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Gallic Acid: Inhibiting Angiogenesis in Adipose Tissue

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GALLIC ACID: INHIBITING ANGIOGENESIS IN ADIPOSE TISSUE

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

In

The Department of Human Ecology

By
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ABSTRACT

Angiogenesis is the process of developing and elongating blood vessels. In obesity, angiogenesis controls the development of the adipose tissue, allowing it to expand as energy stores increase. When angiogenesis is blocked in rodent models, adipose tissue not only stops expanding, but regresses, proving a possible mechanism for weight loss. Nt, a Chinese herbal decoction, and gallic acid, an active antiangiogenic ingredient in Nt, were tested in clinical trials in combination for possible use as a supplement for weight loss. The Nt-gallic acid combination did not cause weight loss or a decrease in food intake in humans, principally due to the inability to achieve adequate serum levels. The highest absorption seen was 19% of 800mg gallic acid, far below the level needed to cause weight loss. In further research, gallic acid was tested in combination with colchicine, a tubulin inhibitor. This combination was hypothesized to act synergistically in an *in vitro* assay to inhibit angiogenesis. The combination inhibited angiogenesis 91% compared to the control, while the addition of the two compounds separately led to an 81% inhibition. In addition, pilot data shows that this combination, when added to a cream base, may aid in the treatment of psoriasis. These studies suggest that gallic acid is capable of inhibiting angiogenesis in humans when adequate therapeutic levels are reached.

CHAPTER 1

INTRODUCTION

Obesity, defined as excess body fat, is gaining prevalence around the world. The estimated direct and indirect costs of obesity are nearly \$100 billion in the United States (Williamson et al., 1998). As of 2002, approximately 65% of adults in the US over the age of 20 were either overweight or obese; likewise, 31% of children between the ages of 6 and 19 were either overweight or obese (Hedley et al., 2004). The largest increases were seen in class III obesity, classified by a body mass index (BMI) of greater than 40 (Freedman et al., 2002). In addition, obesity is more common in the lower socio-economic classes (Freedman et al., 2002; Roberts et al., 2004).

Behavioral, environmental, genetic, and metabolic factors all contribute to the linear increase in the prevalence of obesity (Lyznicki et al., 2001). This rising occurrence of obesity is the result of thousands of years of genetic evolution toward a thrifty use of energy, combined with labor-saving technology, the availability of food to serve psychological needs, and an increased energy density of the foods we consume. Although all of these factors have a link to obesity, other factors may still remain undiscovered. As research continues to expand our knowledge, we will continue to absorb the cost of obesity until an effective treatment is identified.

Obesity increases the risk of developing other diseases that contribute to this health crisis. Excess adipose tissue is associated with insulin resistance and an increase in risk factors for coronary artery disease. This type of heart disease is associated with dysfunction of endothelial cells in response to inflammatory signals. Adipose tissue secretes many adipokines in proportion to its mass which promote this inflammatory response. These include TNF , IL-6, leptin, CRP, PAI-1, resistin, and angiotensin (Lau et al., 2005).

Coronary artery disease and obesity are also associated with high triglycerides, low HDL cholesterol, and small, dense LDL particles (Bray, 2004). Another condition that is closely tied to coronary artery disease and obesity is hypertension. Hypertension increases the workload of the heart, thickens the ventricular wall, and leads to congestive heart failure (Bray, 2004).

Gallbladder disease is yet another major risk attributed to obesity. Obesity is associated with increased cholesterol turn-over resulting in supersaturated bile and the potential to precipitate into gallstones (Bray, 2004).

Perhaps the most well known disease related to obesity is type 2 diabetes mellitus. The risk of developing diabetes continues to increase the longer a person is obese, and also with the central distribution of fat (Bray, 2004). As previously discussed, this is due to insulin resistance resulting from a higher level of fatty acids circulating in the blood. These fatty acids are eventually stored in the liver and muscle as part of the insulin resistance process.

Other manifestations of cardiovascular disease originate from the metabolic disorders that accompany obesity. There is a strong correlation between obesity and a number of different forms of cancer, including cancer of the colon, prostate, gallbladder, and breast (Bray, 2004). Although insulin and IGF-1 are growth factors elevated in obesity thought to be related to the increased risk of cancer in the obese, increased estrogen due to aromatization in fat tissue may also play a role in endocrine related cancers such as cancer of the breast.

There are also several disorders stemming from excess adipose tissue related to the amount of weight the body must carry in an obese state. One of these is a type of sleep apnea, which is caused in the obese by an increase in pressure on the lungs due to the larger amount of fat in the abdomen, which displaces the diaphragm further into the chest cavity (Bray, 2004). In chronic situations this would also lead to other respiratory problems due to the lungs' inability to expand and contract properly.

Likewise, osteoarthritis is another mechanical problem caused by obesity. A strong correlation has been shown between increasing weight and joint problems due to osteoarthritis (Felson et al., 1988). Since many nonweight-bearing joints also display increased osteoarthritis, weight may be only one of many factors contributing to this disease.

The current recommendations for treating obesity involve an initial weight loss of 5-10% followed by 1-2 pounds of weight loss per week until a suitable maintenance goal has been achieved. This plan consists of dietary therapy, increased physical activity, behavior modification, in moderate cases pharmacotherapy, and in extreme cases surgery (Lyznicki et al., 2001). Therapeutic drugs and effective herbal supplements are useful tools to decrease food intake and increase energy expenditure, and several other methods of treating overweight and obesity are now being explored as well (Greenway et al., 2005a; Roberts et al., 2005a; Roberts et al., 2005b; Roberts et al., 2005d). Inhibiting angiogenesis, the process of developing and elongating blood vessels, is one of these promising methods that is being explored for facilitating weight loss.

Angiogenesis

Angiogenesis plays an important role in all tissues during development, from the formation of the fetus during early development through adolescence in humans. Once the body's major phase of growth has ended, angiogenesis is only seen in a few instances. These include wound healing, menstruation in females of child-bearing age, cancer growth, the adverse formation of excessive vasculature seen in the retinopathy, and in other angiogenesis driven diseases including obesity (Folkman, 1982). The capability of adipose tissue to be dynamic in size is an exception, since few other tissues are capable of altering size through angiogenesis.

Adipose tissue must be able to grow and recede based on the availability of and need for fat by peripheral tissues such as skeletal muscle. This dynamic endocrine relationship regulates

the disposition of the adipose tissue as an entire mass. When energy intake surpasses the energy demand of the body, the size of the mass increases. Conversely, when the body signals a need for the release of these fatty acids, the size of the tissue diminishes as the fat droplets release fatty acids into the blood. Each of these situations will be examined in more detail from a vascular perspective.

Folkman, who was studying the growth of cancerous tumors, noted that these tumors were unable to grow past approximately two millimeters in diameter without angiogenesis initiating additional blood supply (Folkman, 1982). The same holds true in adipose tissue (Neels et al., 2004). As the adipocytes begin to swell with lipids in a storage mode, there is a barrier beyond which the cell can no longer expand. This calls for the development of new adipocytes from preadipocytes, which will need an adequate blood supply to provide nutrients to the cells and an adequate vascular network to send fatty acids to peripheral tissues.

This process occurs through a complex system of paracrine signals and environmentally related hypoxic conditions. Acting in tandem, these conditions promote the formation of new adipocytes and endothelial cells from stem cell precursors. This process is amplified by adipocyte metabolism and hormone secretion (Lolmede et al., 2003).

Hypoxia, a lack of oxygen within a tissue, increases the acidity of the interstitial fluid. This acidosis induces angiogenesis due to the lack of adequate vasculature within transplanted fat pads in mouse experiments (Neels et al., 2004). When the fat pads are transplanted, a necrotic core rapidly develops that continues radially from the center of the pad until adequate angiogenesis has occurred. At this point the necrosis is replaced by reforming adipocytes and connective tissue.

It has been hypothesized that this same process occurs on the outskirts of a developing adipose tissue mass as the adipocytes signal the growth mechanism (Crandall et al., 1997).

Minute changes in oxygen and nutrient availability spawn the release of pro-angiogenic adipocytokines that may recruit endothelial cells to expand the preexisting vasculature (Planat-Benard et al., 2004). Another hypothesis asserts that endothelial cells are signaled to develop new vessels, and this process initiates the transformation of preadipocytes into adipocytes (Hausman and Richardson, 2004).

It is currently unclear as to the order in which this process occurs, and multiple studies have been published that support conflicting mechanisms (Fukumura et al., 2003; Hausman and Richardson, 2004). There is some evidence however that the answer to this question may depend on the fat depot location and the stage of development. During fetal development, it is clear that angiogenesis predates adipocyte differentiation (Crandall et al., 1997). This comes as no surprise, since a vascular structure must be established before appreciable development can be seen.

After adolescence, however, the mechanism becomes more difficult to determine. It appears that the location of the adipose tissue will influence which process initiates the other, but more research is needed in order to clearly define this relationship (Neels et al., 2004). In any event, an increase in dietary fat in the blood that surpasses the body's demand will be stored by the adipocytes, and an expanded vasculature must be made to accommodate it.

All of the primary cells needed to fuel this vascular reaction appear to reside within the stroma-vascular structure of adipose tissue (Miranville et al., 2004). This stroma-vascular fraction seems to hold the precursors to both the endothelial cells and the adipocytes. There is also some evidence that the adipocytes themselves can digress towards the progenitor cells and actually redifferentiate into endothelial cells (Planat-Benard et al., 2004). This remarkable capability seems to be localized to white adipose tissue, as brown adipose tissue is not capable of the same transformation.

When adipocytes are placed in a culture conducive to vascular growth, they lose fatty acids, change their morphology to a more preadipocyte-like structure, and become a fibroblast-like cell that attaches to a coverslip (Planat-Benard et al., 2004). The cells then lose the enzymatic characteristics of adipocytes that promote lipolysis, lipogenesis, and adipocyte molecular marker expression. When these cells are cultured in a cellular matrix, they form branched alignments and display markers normally present on endothelial cells (Planat-Benard et al., 2004). Most importantly, these cells do not show the markers of circulating endothelial cells that are derived from macrophages or monocytes, proving that they reside within the adipose tissue.

These endothelial cells, when activated, stimulate revascularization when introduced into the mouse ischemic hind limb model, and this process shows a strong positive correlation with BMI (Miranville et al., 2004; Planat-Benard et al., 2004). Eventually, additional endothelial cells are recruited from other areas to the hypoxic conditions, and this response allows the adipocytes to develop and multiply more rapidly.

The development of endothelial cells can take one of two paths, and it has not until recently been clear which is employed in adipose tissue. One route is by the process of angiogenesis as described above. The other is by a process called neovascularization, which is the recruitment of circulating endothelial cell precursors to the site of new blood vessel formation (Neels et al., 2004). The distinction between the two, although seemingly trivial, is of paramount importance.

The concept of neovascularization would imply a systemic effect of hypoxia induced factors within the body as a whole, but this does not seem to be the case. Neovascularization functions on the broad basis of tissue injury or more localized vastly affected hypoxic and anoxic tissues (Neels et al., 2004). Neovascularization is supplemented by endothelial precursor cells

that circulate in the peripheral blood. These cells, once recruited to the site of injury, will rapidly divide to form new vessels, causing an immense chain reaction of vascularization.

Angiogenesis, in contrast, appears to work in adipose tissue on a much more localized basis, secreting factors that recruit the nearest vasculature to multiply and divide. Angiogenesis results in a much more controlled and precise method of vascular growth since only the nearest cells are affected. This was shown to be the primary mechanism in adipose tissue growth by immunofluorescent staining for endothelial progenitor cells, which was overwhelmingly negative in the vasculature of the adipose tissue (Neels et al., 2004).

Since it is now known that angiogenesis plays a vital role in the expansion of adipose tissue, the potential to inhibit this process can play a major part in preventing weight gain and promoting weight loss. The traditional method of reducing the fat mass involves decreasing caloric intake so the body has to use its fat stores to maintain energy expenditure level. The increasing prevalence of obesity during the last twenty years in the face of national goals to decrease its prevalence is a testimony to the ineffectiveness of this dietary approach to treatment. Inhibiting angiogenesis rather than limiting calories may be more effective since adipose tissue is incapable of receiving an adequate oxygen supply if angiogenesis is inhibited.

There have been many potent angiogenesis inhibitors used in rat and mouse studies to date. One of the most effective angiogenesis inhibitors in mice is TNP-470. It does not seem to produce any adverse side effects in rodents and appears to affect metabolism and food intake rather than behavior (Brakenhielm et al., 2004).

Leptin deficient *ob/ob* mice treated with TNP-470 show a decrease in weight and BMI over a 12 week period (Brakenhielm et al., 2004). This decrease is due to a lack of adipose tissue development with growth but no muscle loss at autopsy. Even more interesting, mice treated with TNP-470 had a significant decrease in body weight and BMI compared to pair-fed

controls. This loss of body weight came from subcutaneous and omental fat depots, and seemed to not affect muscle tissue.

Since most cases of obesity in our society, unlike *ob/ob* mice, are not related to an autosomal recessive mutation, wild type mice have also been studied with TNP-470 administration in combination with a high fat diet (Brakenhielm et al., 2004). The treated mice decreased subcutaneous, omental, and perigonadal fat depots, but had no change in food intake or basal resting metabolic rate. The only significant change was seen in total cholesterol, LDL cholesterol, triglycerides, and serum insulin, all of which decreased. In both the *ob/ob* and *wt* mouse studies, the TNP-470 groups reduced vascularization within the fat pads, and the average volume of the adipocytes decreased approximately 10-fold. Smaller fat cells are associated with improved insulin sensitivity and decreased risk of developing type 2 diabetes mellitus (Gunton et al., 2005; Ravussin and Smith, 2002).

An assay was developed to study the process of angiogenesis in human placental vein tissue, since rodent models may not predict the response of human disease (Gulec and Woltering, 2004; Woltering et al., 2003). This assay can also be used to test antiangiogenic drugs in a patient's tumor before administering chemotherapy drugs, so the best drug can be matched to the individual patient and tumor. We have modified this assay for use in human adipose tissue collected from abdominal surgeries (Greenway et al., 2005c). This *in vitro* assay should better define the action of angiogenesis inhibitors in obesity since it uses human fat tissue.

This assay takes surgically removed abdominal fat, cuts it into pieces approximately two millimeters cubed, and places them in 96 well plates embedded in a fibrin-thrombin clot. After the clot has set for 24 hours, media is added in addition to any test compounds. This media is changed every 48 hours, and an angiogenic index is calculated based on the amount of growth at each time point.

The tissue in each well is visually divided into four quadrants, the number of blood vessels sprouting directly from the fat mass is counted, and a score from one to four is given to each quadrant based on the number of vessels (Figure 1.1). The sum of all the quadrants yields an index plotted over thirteen to fifteen days needed for the assay. By this point significant angiogenesis will have occurred in the control group, and most of the endothelial growth will be in the initial phases of vascular development.

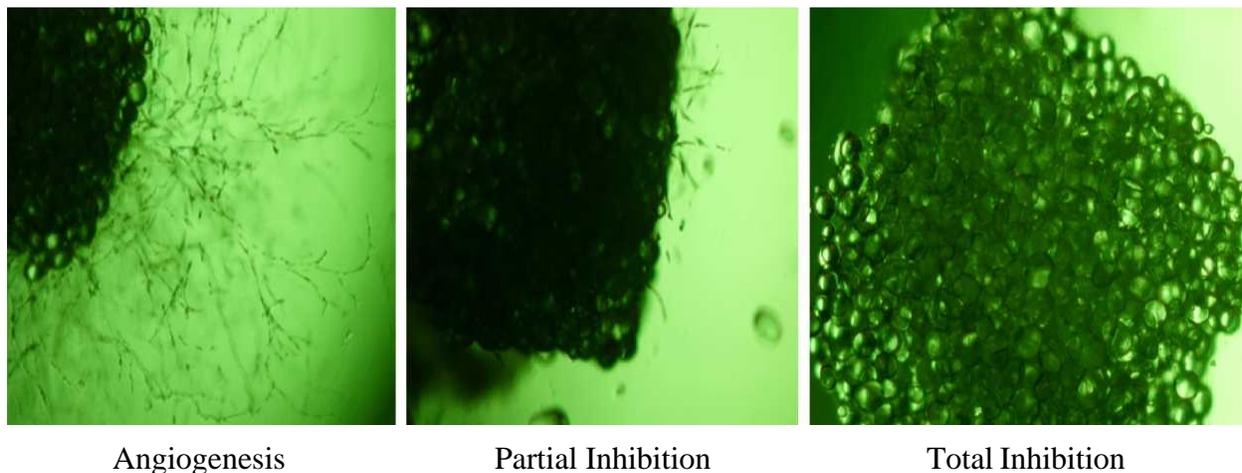


Figure 1.1: Endothelial Vessel Growth in an Angiogenesis Assay. Used with the permission of Frank Greenway.

The decrease in body weight seen in obesity experiments with angiogenesis inhibitors seems to contradict the mechanisms involved in angiogenesis. When the angiogenesis inhibitors are used, one might surmise that development of adipose tissue would cease, and other mechanisms would have to be employed to reduce the fat mass. However, there are several current hypotheses as to why the drastic reduction in fat mass is seen.

The most likely explanation is that the endothelial cells seen in adipose tissue are constantly susceptible to vascular remodeling and are programmed to initiate apoptosis unless the interstitial hormonal environment remains favorable for growth or stability (Rupnick et al., 2002). This hypothesis would suggest that adipose tissue endothelial cells remain in a constant

immature state compared to other weight stable organs. Consequently, the adipose tissue could rapidly expand or contract based on the prevailing metabolic needs of the organism. This would also explain why adipose tissue is capable of growing so rapidly when other tissues remain totally weight stable.

Many factors other than hypoxia are capable of altering the angiogenic capacity of adipose tissue. There are hormones secreted by the adipose tissue itself, and other hormones secreted by the hypothalamus and intestine (Vettor et al., 2005). There is an intricate process of stimulation and inhibition that must be tightly controlled by the tissue. In fact, it is not only the adipocytes that help regulate this process, but also the adipose stromal cells that make up the connective tissue surrounding the fat cells. These two tissues secrete factors that assist in breaking down tissue barriers and promote the propagation of endothelial cells through the tissue.

The first of these factors, matrix metalloproteinases (MMPs), are enzymes that degrade the extracellular matrices surrounding the fat tissue (Hausman and Richardson, 2004). This, in effect, prepares the tissue to be penetrated by new cells. In most tissues, including adipose tissue, there is extremely tight regulation on the molecules and cells that leave the vasculature and enter the tissue. The MMPs help facilitate the entry of new endothelial cells into adipose tissue by loosening the grasp of the matrix on fat cells.

The plasminogen enzymatic system also is another class of enzymes that participates in the stimulation of angiogenesis. These enzymes help to break down the basement membrane surrounding the vasculature separating the artery from the adipose tissue (Hausman and Richardson, 2004). Endothelial cells are connected by tight junctions, which allow only the smallest nutrients and particles through. Endothelial cells are further encapsulated by a basement membrane which prevents unwanted cells from penetrating the tissue.

This system is modulated by plasminogen activator inhibitor-1 (PAI-1), and high PAI-1 levels are associated with hypercoagulability. Mice with mutations in the PAI-1 gene have shown decreases in adipose endothelial cells, but interestingly no decrease in adipose mass or development (Hausman and Richardson, 2004). Although it can be assumed that this is due to a primary effect of PAI-1 on the vasculature, there must be additional underlying mechanisms that are affecting this system.

The two factors discussed above are vital in allowing the vasculature to develop, but there must be a signal that stimulates their secretion more reliably than insulin or a lack of leptin. Indeed there is, and this compound is called vascular endothelial growth factor (VEGF). VEGF is the principal factor that controls angiogenesis in adipose and many other tissues. Its secretion causes a chain of events the severity of which depends on the amount of VEGF secreted (Claffey et al., 1992).

VEGF is a protein whose expression is stimulated by protein kinase C- and A- mediated pathways (Claffey et al., 1992). In endothelial cells, it is the action of VEGFR-2 receptor that plays a pivotal role in modulating the physiological and pathological changes associated with angiogenesis induction (Fukumura et al., 2003). Angiogenesis induction causes the activation of the MMPs and PAI-1, which result in angiogenesis. VEGF is positively correlated with BMI and visceral fat in addition to the induction of angiogenesis (Miyazawa-Hoshimoto et al., 2003).

Many other hormones help regulate this process, and the mechanisms of several are still not fully understood. What is clear at this point is that inhibiting angiogenesis in rodents leads to a chain of events that result in weight loss and reliable weight maintenance. Although the compounds most effective in rodents have been discussed, these compounds do not always inhibit angiogenesis in humans. However, gallic acid inhibits angiogenesis in rodents and in human assays *in vitro* (Greenway et al., 2005b; York et al., 2005).

Nt and Gallic Acid

Nt, a traditional Chinese medicinal herbal supplement containing rhubarb, has long been used in China to help treat and prevent obesity. Nt is made by soaking the leaves and roots of five herbs, freeze drying the liquid to a solid and using the solid as the supplement. The makeup of this herbal decoction is 40% rhubarb root and stem, 13.3% astragalus root, 13.3% red sage root, 26-27% tumeric, and 6-7% dried ginger. It was delivered orally in capsules during the human trials.

Nt in Chinese studies caused weight loss in rats when orally gavaged. In three separate experiments obese rats lost an average of 30-40% of their weight compared to the untreated controls. Large drops in triglycerides, total cholesterol, and LDL cholesterol were seen, accompanied by an increase in HDL cholesterol (unpublished data; (Greenway et al., 2005b). These encouraging preliminary results were confirmed in the United States.

York *et al* performed a two month weight loss study in Wistar rats prior to the use of Nt in human clinical trials (York et al., 2005). Two doses of Nt were studied along with a positive control (fenfluramine), a pair-fed group, and the saline-treated control. The experimental and pair-fed groups gained between 25% and 35% less body weight compared to the controls, validating the Chinese studies and showing that Nt's mechanism of action lies in decreasing food intake. No adverse effects were seen with the exception of occasional loose stools, supporting the safety of Nt and encouraging initiation of a clinical trial.

A two month human pilot trial was performed by Greenway *et al* to examine the effects of Nt on food intake and body weight (Greenway, 2005). The doses of Nt given were 1/6 and 1/12 the doses given to the rats in the previous study, as converted using Kleiber's 0.75 mass exponent equation (Heusner, 1982). The treated groups did not show any significant change in food intake or weight loss, presumably due to the low doses employed. Unfortunately, there

were treatment emergent adverse events related to loose stools. These gastrointestinal side effects were most likely associated with the sennosides found in the herbal extracts. These side effects prevented using higher doses of Nt in humans.

Concurrently with these experiments, an active ingredient in Nt was identified. Through successive fractional and mass spectrometry, gallic acid was found to be an active compound in rhubarb, the main ingredient in Nt (unpublished data). Gallic acid is found in many other herbs as well, and has been shown in rat studies to inhibit food intake (Glick, 1981; Liu et al., 2005) and *in vitro* to inhibit angiogenesis (Liu et al., 2005). When rhubarb and Nt were tested in the angiogenesis assay described earlier, both were potent angiogenesis inhibitors.

The mechanism of action by which Nt causes weight loss was not clear, until angiogenesis inhibition was demonstrated. Several experiments in the 1960s through the 1980s studied oral and parenteral gallic acid in rats (Glick, 1981; Glick and Joslyn, 1970b; Joslyn and Glick, 1969; van der Heijden et al., 1986). The consistent finding in these studies was the reduction in food intake during the first several days of treatment. The rats given gallic acid gained less weight than the control group, but the weight gain in both groups increased in parallel.

Several early studies of gallic acid in rodents raised concerns regarding anemia and liver toxicity in the rats. Thus, a formal toxicology study was needed to demonstrate that the decreased food intake was not due to illness induced by gallic acid.

Studies were performed in 2001 in both mice and rats demonstrating the no observed adverse event level (NOAEL). There was an absence of carcinogenicity or toxicity at doses effective in decreasing food intake (Niho et al., 2001; Rajalakshmi et al., 2001). These studies were consistent with the studies in rats done by York *et al.* A dose-response curve of gallic acid on angiogenesis was performed using the angiogenesis assay previously described. Gallic acid

completely inhibited angiogenesis at 10^{-3} M (Greenway et al., 2005b). Partial inhibition was seen at 10^{-4} M.

Since the human pilot study by Greenway *et al* was unable to provide efficacious levels of Nt without side effects, the first goal of this thesis is to initiate a human trial to study Nt in combination with additional gallic acid. This should curb any increase in side effects, since gallic acid lacks the sennosides seen in Nt. Research involving gallic acid given in tea has shown that the level absorbed reaches 10^{-6} M, which would presumably be increased to effective levels by giving several log orders more of gallic acid (Shahrzad et al., 2001). Delivery of effective levels of gallic acid should be accompanied by a decrease in food intake and body weight.

The pharmacokinetic profile of gallic acid will also be examined as the second phase of this study. This will give definitive data concerning the absorption rate and serum levels of the gallic acid using timed blood draws and urine collection. Knowing the pharmacokinetics will determine optimal dosing and insure that toxic build-up of gallic acid will not occur.

Gallic Acid and Colchicine

Another possible strategy for therapeutic use of gallic acid would be to combine it with other compounds known to inhibit angiogenesis by a different mechanism. This will be the second focus of this thesis. Such a combination of antiangiogenic drugs could be synergistic and a more effective treatment of angiogenesis driven diseases. Based on blood vessel morphology in the assay using human fat, colchicine inhibits angiogenesis by a mechanism different than gallic acid (Chen et al., 2003; Levy et al., 1991). Thus, the combination of colchicine and gallic acid may be a superior treatment for angiogenesis-driven diseases.

Colchicine is an alkaloid extracted from a plant grown in Asia Minor. It has been used to treat a variety of diseases including liver cirrhosis, Mediterranean fever, and scleroderma

(Thomas et al., 1989). Colchicine has also been used continuously for the treatment of gout since the sixth century. Colchicine inhibits microtubule formation in cells that rely on complex intracellular structures to survive, including endothelial cells (Levy et al., 1991). Microtubule inhibition halts the elongation of endothelial cells, resulting in a breakdown of the intracellular and extracellular matrix.

While this effect may seem detrimental, there are diseases that can be controlled by this inhibition at concentrations that do not adversely affect healthy tissue. One such condition is psoriasis, where the epidermal tissue differentiation cycle time is reduced, resulting in increased tissue density and thickness (Kaidbey et al., 1975). Thick scaly patches of skin develop on the body with increased redness due to angiogenesis supplying the newly formed cells with oxygen and nutrients.

Colchicine has been used with some success in treating psoriasis as an ointment rather than orally or intravenously. Topical colchicine administration avoided side effects such as nausea, vomiting, or abdominal cramping associated with oral parenteral use (Kaidbey et al., 1975). The most effective dose of colchicine in these studies was a 1% concentration in a hydrophilic ointment, although hydrophilic petrolatum based ointments worked nearly as well.

The purpose of combining gallic acid and colchicine into a topical ointment is to inhibit angiogenesis by two different mechanisms. The gallic acid will act by inhibiting the recruitment and proliferation of the endothelial cells, while the colchicine will stop microtubule formation and adhesion capability within these cells. This dual action may result in the ability to use these herbal extracts at lower concentrations than what would be needed for treatment with either used alone. This potential synergism may widen the therapeutic potential of angiogenesis inhibitors.

CHAPTER 2

THE SAFETY AND EFFICACY OF A DIETARY HERBAL SUPPLEMENT AND GALLIC ACID FOR WEIGHT LOSS

Introduction

Nt is an herbal supplement derived from a water extract of 40% rhubarb root and stem (*radix et rhizoma rhei*), 13.3% astragalus root (*radix astragali*), 13.3% red sage root (*radix salviae miltiorrhizae*), 26-27% turmeric (*rhizoma curcumae longae*), and 6-7% dried ginger (*rhizoma zingiberis officinalis*). Nt caused weight loss and reduced weight gain in rodents in China (Wei K, 2003). and its ability to suppress weight gain in rodents was confirmed by York et al. in the USA (York et al., 2005).

A pilot study testing the efficacy and safety of Nt to induce weight loss in humans produced diarrhea as a result of sennosides, herbal laxatives present in the Nt, but was otherwise well tolerated (Greenway, 2005). Nt was demonstrated to also contain gallic acid, a compound known to give weight loss and reduce food intake in rodents when given either orally or parenterally (Glick, 1981). Therefore, in an effort to reduce the side effects of Nt and preserve weight loss efficacy, a mixture of Nt (20% by weight) with herbal gallic acid derived from gall nuts (80% by weight) was tested for safety and efficacy in treating human obesity.

Gallic acid is generally recognized as safe (GRAS) in the form of gallotannins by the FDA and has been used as an antioxidant in food. Toxicology studies in rodents show that the no-observed-adverse-effect-level (NOAEL) is 120 mg/kg, a dose equivalent to 2.9 grams in a 70 kg man (Niho et al., 2001). Gallic acid is available from herbal sources that can be used to increase the concentration in Nt. The human equivalent amount of gallic acid in the low dose of Nt shown to be effective for weight loss in rodents is 1.2 grams per day, less than half the NOAEL (Heusner, 1982).

An 8-week pilot study was performed at the Pennington Center in eight healthy females with a BMI between 25 and 35 kg/m² on no regular medications except birth control or hormone replacement therapy. Subjects were randomized to two placebo capsules three times a day or two capsules each containing 200 mg of gallic acid from an herbal source and 50 mg of crude Nt extract given three times a day, for a total of 1.2 grams of gallic acid and 300 mg of crude Nt extract per day. This dose of gallic acid was less than 50% of the NOAEL and the amount of Nt was only 5% of the dose shown to give loose stools. Weight loss in the Nt-gallic acid group was 3% of body weight compared to 1.8% in the placebo group (p=0.38). Compliance by pill count was >98% and there were no significant changes in blood pressure, pulse, complete blood counts or chemistry panel values throughout the trial. There were no adverse events felt to be related to the treatment and there were no changes in the physical examinations or electrocardiograms.

Results of the pilot study were used to power a 6-month clinical trial testing the safety and efficacy of two doses of the Nt-gallic acid mixture. One dose was the same as that used in the pilot trial, i.e. 1.2 grams of gallic acid per day, and the second dose contained 2.4 grams of gallic acid per day, below the 2.9 grams per day determined to be the NOAEL calculated from the metabolic mass equation using the data from rats (Heusner, 1982).

Materials and Methods

Main Study Design

One hundred five healthy volunteers between the ages of 18 and 60 years of age with a BMI between 25 and 35 kg/m², on no chronic medication other than oral contraceptives or hormone replacement therapy, participated in this 24-week study. Subjects had height, weight and blood pressure measured, pulse taken, and a CBC (hemoglobin, hematocrit, mean corpuscular volume, platelets, white blood cell count, neutrophil number, and eosinophil number) and chemistry panel (creatinine, uric acid, potassium, glucose, albumin, calcium, magnesium,

iron, creatine phosphokinase, alanine leucine transaminase, alkaline phosphatase, total cholesterol, triglycerides, high density lipoprotein cholesterol, and low density lipoprotein cholesterol) done at screening. A registered dietitian at baseline instructed subjects in a diet 700 kcal/d below weight maintenance requirements based on the World Health Organization formula (World Health Organization, 1990). At baseline, an electrocardiogram, medical history and physical examination were done, and women had a pregnancy test performed.

Subjects were randomized to two doses of an Nt mixture (20% Nt and 80% gallic acid) or placebo. The Nt enriched with gallic acid was given at either 1.2 grams of gallic acid and 300 mg of Nt per day or at 2.4 grams of gallic acid and 600 mg of Nt per day vs. placebo in a 1:1:1 ratio. Capsules contained 200 mg of gallic acid and 50 mg of Nt or placebo. Subjects took 2 capsules 3 times a day. On each visit (weeks 0, 2, 4, 8, 12, 16, 20, and 24), subjects were weighed, had their blood pressure measured, their pulse taken, their medications dispensed, medication bottles returned, and subjects were questioned about any adverse events. The physical exam, CBC, chemistry panel, pregnancy test and electrocardiogram were repeated on week 24 at the end of the study.

Food Intake

Subjects had food intake evaluated with a universal eating monitor at screening and at week two of the study. Subjects visited the food intake laboratory after an overnight fast during which only water was allowed. Subjects completed Visual Analogue Scales (VAS) of appetite and a questionnaire about factors that might affect taste such as colds or allergies. They were then given 2 pills to swallow and the VAS was repeated after 1 hour. An hour after receiving the pills the subjects were given a meal of sandwich quarters, chips, and cookies larger than they could reasonably eat, and they were allowed to eat as much or as little as they wished for 20 minutes. At the completion of the meal, subjects completed the VAS again to conclude a pre-

and post-meal evaluation. The subjects were given placebo on the first visit and the treatment to which they were randomized on week 2.

Pharmacokinetic Sub-Study

Five healthy men between the ages of 18 and 60 years with a BMI between 20 and 35 kg/m² were enrolled. The subjects took no regular medication and were not lactose intolerant. Subjects had a medical history, physical examination and chemistry panel performed at screening. All subjects received a gallic acid-free diet of white bread, cheese, butter, and water or milk for the day prior to and the day of each pharmacokinetic test lasting through the end of a 24 hour urine collection.

The first two subjects had two pharmacokinetic tests one week apart. The first subject had one test with gallic acid from gall nut extract 800 mg after an overnight fast and the other test was with gallic acid from gall nut extract 800 mg along with a 570 kcal meal consisting of a grilled cheese sandwich and a glass of milk. The second subject had one test with gall nut extract 800 mg combined with Nt 200 mg after an overnight fast and the other test was with gallic acid from gall nut extract 800 mg combined with Nt 200 mg along with the same 570 kcal meal. The mixture of 800 mg gallic acid from gallnut extract and 200 mg Nt was the same as the high dose of the Nt-gallic acid mixture used in the trial.

Subjects reported fasting on the morning of the test and took test medication orally. Ten cc blood samples were collected at times 0, 30, 60, 90, 120, 150, 180, 240, 360, and 480 minutes. The blood was collected in heparinized tubes, centrifuged, plasma separated and frozen until analyzed on high-pressure liquid chromatography (HPLC) for gallic acid and gallic acid metabolites.

The second two subjects had one test day after an overnight fast with gallic acid from gall nut extract 800 mg without a meal. The fifth subject had one test day after an overnight fast in

which he let 800 mg of gallic acid from gall nut extract contained in hard candy dissolve in his mouth. All subjects emptied their bladders at time 0, and an aliquot of the urine was frozen for later analysis. All urine was collected for the 24 hours following the gallic acid ingestion. The urine volume was measured and an aliquot was frozen for later measurement of gallic acid and gallic acid metabolites.

Statistics

The study was designed to extend over 24 weeks with interim analysis at 8 weeks. Thus, the study was powered for two endpoints, the interim endpoint at 8 weeks and the 24-week endpoint. The first power calculation for the 8-week time point was based on a standard deviation of 4 kg, a value derived from Pennington weight loss studies at 8 weeks. Since the expected difference between the 2.4 gram gallic acid group and placebo was 3 kg at 8 weeks based on the pilot study, there was an 80% power to detect this difference at 8 weeks with 29 subjects finishing in each group at $p < 0.05$. Allowing for a 20% dropout rate, one needed to enroll 35 subjects per arm.

The second power calculation was for the 24-week time point and was based on a standard deviation of 6.5 kg, a value derived from Pennington weight loss studies at 24 weeks. Since the expected difference between the 2.4 gram gallic acid group and placebo was 5 kg at 24 weeks based on the pilot study, there was an 80% power to detect this difference at 24 weeks with 28 subjects finishing in each group at $p < 0.05$. Allowing for a 20% dropout rate, one needed to enroll 35 subjects per arm.

Repeated measures analysis of variance was used to compare weight loss in the Nt-gallic acid and placebo groups. The interim analysis was performed at 8 weeks and a final analysis at 24 weeks. The primary study endpoint was body weight. Secondary endpoints were food intake and the safety measures (laboratory, adverse events and electrocardiograms). The study was

powered to detect a 3 kg difference at 8 weeks and a 5 kg difference at 24 weeks at 80% power with an alpha of 0.05.

Results

Efficacy

Weight Loss

The planned interim analysis was performed at 8 weeks. Baseline weights in the placebo, low dose and high dose groups were 83.3 ± 2.05 kg (SEM), 84.1 ± 2.53 kg, and 82 ± 2.18 kg respectively. The placebo group behaved as expected with an average weight loss of 0.7% body weight (0.6 ± 0.42 Kg, SEM). Subjects receiving the low dose Nt-GA mixture (1.2 gm Nt – 300 mg gallic acid) lost 1.2% body weight (2.6 ± 1.3 kg) that was statistically different than placebo ($p < 0.05$). Subjects on the high dose Nt-GA mixture (2.4 gm Nt - 600 mg gallic acid) lost 0.6 % body weight (0.46 ± 0.74 kg), an amount that was not different from placebo.

After all subjects were enrolled, the interim analysis showed no evidence of efficacy, so the trial was terminated. Thus, the number of subjects completing the trial was less than anticipated. At the end of 24 weeks, there were 19 subjects in the high dose group, 20 subjects in the low dose group and 25 subjects in the placebo group. The final weight loss at 24 weeks was 1.05 ± 0.54 (SEM) kg in the placebo group, 2.01 ± 1.18 kg in the low dose group and a gain of 0.54 ± 0.79 kg in the high dose group. The difference between the high and low dose groups was statistically significant ($p = 0.044$), but weight loss in the Nt-GA groups was not significantly better than placebo (Figure 2.1).

Food Intake

Food intake was measured in the eating laboratory at Weeks 0 and 2. One-way analysis of variance (ANOVA) indicated that the three groups' food intake (kcal) did not differ significantly at Week 0, $F(2, 102) = 1.37$, $p = 0.26$. Dependent t-tests indicated that food intake

(kcal) decreased significantly from Week 0 to Week 2 for the high dose group only, $t(32) = 2.33, p < 0.05$. Nevertheless, independent samples t -tests indicated that the food intake difference scores (food intake at Week 2 minus Week 0) did not differ between the placebo and low dose group, $t(70) = -0.39, p = .70$, or the placebo and high dose group, $t(67) = 67, p = .73$.

The change in food intake from Week 0 to Week 2 by group is illustrated in Figure 2.2.

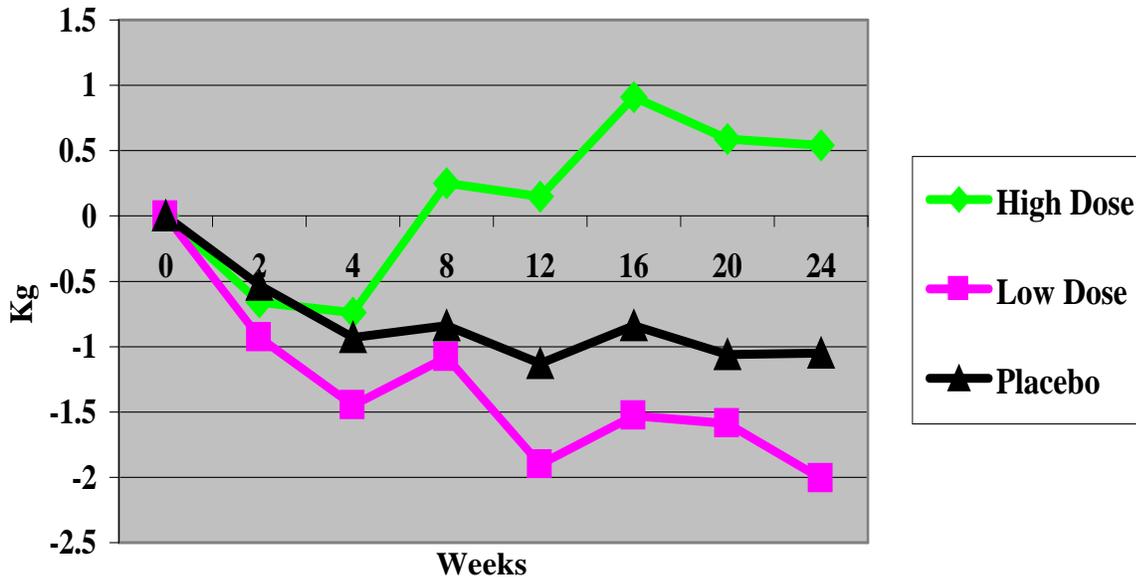


Figure 2.1. Nt-Gallic Acid 24-Week Weight Loss.

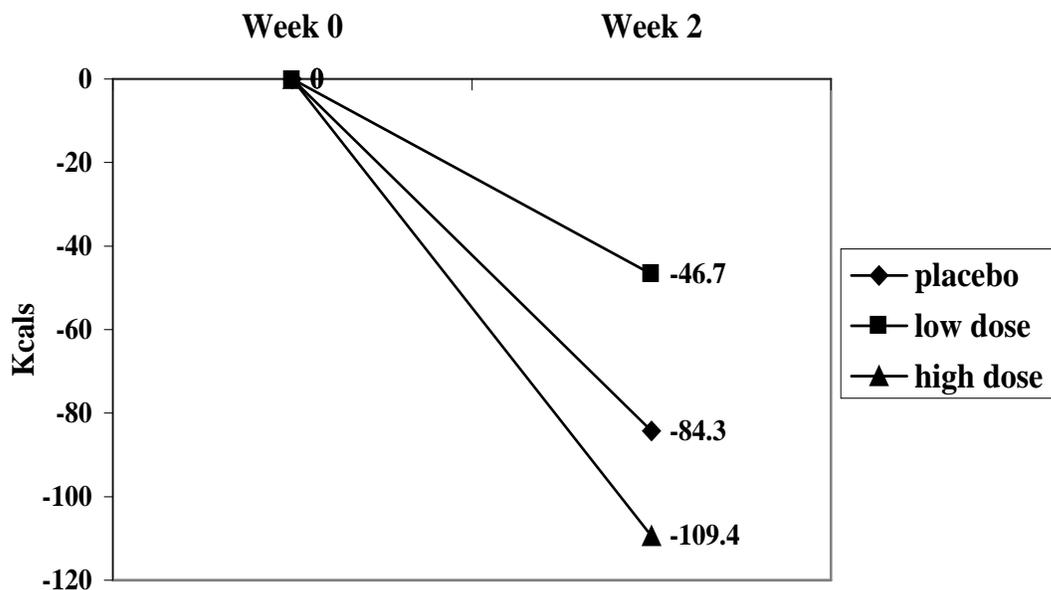


Figure 2.2. Change in Food Intake (kcal) from Week 0 to Week 2 by Group.

Safety

Blood Pressure and Pulse Rate

Systolic blood pressure rose 1.9 ± 2.3 (SD) mm Hg in the placebo group, 1.7 ± 2.1 mm Hg in the high dose group and fell 1 ± 1.8 mm Hg in the low dose group. Diastolic blood pressure rose 1.9 ± 1.5 mm Hg in the high dose group, 0.43 ± 1.9 mm Hg in the low dose group and 0.8 ± 1.3 in the placebo group. The pulse rate rose 2.6 ± 2.5 bpm in the high dose group, 3.2 ± 2.4 bpm in the low dose group and 1.0 ± 1.9 in the placebo group. None of these differences were statistically significant.

Complete Blood Counts, Chemistry Panel, Liver Function and Lipids

Hemoglobin and hematocrit fell significantly in the high dose Nt-gallic acid group compared to the placebo group: -0.51 ± 0.21 vs. -0.02 ± 0.12 gm (SEM) and -1.9 ± 0.6 vs. -0.62 ± 0.65 % respectively ($p < 0.05$). Uric acid dropped more in the high dose group than in the low dose or placebo groups; -0.71 ± 0.24 vs. -0.05 ± 0.12 vs. -0.16 ± 0.12 mg/dL respectively ($p < 0.05$). There were no other dose related changes in the laboratory testing. These laboratory values were fluctuations within the normal range and none of the changes were clinically significant.

Compliance

Pill counts showed that the subjects in the low dose group took $94.8 \pm 0.8\%$, subjects in the placebo group took $93 \pm 0.8\%$ and the subjects in the high dose group took $92.2 \pm 1.5\%$ of the pills prescribed. These compliance numbers did not differ between the groups.

Pharmacokinetics

The subject who took Nt-gallic acid (200 mg – 800 mg) on two occasions, once fasting and once with food had a peak gallic acid concentration in the plasma of 10 uM without food at 4 hours and a peak plasma concentration of 8 uM with food at 2 hours (Figure 2.3). The subject

who took gallic acid 800 mg on two occasions, once fasting and once with food had a peak gallic acid concentration in plasma of 2 uM without food at 2 hours and a peak plasma concentration of 7 uM with food at 4 hours (Figure 2.4). The three subjects who had 800 mg of gallic acid orally when fasting had a peak plasma concentration of gallic acid in the plasma of 4 uM at 2.5 to 3 hours (Figure 2.5). The one subject who let candy containing 800 mg of gallic acid dissolve in his mouth fasting had a peak plasma concentration of gallic acid of 9 uM at 2 hours (Figure 2.6). Measurement of gallic acid in the urine revealed that the capsules of gallic acid with or without Nt had about 6% absorption. Eating increased absorption to the 9-12% range. By allowing for complete dissolution and allowing for possible trans-mucosal absorption using candy containing gallic acid dissolved in the mouth increased absorption further to 19% (Figure 2.7).

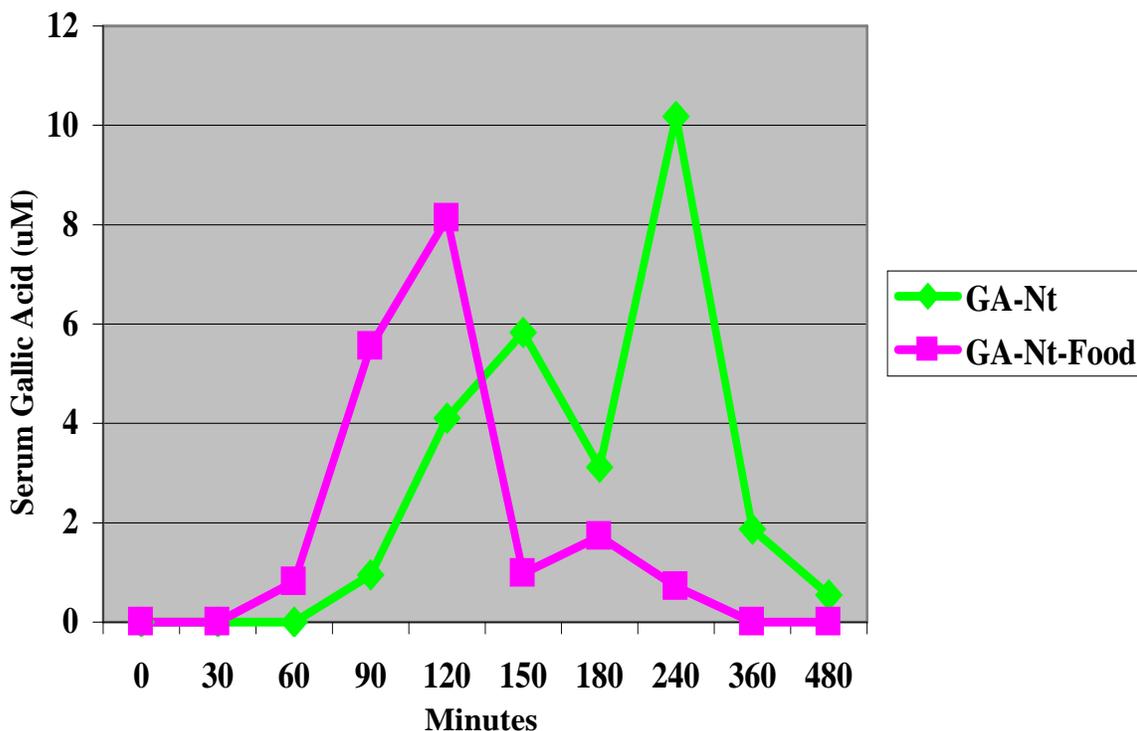


Figure 2.3. Gallic Acid-Nt With and Without Food. Gallic Acid 800 mg with Nt 200 mg with and without Food in 1 subject on 2 occasions.

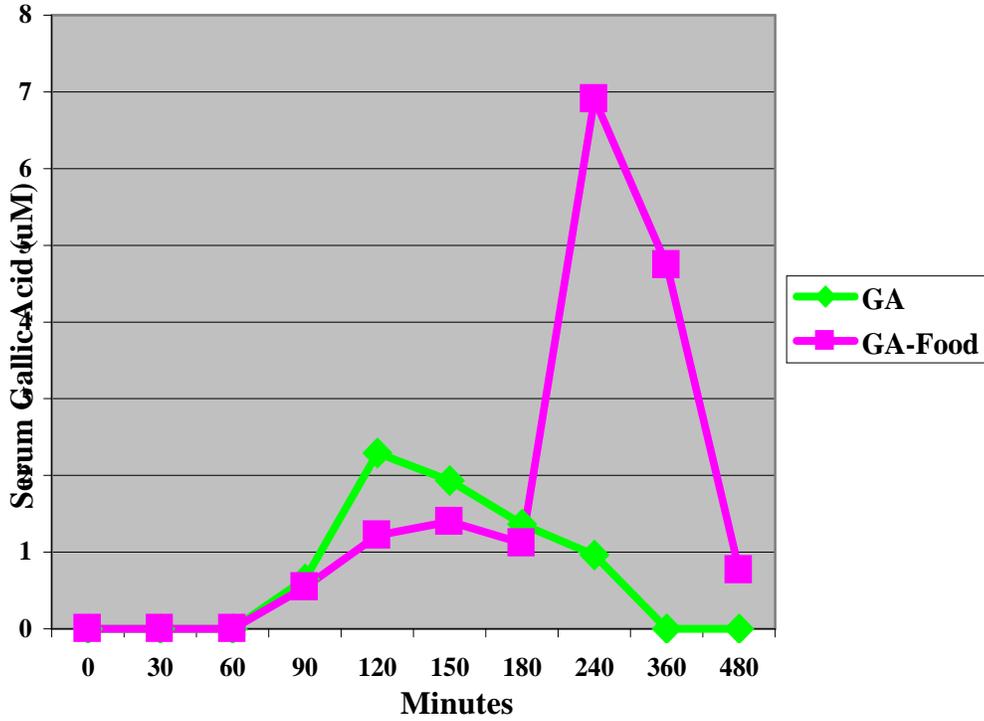


Figure 2.4. Gallic Acid With and Without Food. Gallic Acid 800 mg with and without food in one subject on two occasions.

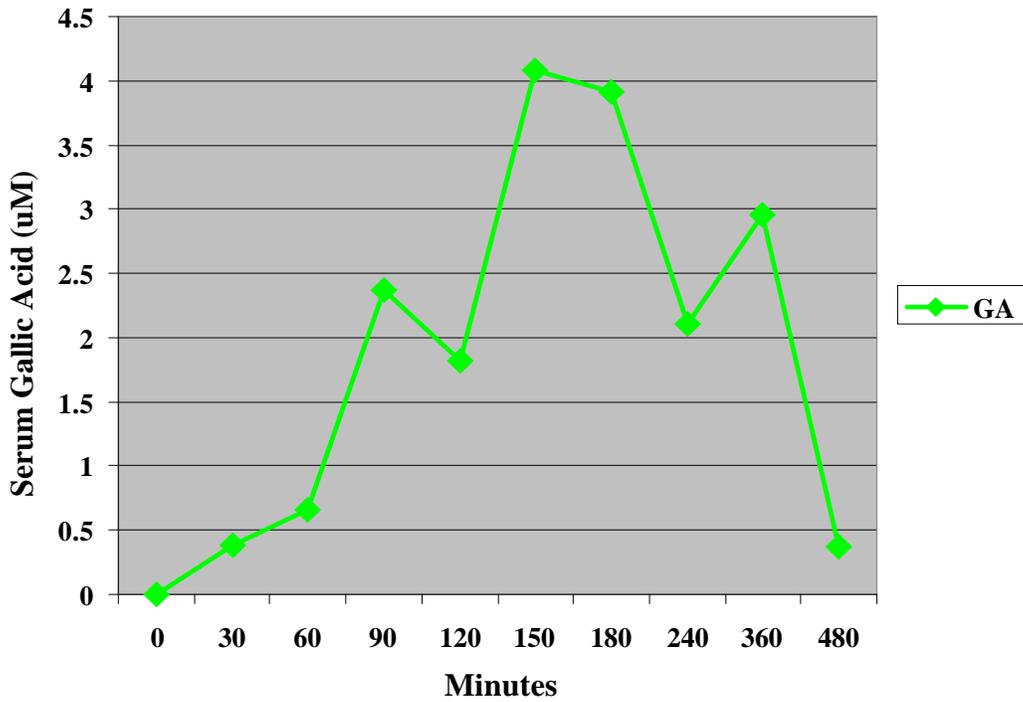


Figure 2.5. Oral Administration of Gallic Acid. Serum levels of gallic acid in three subjects after oral ingestion of 800 mg.

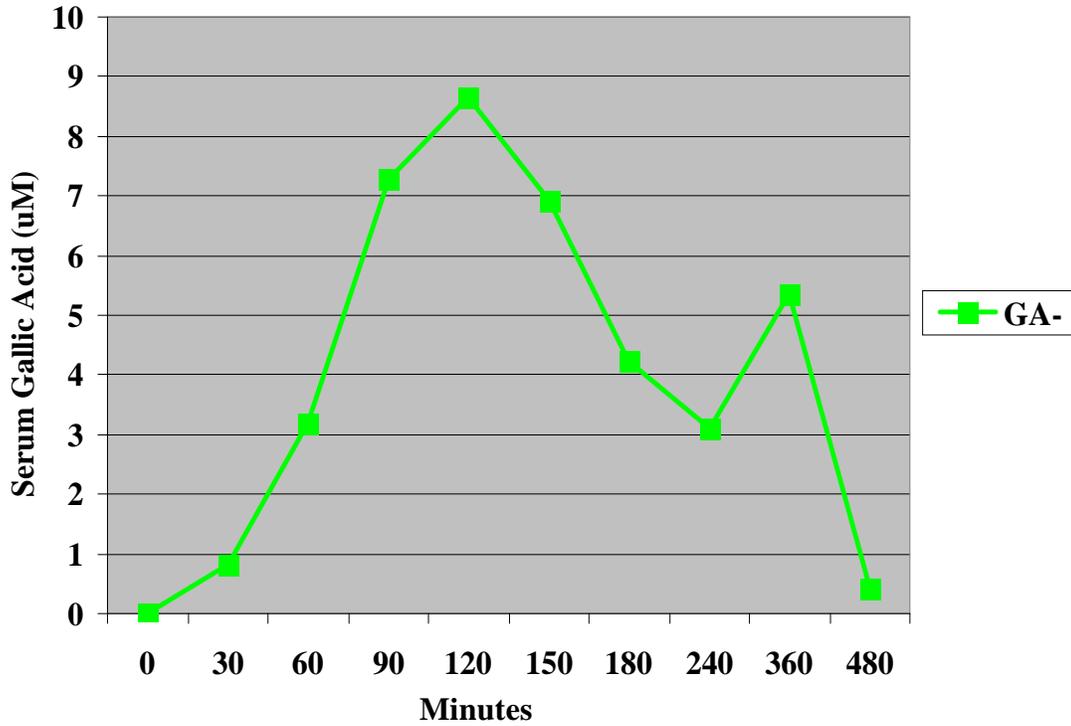


Figure 2.6. Gallic Acid Administered in Candy. Serum levels of gallic acid in one subject after letting candy with 800 mg of gallic acid dissolve in the mouth fasting.

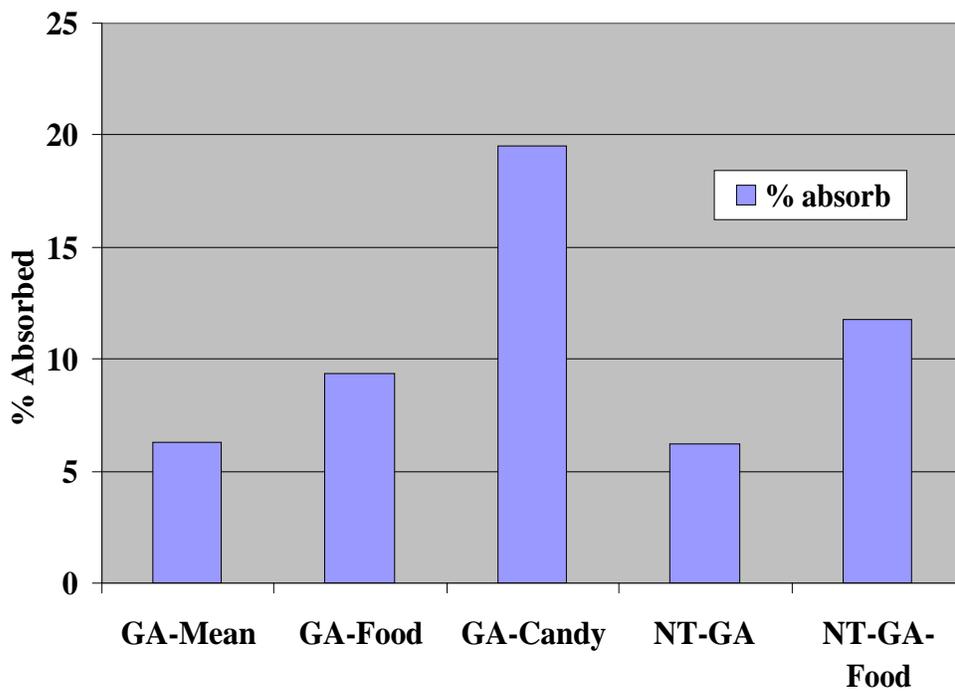


Figure 2.7. The Percent of Gallic Acid Absorbed Orally. The percent absorption of Gallic Acid from the gastrointestinal tract and the effect of food on absorption.

Discussion

When gallic acid was fed to rats at levels of 2%, 4%, 5%, 6%, 8% and 10% of diet, food intake was depressed and there were no deaths up to 5% of the diet. At 5% of diet, gallic acid depressed food intake by 50% with a similar reduction in body weight compared to control (Joslyn and Glick, 1969). There was a marked increase in hepatic fat in rats fed a diet with 5% gallic acid, 36% vs. 7% of dry weight, which was not seen with similar levels of tannic acid (Glick and Joslyn, 1970b). Fecal protein excretion increased with feeding of the diet supplemented with tannic acid, but was not significantly different from control with diets supplemented with gallic acid (Glick and Joslyn, 1970a). Gallic acid was also shown to decrease food intake when infused intraperitoneally as a 2% solution. Thus, the mechanism by which gallic acid decreases food intake involves more than taste aversion or gastrointestinal factors (Glick, 1981).

These studies with gallic acid along with evidence that Nt contained gallic acid encouraged us to explore the use of herbal gallic acid combined with Nt for the treatment of obesity in humans. The no observed adverse effect level (NOAEL) in rodents is 120 mg/kg, a dose equivalent to 2.9 grams per day in a 70 kg man using the metabolic mass equation (Heusner, 1982; Niho et al., 2001). The daily doses of the herbal gallic acid used in this study were up to 2.4 grams, well below the NOAEL in these obese subjects. The doses of Nt used in this study did not exceed 600 mg, one tenth of the dose that gave loose stools in a clinical trial of Nt.

The Nt-gallic acid mixture was well tolerated and loose stools were not a problem. Disappointingly, the combination did not give weight loss. In fact, the high dose gave less weight loss than the placebo. This was surprising in view of the experience in rodents in which food intake was suppressed.

A human pharmacokinetic study of gallic acid by Shahrzad et al. demonstrated that 40% of a 50 mg dose of gallic acid was absorbed and achieved a peak serum concentration between 1.8 and 2.1 micromolar (1.8-2.1 times 10^{-6} molar) at 1 hour and 10 minutes (Shahrzad et al., 2001). We developed an HPLC method to measure gallic acid and did pharmacokinetic studies with the high dose of Nt-gallic acid (Nt 200 gm and gallic acid 800 mg) and with the gallic acid it contained. Only 6% was absorbed giving a peak serum concentration of 4 micromolar (4 times 10^{-6} molar). Food appeared to increase absorption, and letting hard candy containing 800 mg of gallic acid dissolve in the mouth further increased absorption.

Even in the best of circumstances the absorption never exceeded 19% and the plasma levels never exceeded 10 micromolar (10 times 10^{-6} molar). This suggests, but does not prove, that gallic acid absorption is limited in humans by an active transport system that becomes saturated far below the levels needed to get levels of gallic acid into the plasma that are adequate to suppress food intake.

Unfortunately, the Nt - gallic acid pilot data that looked so encouraging for weight loss appears to have been misleading due to the small numbers of subjects which precluded obtaining a statistically significant result. The information generated by this trial is still important to report. The animal data were very encouraging that gallic acid would be effective and safe for weight loss, and it is likely that someone else would have explored this possibility. This trial gives evidence that gallic acid will not be an effective oral supplement for the treatment of human obesity.

CHAPTER 3

GALLIC ACID AND COLCHICINE INHIBIT ANGIOGENESIS SYNERGISTICALLY

Introduction

Colchicine, a tubulin inhibitor, inhibits angiogenesis at 10^{-8} M and higher concentrations (Stafford et al., 2005). Gallic acid is another inhibitor of angiogenesis derived from gallotannins which are natural plant products (Liu et al., 2005). Gallotannins inhibit angiogenesis by blocking the effect of VEGF on endothelial cells (Lee et al., 2004). 4-O-methylgallic acid and the gallotannin, penta-O-galloyl-beta-D-glucose inhibit the angiogenesis stimulated by basic fibroblast growth factor in addition to blocking endothelial cell VEGF production (Huh et al., 2005; Jeon et al., 2005). Tannic acid also selectively inhibits the interaction of CXCL 12/stromal cell-derived factor-1 alpha with its receptor, CXCR4, an interaction that participates in angiogenesis (Chen et al., 2003). Thus, since these two inhibitors act through different mechanisms, combining them might be synergistic or additive for inhibiting of angiogenesis. We hypothesize that gallic acid and colchicine will inhibit angiogenesis when combined.

Materials and Methods

Tissue Preparation

Fat removed at surgery was cut into fragments approximately 1 mm thick and 2 mm in diameter. These fat fragments were placed in 96-well plates as described below.

Preparation of the 96-well Plates

The 96-well plates were loaded with human thrombin solution (0.05 IU in 4mL/well).

Loading the 96-well Plates

The fat fragments were placed in the prepared 96-well plates and covered with 100 μ l clotting media. The clotting media consisted of fibrinogen (3 mg/ml) (Sigma Chemical Co., St. Louis, Missouri) and epsilon amino caproic acid (0.5%) (Sigma Chemical Co., St. Louis,

Missouri) in human placental vein angiogenesis model (HPVAM) media. HPVAM media consists of Medium 199 (GibcoBRL, Gaithersberg, Maryland), and antibiotic/antimycotic solution (100 U penicillin, 100 U streptomycin sulfate and 0.25 mcg amphotericin beta per ml) (GibcoBRL, Gaithersberg, Maryland). The mixture was allowed to clot by incubation in 6% CO₂, 94% air at 37°C in a humidified incubator. After the media had gelled overnight, the fat-containing clot was supplemented with 100 mcL HPVAM media containing 20% fetal bovine serum (GibcoBRL, Gaithersberg, Maryland). The total volume of each well was 200 mcL.

Angiogenic Evaluation

All wells were evaluated for angiogenic sprouting by examination at 40X and 200X magnification by an unbiased observer using an inverted microscope on Mondays, Wednesdays, and Fridays. Angiogenesis was defined by two criteria. First, the number of wells (as a percentage of the total plated) that developed new vessel growth was termed initiation. This was defined by the presence of at least three angiogenic sprouts of approximately 0.5 mm in length growing from the periphery of the fat tissue. The degree of angiogenic response was assessed using a semi-quantitative visual rating scale (Bosman, 1994). The tissue was visually divided into four quadrants. Each quadrant was given a numeric score from 0-4 based on the neovessel's length, density and percentage of the quadrant's circumference involved with the angiogenic response. Numeric results from the four quadrants were summed and expressed as an angiogenic index (AI, 1-16). This rating scale has been validated by Hornick et al using multiple independent observers (Hornick et al., 2003).

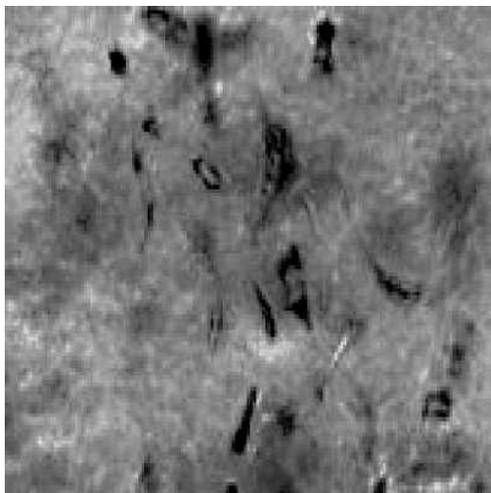
Experiments

Colchicine was tested at concentrations of 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰ and 10⁻¹¹ M. Gallic acid was tested at concentrations of 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ M. Colchicine and gallic acid in combination were tested at the concentrations of colchicine 10⁻⁹ M with gallic acid 10⁻⁴

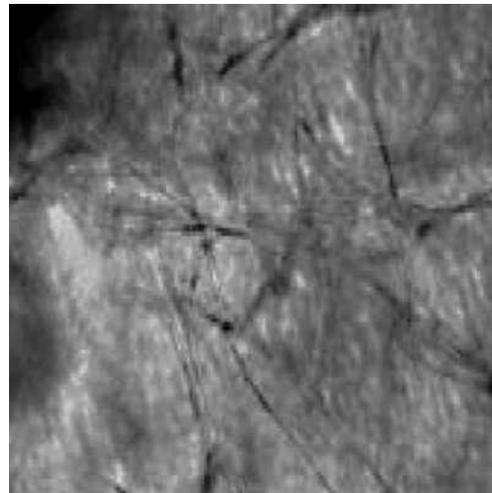
M, colchicine 10^{-10} M with gallic acid 10^{-5} M, and colchicine 10^{-11} M with gallic acid 10^{-6} M, since these concentrations were physiologically achievable.

Results

Preliminary assays showed that the optimal concentration for colchicine to test for synergism was 10^{-10} M and gallic acid 10^{-5} M. These concentrations, when combined, were partially inhibitory compared to combinations of colchicine 10^{-9} M and gallic acid 10^{-4} M, which were completely inhibitory, and colchicine 10^{-11} M and gallic acid 10^{-6} M, which did not inhibit angiogenesis compared to the control. Using intermediate concentrations allowed a better interpretation of possible synergism, since each concentration alone was able to block angiogenesis compared to the control, but the combination did not result in complete inhibition. In addition, higher levels of colchicine resulted in beading of the blood vessels in the assay, possibly leading to eventual tissue injury (Figure 3.1). This beading was not seen at inhibitory concentrations of gallic acid, another indication that these two compounds had different mechanisms by which they inhibited angiogenesis.



Colchicine



Control

Figure 3.1. Vascular Beading Caused by Colchicine.

The results of the assay in which colchicine, gallic acid and the combination was compared to control is shown in Figure 3.2. Colchicine 10^{-10} M inhibited angiogenesis 31% compared to the control, while gallic acid 10^{-5} M inhibited angiogenesis 50% compared to the control. Consequently, if a strictly additive effect were to be seen, the combination group would give an 81% reduction in angiogenesis. However, the combination of colchicine 10^{-10} M and gallic acid 10^{-5} M inhibited angiogenesis by 91% compared to the control, indicating that these two angiogenesis inhibitors act in a synergistic rather than a strictly additive manner.

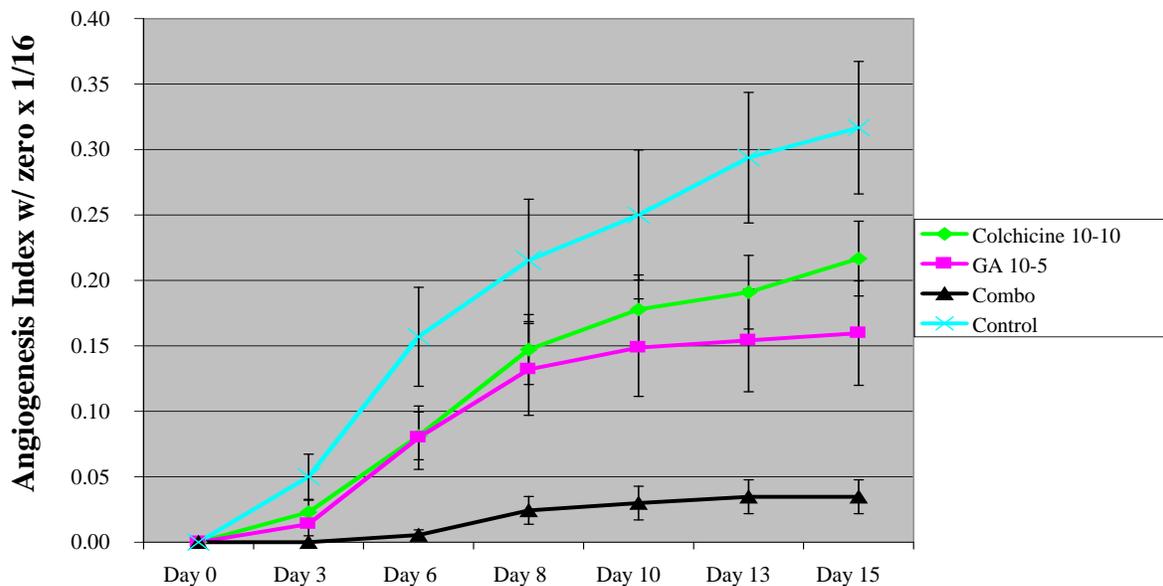


Figure 3.2. Colchicine and Gallic Acid Inhibit Angiogenesis Synergistically.

Discussion

As hypothesized, colchicine and gallic acid were synergistic in inhibiting angiogenesis. Angiogenesis plays a role in the etiology of many diseases, and is involved in the pathogenesis of psoriasis (Leong et al., 2005). In fact, topical colchicine has been used as a treatment for psoriasis (Kaidbey et al., 1975), and gallic acid has been included in topical psoriasis treatments as well (Bosman, 1994). In fact, pilot work in our clinic with 0.2 M gallic acid cream gave resolution of long standing psoriasis lesions over 16 weeks (Figure 3.3). Therefore, it is likely

that the combination of gallic acid and colchicine will be even more effective than either of the separate components, and since lower doses can be used in the combination, the potential for adverse events, which was low when the components were used separately, will be further reduced.

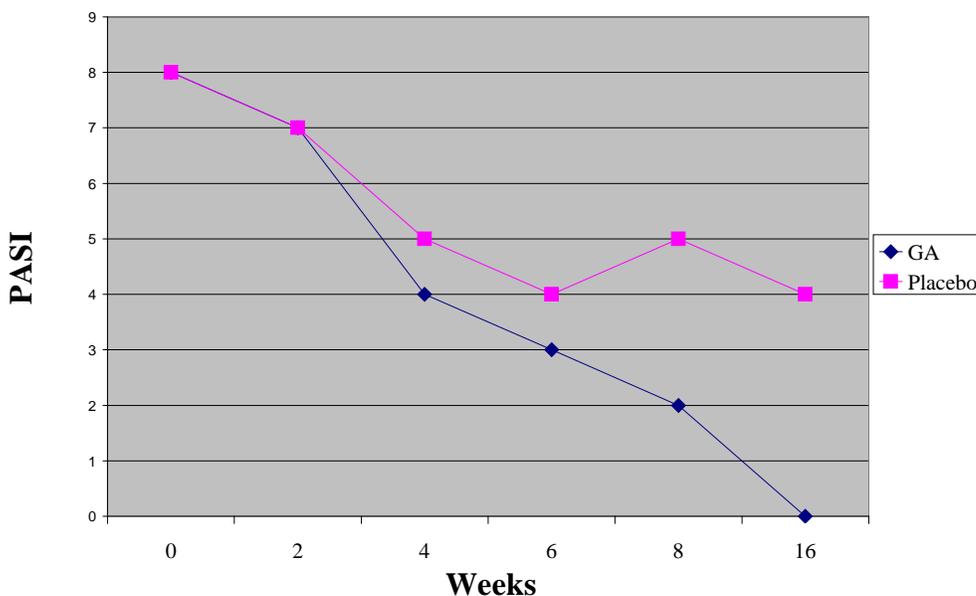


Figure 3.3. Psoriasis Area and Severity Index. Psoriasis Area and Severity Index scores over 16 weeks of topical treatment for bilateral psoriasis with 0.2 M gallic acid cream applied twice a day.

Inhibitors of angiogenesis have been shown to reverse animal models of obesity (Rupnick et al., 2002). Therefore, it is likely that local application of angiogenic inhibitors will cause localized fat reduction for cosmetic purposes. If this is borne out in subsequent clinical trials, colchicine and gallic acid may be used for spot fat reduction in much the same way that creams containing beta-adrenergic stimulators have been used to reduce the size of the hips, thighs, waist and to smooth the skin of the buttocks and thighs in women (Caruso et al., 2005; Greenway et al., 1995).

A pharmacokinetic study of oral gallic acid suggested that an 800 mg dose gives serum levels between 4×10^{-6} and 1×10^{-5} M without any sign of toxicity (Roberts et al., 2005c).

Colchicine doses between 0.5 and 1.5 mg, doses used routinely for the prevention of gout, give peak serum levels of approximately 1×10^{-6} M (Girre et al., 1989). Therefore, it may be possible that the combination of gallic acid and colchicine in the concentrations shown to be synergistic in the fat assay could be achieved by oral administration in humans. If this were so, the combination might represent a safe oral obesity drug for non-pregnant women.

We conclude that gallic acid 10^{-5} M combined with colchicine 10^{-10} M inhibits angiogenesis synergistically. This combination may be an effective topical treatment for angiogenesis driven diseases like psoriasis and possibly represent a safe oral treatment for angiogenesis driven diseases like obesity.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Although initial animal data demonstrated the potential weight loss efficacy of Nt, subsequent human trials proved that Nt, even in combination with gallic acid, was not successful in decreasing food intake and body weight in humans. The second phase of the human trial, as reported in this thesis, evaluated the pharmacokinetics of gallic acid. When several forms and doses of gallic acid were given orally, the absorption rate never rose above 19%, indicating that the transport mechanism for gallic acid across the gastrointestinal tract in humans may be saturated. This could explain why impressive weight loss was seen in rodents but did not carry into humans.

Although methods to increase oral absorption of gallic acid may be available, additional research should be done to evaluate the saturation point of this mechanism. If the system is indeed saturated, gallic acid may be more effective when given parenterally, although an indication for obesity would almost definitely be ruled out. Preliminary reports have shown that gallic acid may have use in treating certain forms of cancer when given intravenously, however no formal studies have been performed in humans to validate this pilot data obtained in rodents.

Another possible way to use gallic acid therapeutically is to combine it with other angiogenesis inhibitors that act through different mechanisms to increase the combined effectiveness through synergism. This strategy was examined in the second research goal of this thesis when gallic acid was combined with colchicine in an *in vitro* assay. The compounds, in combination, clearly showed greater inhibition of angiogenesis than either the control or each compound individually. Using such a combination may circumvent the problems seen with transport saturation when using gallic acid alone. More importantly, this study demonstrates that gallic acid and colchicine may have uses in other conditions driven by angiogenesis that do not

require intestinal absorption, psoriasis being one example. More research should be done to develop a topical treatment for psoriasis with greater efficacy based on the synergistic interaction of gallic acid and colchicine to inhibit angiogenesis.

The original intent of this thesis was to evaluate the efficacy Nt and gallic acid in treating obesity. As often happens in research, new information was discovered that may lead to treatments for diseases that currently lack adequate therapeutic alternatives. These findings further validate the importance of research into angiogenesis and its mechanisms. A better understanding of angiogenesis may lead to more effective treatment of conditions from cancer to obesity when inhibited, and revascularization after heart attacks or repair of tissue injury when stimulated. Such wide ranging effects may address disease in multiple organ systems and benefit different diseases simultaneously, improving health and quality of life.

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VITA

Andrew Thaxton Roberts was born on January 31, 1982, to Bardie and Diane Roberts in Baton Rouge, Louisiana. Andrew grew up having a fascination with learning, especially in the realm of science. He excelled in the sciences through grade school and graduated from Baton Rouge Magnet High School in May of 2000 with a great desire to pursue a degree in science. After enrolling in the College of Basic Sciences at Louisiana State University, Andrew pursued a position within Pennington Biomedical Research Center to give him more hands-on experience in clinical research. He received his Bachelor of Science in August of 2004 and immediately began working towards his master's degree in the field of nutrition. He hopes to pursue a career in the business sector of clinical research and development once he finishes his degree.