in labelling can be partially accounted for on the basis of tritiated thymidine availability. The cells utilized within the *in vitro* method were exposed to a constant concentration of labelled thymidine for one full hour, as opposed to a relatively short and highly variable pulse *in vivo* [78]. Because incubating the aortic tissue sections for an hour assures that the cells are exposed to a constant concentration of tritiated thymidine, this endows the *in vitro* method with a more reproducible specific activity than the *in vivo* method. One study [79] reported that administration of tritiated thymidine via injection results in a sharp increase, then a decline during the first few minutes, followed by an exponential decay with a half-life of 9.5 minutes. In addition, administration via the *in vivo* method results in the labelled thymidine being catabolized in various organs (spleen, etc.) and other tissues of the body, thereby releasing free tritium. Because aortic tissue does not catabolize labelled thymidine *in vitro*, the *in vitro* method essentially avoids the problem of tritium loss or

incorporation of label into other cell constituents.

The *in vitro* method also avoided the potential of monocytes becoming labelled in the bone marrow, thereby preventing the possibility of labelled monocytes from migrating to the site of the lesion to be erroneously counted as proliferating smooth muscle cells.

Labelling techniques (using radioautography or liquid scintillation methods) cannot preferentially limit or preclude the uptake of tritiated thymidine to specific cell populations that are more actively dividing. It has been shown that under hyperlipidemic conditions, cells other than vascular smooth muscle cells (i.e. endothelial cells) have an increased propensity to proliferate [77,80]. This became evident when some endothelial cells became labelled, indicating that endothelium plays a role in proliferation under hyperlipidemic conditions [80]. Since the vast majority of cellular proliferation occurs within the lesion itself, and because in this study smooth muscle cells were selectively induced to proliferate via the peanut oil - butylated hydroxy anisole (BHA) diet, then the bulk of dividing cells taking up labelled precurser will be PTAH positive smooth muscle cells. Although in this study a smooth muscle cell rich lesion was employed to measure the influence of quercetin rutinoside, background labelling did occur in endothelial cells in both groups, however, this was a proportionally smaller fraction, and no attempt was made to quantify this background uptake.

The labelling index of atherosclerotic lesions using radioautography requires the calculation of the ratio of labelled nucleated cells to the total number of nucleated cells in the histological section. The absolute accuracy of such a measurement is limited by the number of five

micron tissue sections that can be taken from each lesion. It is statistically difficult to accurately index cell proliferation in small lesions using radioautographic methods due to the small section of tissue being examined [77]. As well, the large variability of cell proliferation between samples of lesions creates a certain discrepancy due to the difficulty of counting sufficient sections to obtain statistically reliable data of all parts of the lesion. Obtaining statistically reliable data of enough representative lesions can also hinder the accuracy of indexing cell proliferation. It becomes obvious that the *in vitro* method is more accurate over the *in vivo* histological method in terms of overcoming the potential sampling deficiency because it quantifies DNA synthesis in terms of the whole lesion.

Caution must however be exercised in the evaluation of DNA synthesis as an index of cell proliferarion, [81] as not all cells synthesizing DNA or incorporating tritiated thymidine into DNA go on to divide. Nevertheless, the measurement of DNA synthesis by determining tritiated thymidine incorporation still remains the method of choice by most investigators to measure cell proliferation and remains superior to the detection of mitotic figures and radioautography.

Although the results of this experiment appear not to substantiate the hypothesis put forward by Becker et al. [7], namely that quercetin rutinoside may be one factor in tobacco smoke responsible for it being a risk factor for atherosclerosis, the results must be interpreted with caution and remain speculative until further research is conducted into the role of quercetin rutinoside in this and other types of atherosclerosis.

The reasons for the discrepancy between the *in vivo* results and the *in vitro* findings of Becker et al. [7] are unknown, this discrepancy together with the many conflicting reports in the literature point to the need for a greater understanding of the role of quercetin rutinoside in altering the activity of mammalian cells.

SUMMARY

1. The difficulties encountered in testing a hypothesis based on *in vitro* data within an *in vivo* model were numerous.
2. Many factors could have potentially affected the biological activity of this flavonoid. These include the different pH values of the gastrointestinal tract and blood as well as the conjugation with glucuronides, sulfates, etc.
3. The biphasic or differential effects of quercetin rutinoside reported in the literature [19] may have exerted a slight inhibitory effect on cell proliferation. Smaller doses tend to stimulate cell proliferation whereas higher doses inhibit cell proliferation. There are many plant compounds that are biologically active in rather low concentrations, this applies specifically to the compounds of phenolic structure and preferentially to the flavonoids [23]. It is reasonable to speculate that if 20 mg of quercetin rutinoside were absorbed into the blood stream of a 3 kg rabbit, it would constitute a *large* dose, and thus possibly result in the inhibition of cell proliferation.

REFERENCES

1. Ross, R. The pathogenesis of atherosclerosis - an update. New England Journal of Medicine 314: 488, 1986.
2. Thomas, W.A., Kim, D.N. Atherosclesosis as a hyperplastic and/or neoplastic process. Laboratory Investigation 48: 245, 1983.
3. Campbell, G.R., Campbell, J.H. Smooth muscle phenotypic changes in arterial wall homeostasis: implications for the pathogenesis of atherosclerosis. Experimental and Molecular Pathology 42: 155, 1985.
4. Burke, J.M., Ross, R. Synthesis of connective tissue macromolecules by smooth muscle. International Review of Connective Tissue Research 8: 152, 1979.
5. Schwartz, S.M. Cellular Mechanisms in atherosclerosis. Annals of the New York Academy of Sciences 454: 321, 1985.
6. Arteriosclerosis 1981. Report of the Working Group on Arteriosclerosis of the National Heart, Lung, and Blood Institute, Vol. 1. U.S. Department of Health and Human Services. Public Health Service. National Intsitute of Health.
7. Becker, C.G., Hajjar, D.P., Hefton, J.M. Tobacco constituents are mitogenic for arterial smooth muscle cells. American Journal of Pathology 120: 1, 1985.
8. Wright, H.E., Burton, W.W., Berry, R.C. Soluble browning reaction pigments of aged Burley tobacco. 1. The nondialyzable fraction. Archives of Biochemistry and Biophysics 86: 99, 1960.
9. Stedman, R.L., Chamberlain, W.J., Miller, R.L. High molecular

weight pigment in cigarette smoke, Chemistry and Industry 1560, 1966.

10. Stedman, R.L., Benedict, R.C., Dymicky, M., Bailey, D.G. Composition studies on tobacco XXXVIII, Evidence for the origin of the high molecular weight, acidic pigment in cigarette smoke condensate. Beitrage zur Tabakforsch 5: 97, 1969.

11. Stedman, R.L. The chemical composition of tobacco and tobacco smoke. Chemical Reviews 68: 179, 1968.

12. Dillon, L., Glenn, F., Becker, C.G. Induction of acalculous cholecystitis and pneumonitis in dogs following inhalation of constituents of cigarette smoke condensate. American Journal of Pathology 109: 257, 1982.

13. Becker, C.G., Dubin, T. Activation of factor XII by tobacco glycoprotein. Journal of Experimental Medicine 146: 464, 1977.

14. Becker, C.G., Dubin, T., Glenn, F. Induction of acute cholecystitis by activation of factor XII. Journal of Experimental Medicine 151: 88, 1980.

15. Becker, C.G., Dubin, T., Wiedemann, H.P. Hypersensitivity to tabacco antigen. Proceedings of the National Academy of Sciences, U.S.A. 73: 1716, 1976.

16. Gotzos, V., Spreca, A., Conti, G. Action de O-(B-hydroxyaethyl)-rutosidea (HR) sur les fibroblastes de l'embryon de poulets cultives *in vitro*. Archives d'Anatomie Microscopique 60: 142, 1971.

17. Choy, J.W., Becker, C.G., Siskind, G.W. Effects of tobacco glycoprotein (TGP) on the immune system I. TGP is a T-independent B cell mitogen for murine lymphoid cells. Journal of Immunology

134: 3193, 1985.

18. Capelli, B., Conti, G., Laszt, L. Action du facteur P. sur les arteres de l'embryon de poulet cultivee *in vitro*. Angiologica 5: 28, 1968.

19. Huot, J., Nosal, G., Radouco-Thomas, C. Effets des flavonoides et de l'actinomicine D sur la proliferation et le developpement *in vitro* des cellules normales et neoplastiques. International Journal of Clinical Pharmacology 5: 257, 1971.

20. Chamley-Campbell, J.H., Campbell, G.R., Ross, R. Phenotype-dependent response of cultured aortic amooth muscle to serum mitogens. Journal of Cell Biology 89: 382, 1981.

21. Fowler, S., Shio, H., Wolinsky, H. Subcellular fractionation and morphology of calf aortic smooth muscle cells. Journal of Cell Biology 75: 166, 1977.

22. Chamley-Campbell, J.H., Campbell, G.R. What controls smooth muscle phenotype? Atherosclerosis 40: 348, 1981.

23. Kuhnau, J. The Flavonoids. A class of semi-essential food components: their role in human nutrition. World Review of Nutrition and Dietetics 24: 121, 1976.

24. Weiss, A. Uber das Rutin, vorgetragen beim apotheser-Gremium fur Mittelfranken in Baiern. Pharmaceutisches Central-Blatt 13: 903, 1842.

25. Krewson, C.F., Naghski, J. Occurrence of rutin in plants. American Journal of Pharmacy 125: 190, 1953.

26. Griffith, J.Q., Krewson, C.F. Rutin and Related Flavonoids: Chemistry, Pharmacology and Clinical Applications. Mack Publishing

Co., 1955.

27. Ivancevic, I., Knezevic, M. Die wirkung des rutins auf die durch vitamin D_2 hervongerufenen gewebsschadigungen. Archives of International Pharmaco-dynamics 86: 414, 1951.

28. Shaikemeleva, U.S. Effect of rutin on cholesterol concentration in canine lymph, blood and tissues. Byulleten' Eksperimental' noi Biologii i Meditsiny 95(3): 37, 1983.

29. Lobova, T.M. The effect of glutathione and vitamins B_1, B_{12}, rutin on the hepatic lipid content in experimental atherosclerosis. Patologisheskaia Fiziologiia i Eksperimentalnaia Terapiia 6: 36, 1962.

30. Heinitz, M. Experimentelle Untersuchungen zur Beeinflussung der Futterungsatherosklerose bei Huhnern durch intravenos applizierte definierte Wirkstoffe. Arztliche Forschung 17: 181, 1963.

31. Tseitina, A.Y., Lapina, S.A. The effect of vitamin P (rutin) on the blood serum cholesterol content and ascorbic acid level in the organs of experimental animals. Voprosy Pitanniia 23: 69, 1964.

32. Voskresensky, O.N., Bobyrev, V.N. The effect of ascorbic acid and rutin on the development of experimental peroxide atherosclerosis. Farmakologiia i Toksikologiia 42(4): 382, 1979.

33. Papageorge, E., Adair, F. Blood levels of certain constituents in normal adults before and after ingestion of rutin. Federation Proceedings 6: 283, 1947.

34. Papageorge, E., Mitchell, G.L. The effect of oral administration of rutin on blood, liver and adrenal ascorbic acid and liver and adrenal cholesterol in guinea pigs. Journal of Nutrition 37: 538,

1949.

35. Friedman, M. In: Pathogenesis of Coronary Artery Disease. McGraw Hill, 1969.

36. Weigensberg, B.I., Lough, J., More, R.H. Biochemistry of atherosclerosis produced by cholesterol feeding, thrombosis and injury. Experimental and Molecular Pathology 37: 175, 1982.

37. Shanno, R.L. Rutin: a new drug for treatment of increased capillary fragility. American Journal of Medical Science 211: 539, 1946.

38. Winternitz, M.C., Thomas, R.M. The Biology of Arteriosclerosis. Springfield, Ill.: Charles C. Thomas, 1938.

39. Barger, A.C., Beeuwkes III, R., Lainy, L.L. Hypothesis: vasa vasorum and neovascularization of human coronary arteries. New England Journal of Medicine 310: 177, 1984.

40. Baba, S., Furuta, T., Horie, M., Nakagawa, H. Studies on drug metabolism by use of isotopes XXVI: Determination of urinary metabolites of rutin in humans. Journal of Pharmaceutical Sciences 70: 780, 1981.

41. Griffiths, L.A., Barrow, A. Metabolism of flavonoid compounds in germ-free rats. Biochemistry Journal 130: 1161, 1972.

42. Murray, C.W., Booth, A. Absorption and metabolism of rutin and quercetin in the rabbit. Journal of the American Pharmaceutical Association, XLIII: 364, 1954.

43. Booth, A.N., Murray, C.W. The metabolic fate of rutin and quercetin in the animal body. Journal of Biological Chemistry 223: 256, 1956.

44. Griffiths, L.A., Barrow, A. The fate of orally and parenterally administered flavonoids in the mammal. The significance of biliary excretion. Angiologica 9: 167, 1972.

45. Levi, R., Zavecz, J.H., Burke, J.S., Becker, C.G. Cardiac and pulmonary anaphylaxis in guinea pigs and rabbits induced by glycoprotein isolated from tobacco leaves and cigarette smoke condensate. American Journal of Pathology 106: 318, 1982.

46. Alonso, D.R., Becker, C.G. Cigarette smoke condensate causes focal myocardial necrosis and pulmonary injury in rabbits. Federation Proceedings 40: 758, 1981.

47. Kitchevsky, D. Experimental atherosclerosis. In: Lipid Pharmacology. Ed. R. Paoletti, Academic Press, New York, 1963.

48. Clarkson, T.B. Animal models for atherosclerosis. North Carolina Medical Journal 32: 88, 1971.

49. Malinow, M.R., Maruffo, C.A., Perley, A.M. Aortic and coronary atherosclerosis induced in primates in the absence of hypercholesterolemia. Circulation 32 (Suppl II): 141, 1965 (Abstract).

50. Beckel, F. Atherogenesis in rabbits fed simulated human diet. Archives of Pathology 77: 566, 1964.

51. Duff, G.L. The nature of experimental cholesterol arteriosclerosis in the rabbit. Archives of Pathology 22: 161-182, 1936.

52. Duff, G.L., McMillan, G.C., Ritchie, A.C. The morphology of early atherosclerotic lesions of the aorta demonstrated by the surface in rabbits fed cholesterol. American Journal of Pathology 33: 845-860, 1957.

53. Parker, F., Odland, G.F. A correlative histochemical, biochemical

and electron microscopic study of experimental atherosclerosis in the rabbit aorta with special reference to the myointimal cell. American Journal of Pathology 48: 201, 1966.

54. Imai, H., Lee, T., Pastori, S., Panlilio, E. Atherosclerosis in rabbits. Experimental & Molecular Pathology 5: 273, 1966.

55. Geer, J., Haust, M.D. Smooth muscle cells in atherosclerosis. In Monographs on Atherosclerosis 2: 23, Karger, Basel 1972.

56. Still, W.J.S. An electron microscopic study of cholesterol atherosclerosis in the rabbit. Experimental & Molecular Pathology 2: 491, 1963.

57. Constantinides, P., Booth, J., Carlson, G. Production of advanced cholesterol atherosclerosis in the rabbit. Archives of Pathology 70: 712, 1960.

58. Mills, G.L., Taylaur, C.E. The distribution and composition of serum lipotroteins in eighteen animals. Comparative Biochemistry and Physiology 40B: 492, 1971.

59. Shore, B., Shore, V. Rabbits as a model for study of hyperlipidproteinemia and atherosclerosis. Atherosclerosis Drug Discovery. In: Advances in Experimental Medicine and Biology. C.E. Day (ed), Plenun Press, New York, 1975.

60. Shore, B., Shore, V., Hart, R.G. Changes in apolipoproteins and properties of rabbit very low density lipoproteins on induction of cholesterolemia. Biochemistry 13: 1579, 1974.

61. Vesselinovitch, D., Getz, G.S., Hughes, R.H., Wissler, R.W. Atherosclerosis in the Rhesus monkey fed three food fats. Atherosclerosis 20: 303, 1974.

62. Ehrhart, L.A., Holderbaum, D. Aortic collagen, elastin and non-fibrous protein synthesis in rabbits fed cholesterol and peanut oil. Atherosclerosis 37: 423, 1980.

63. Kritchevsky, D., Tepper, S.A., Vesselinovitch, D., Wissler, R.W. Cholesterol vehicle in experimental atherosclerosis. II. Peanut oil. Atherosclerosis 14: 54, 1971.

64. Kritchevsky, D., Davidson, L.M., Weight, M., Kriek, N.P.J., du Plessis, J.P. Influence of native and randomized peanut oil on lipid metabolism and aortic sudanophilia in the vervet monkey. Atherosclerosis 42: 53, 1982.

65. Kritchevsky, D., Tepper, S.A., Kim, H.K., Story, J.A., Vesselinovitch, O., Wissler, R.W. Experimental atherosclerosis in rabbits fed cholesterol-free diets. 5. Comparison of peanut, corn, butter and coconut oils. Experimental and Molecular Pathology 24: 389, 1976.

66. Vesselinovitch, O., Wissler, R.W., Schaffner, T.J., Borensztajn, J. The effect of various diets on atherogenesis in Rhesus monkeys. Atherosclerosis 35: 190, 1980.

67. Kritchevsky, D., Tepper, S.A., Vesselinovitch, E., Wissler, R.W. Cholesterol vehicle in experimental atherosclerosis. 13. Randomized peanut oil. Atherosclerosis 17: 237, 1973.

68. Wilson, R.B., Middleton, C.C., Sun, G.Y. Vitamin E, antioxidants and lipid peroxidation in experimental atherosclerosis of rabbits. Journal of Nutrition 108: 1858, 1978.

69. Harkness, J.E., Wagner, J.E. In: The Biology and Medicine of Rabbits and Rodents. Lea and Febiger, Philadelphia, 1977.

70. Pfeifer, K., Mehnert, W.-H., Huelsman, W. Vergliechende tierexperimentelle untersuchungen zur frage der konkrement-bildenden wirkung verschiedener rutinpraparate. Deutsches Gesundheitswesen 25: 386, 1970.

71. Cavallero, C., Turolla, E., Ricevuti, G. Cell proliferation in atherosclerotic plaques of cholesterol fed rabbits. Part I. Colchicine and H^3 thymidine studies. Atherosclerosis 13: 9, 1971.

72. McMillan, G.C., Horlick, L., Duff, G.L. Cholesterol content of aorta in relation to severity of atherosclerosis. Archives of Pathology 59: 289, 1955.

73. Munro, B.A., Visintainer, M.A. In: Statistical Methods in Health Care Research. J.B. Lippencott, Philadelphia, 1986.

74. Constantinides, I. In: Experimental Atherosclerosis. Elsevier, New York, 1965.

75. Stemerman, M.B. Effects of moderate hypercholesterolemia on rabbit endothelium. Arteriosclerosis 1: 25, 1981.

76. Spraragen, S.C., Bond, V.P., Dahl, L.K. Role of hyperplasia in vascular lesions of cholesterol-fed rabbits studied with thymidine-H^3 autoradiography. Circulation Research 11: 334, 1962.

77. McMillan, G.C., Stary, H.C. Preliminary experience with mitotic activity of cellular elements in the atherosclerotic plaques of cholesterol fed rabbits studied by labelling with tritiated thymidine. Annals of the New York Academy of Science 149: 699, 1968.

78. Potter, V.R. Metabolic products formed from thymidine. In:The Kinetics of Cellular Proliferation. Ed. by Stohlman, F., New York,

1959.

79. Staroscik, R.N., Jenkens, W.H., Mendelsohn, M.L. Availability of tritiated thymidine after intravenous administration. Nature 202: 456, 1964.

80. Hassler, O. Arterial cell renewal under hyperlipidemic conditions. Virchows Archiv Pathological Anatomy and Histopathology 352: 33, 1971.

81. Pelc, S.R. Incorporation of labeled precursors of DNA in non dividing cells. In: L.F. Lamerton and R. Fray (Eds.), Cell Proliferation, Philadelphia, PA, 1963, pp. 94-109.

82. Weigensberg, B.I., Schwarz, D., Alavi, M., Moore, S. Effect of quercetin rutinoside on cholesterol atherosclerosis in rabbits. Clinical and Investigative Medicine, Suppl. Vol. 9: 33, 1986.

TABLE 1: SUMMARY OF BECKER ET AL. [7] *IN VITRO* WORK: EFFECTS OF TOBACCO CONSTITUENTS AND POLYPHENOLS ON CULTURED AORTIC SMOOTH MUSCLE CELL PROLIFERATION EXPRESSED AS PERCENT INCREASE OVER CONTROL (AS MEASURED BY INCORPORATION OF TRITIATED THYMIDINE). PREINCUBATION TIME: 30 MINUTES.

Tobacco Constituent/Polyphenol	% Smooth Muscle Proliferation Increase Over Control
1. Control	
50 ug/ml	10
100 ug/ml	10
2. Quercetin[1]	
25 ug/ml	143.3
50 ug/ml	372.4
3. Chlorogenic Acid[1]	
25 ug/ml	28.7
50 ug/ml	189.2
4. Platelet Derived Growth Factor - type 1	
2 units	138
5. Tobacco Glycoprotein	
50 ug/ml	112
100 ug/ml	108
6. Quercetin Rutinoside Pre-incubated*	
50 ug/ml	85
100 ug/ml	83
7. Quercetin Rutinoside - BSA	
50 ug/ml	65
100 ug/ml	96
8. TAR	
50 ug/ml	58
100 ug/ml	63
9. Quercetin Rutinoside Unconjugated*	
50 ug/ml	25
100 ug/ml	65

[1] Polyphenols were incubated with 4×10^5 cells/well (24 wells/plate), with the remaining tobacco constituents incubated with 2×10^5 cells/well.

Standard error of the mean for each point averaged is 12%.

*Standard error of the mean for each point averaged is 8%.

TABLE 2: SUMMARY OF BECKER ET AL. [7] *IN VITRO* WORK: EFFECTS OF TOBACCO CONSTITUENTS AND POLYPHENOLS ON CULTURED AORTIC SMOOTH MUSCLE CELL PROLIFERATION EXPRESSED AS THE NUMBER OF CELLS x 10,000 (INCUBATION TIME: 84 HOURS).

Tobacco Constituent/Polyphenol	No. of SMC x 10,000
1. Control	6.75
2. Tobacco Glycoprotein 50 ug/ml	18.0
3. TAR 50 ug/ml	16.5
4. Quercetin Rutinoside - BSA 50 ug/ml	16.0
5. Quercetin 50 ug/ml	14.0
6. Chlorogenic Acid 50 ug/ml	11.0

All constituents, at a concentration of 50 ug/ml, were incubated with 2 x 10^4 cells/well.

Standard error of the mean for each point averaged is 15%.

TABLE 3: DIET COMPOSITION AND TREATMENT

Group	Control	Experimental
	g	g
Cholesterol	0.5	0.5
BHA	0.06	0.06
Peanut Oil	6.0	6.0
Ethanol	1.0	1.0
Quercetin Rutinoside	0.0	2.0
Food pellets	52.44	50.44
Total daily ration	60	60
Extra food pellets	ad lib	ad lib
Water	ad lib	ad lib

TABLE 4: MORTALITY, BODY AND ORGAN WEIGHTS

GROUP 1 - Control Group

	Initial Body Wt g	Final Body Wt g	% Change	Liver Wet Wt g	Spleen Wet Wt g	Aorta Delip Wt g
Mean	2577	2989	+18.3	140.9	2.80	91.6
SD	374	469	24.6	25.2	1.72	15.2
SE	100	125	6.6	6.7	0.46	4.1
N	15	15	15	15	15	15

MORTALITY RATE: 1 DEATH/15 RABBITS i.e. 7%

GROUP 2 - Experimental Group

	Initial Body Wt g	Final Body Wt g	% Change	Liver Wet Wt g	Spleen Wet Wt g	Aorta Delip Wt g
Mean	2990	2989	- 1.26	144.2	4.31	96.0
SD	411	335	15.10	35.6	3.23	24.3
SE	84	68	3.08	7.4	0.67	5.1
N	25	25	25	24	24	24

MORTALITY RATE: 14 DEATHS/40 RABBITS ON DIET i.e. 35%

STATISTICAL DIFFERENCE BETWEEN RESULTS OF GROUP 1 AND GROUP 2

t	3.1666	-0.0049	-2.3446	0.3324	1.8552	0.6697
p	<0.005	>0.995	<0.025	>0.70	>0.05	>0.50

TABLE 5: EXTENT OF AORTIC AND PULMONARY ATHEROSCLEROSIS

GROUP 1 - Control Group

	Percent Surface Area Covered with Lesion				
	Arch	Thoracic	Abdominal	Whole Aorta	Pulmonary Artery
Mean	57.9	14.4	9.7	20.9	20.5
+/- SD	24.0	21.4	12.1	18.4	16.4
+/- SE	6.4	5.7	3.2	4.9	4.4
N	1	1	1	1	1

GROUP 2 - Experimental Group

	Percent Surface Area Covered with Lesion				
	Arch	Thoracic	Abdominal	Whole Aorta	Pulmonary Artery
Mean	56.2	15.0	7.6	20.7	30.6
+/- SD	33.1	17.4	11.7	16.8	25.6
+/- SE	6.7	3.5	2.3	3.4	5.2
N	2	2	2	2	2
STATISTICAL DIFFERENCE BETWEEN RESULTS OF GROUP 1 AND GROUP 2					
t	-0.1859	0.0878	-0.5096	-0.0355	1.4783
P	>0.80	>0.90	>0.60	>0.95	>0.10

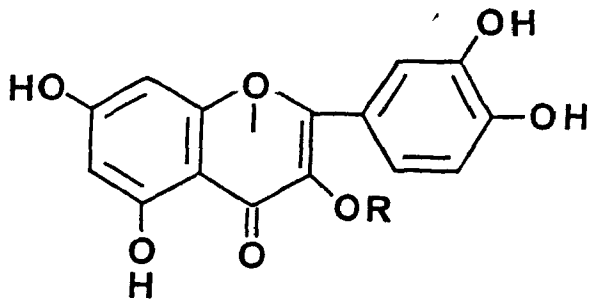
TABLE 6: TRITIATED THYMIDINE INCORPORATION INTO NEWLY SYNTHESIZED DNA AND TOTAL CHOLESTEROL

	TRITIATED DNA SYNTHESIS DPM per mg Delip Tissue				TOTAL CHOLESTEROL		
	Aorta Normal	Aorta Lesions	Aorta Mixed*	Pulm Mixed*	Aorta mg/100 mg Dry Wt	Pulm mg/100 mg Dry Wt	Serum mg/100 ml
GROUP 1 - Control Group							
Mean	518	4474	1128	3256	5.33	5.93	2534
SD	234	2539	892	1439	4.83	3.08	1711
SE	45	598	257	480	1.34	0.97	434
N	28	19	13	10	14	11	13
GROUP 2 - Experimental Group							
Mean	453	3481	1173	3779	4.55	6.11	2429
SD	275	2583	1429	2709	3.92	3.89	1781
SE	65	593	337	699	0.82	0.97	371
N	19	20	19	16	24	17	24
STATISTICAL DIFFERENCE BETWEEN RESULTS IN GROUP 1 AND GROUP 2							
t	-0.83388	-1.17877	0.10659	0.61616	-0.49670	0.27980	-0.17129
P	>0.40	>0.20	>0.90	>0.50	>0.60	>0.70	>0.80

*Mixed: lesion and non-lesion areas that could not be definitively separated.

Figure 1: Molecular Structure of Quercetin Rutinoside and Quercetin

Quercetin Rutinoside



R = glucose and rhamnose

Quercetin

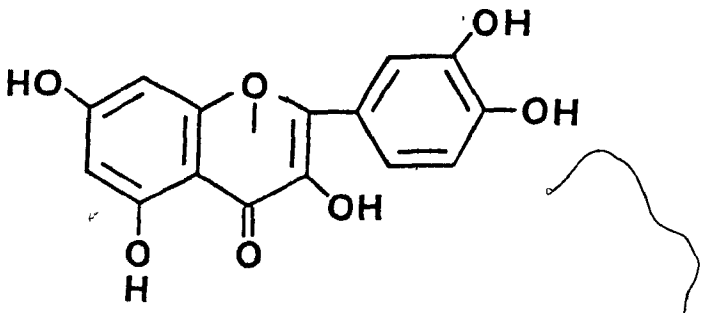


Figure 2

Figure 3

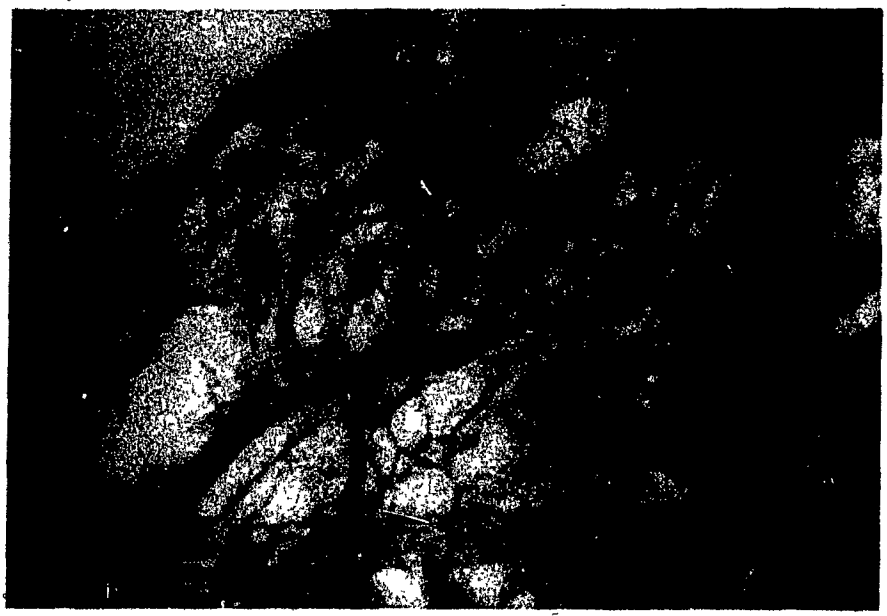


Fig. 2-5

Photomicrographs of some typical lesions of average severity taken from the aortae of rabbits on a cholesterol - peanut oil - BHA - ethanol enriched diet. These lesions are characterized by abundant cellular proliferation with considerable intercellular fibroproteins (collagen and elastin) and moderate amounts of extracellular lipid both in the intima and media. The moderate amounts of intercellular fibrillar material render the lesions reasonably dense. Masson Trichrome and Phosphotungstic acid-hemotoxylin positive spindle shaped cells can be easily distinguished underlying the intima. No difference with regard to the severity or fibrocellular morphology was observed between the treated and control aortae. The differences between the four micrographs reveal the inherent variability from rabbit to rabbit. Fig. 2-4: Masson Trichrome and Fig. 5: Phosphotungstic acid-hemotoxylin; x 1000. Note that the higher magnification makes the section look relatively thicker than it is.

Figure 4

Figure 5