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A Proposed Method for Noninvasive Assessment of Endothelial Damange

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A PROPOSED METHOD FOR NONINVASIVE ASSESSMENT OF ENDOTHELIAL DAMAGE

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Abstract

A PROPOSED METHOD FOR NONINVASIVE ASSESSMENT OF ENDOTHELIAL DAMAGE

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Transdermal microvascular studies of endothelial cell function have typically used iontophoresis to facilitate acetylcholine absorption, but iontophoresis introduces an important confounding stimulus that can alter the behavior of the microvasculature. This study examines a non-iontophoretic technique for transdermal microvascular studies using acetylcholine and nitroglycerin and demonstrates a relatively impaired vasodilatory response to these substances in a population with known microvascular pathology.

Ten subjects without known vascular disease or diabetes were recruited for laser Doppler flowmetry (LDF) monitoring. Topical acetylcholine chloride, nitroglycerin, and placebo were applied to subjects' foreheads directly below LDF probes. Readings increased by averages of 406% (245%-566%) and 36% (26%-46%), respectively, at the acetylcholine and placebo sites ($p=0.005$ by Wilcoxon Signed Rank Test (WSRT)); and they increased by 365% (179%-550%) at the nitroglycerin site ($p=0.005$ by WSRT versus placebo; $p=0.6$ versus acetylcholine).

Ten diabetic subjects were also monitored. Mean percent increases in blood flow were 156% (91%-221%) and 116% (79%-153%), respectively, at the acetylcholine and nitroglycerin sites, vs. 21% (CI 4-37%) at the placebo site ($p=0.005$ by WSRT for placebo versus each active site). Diabetics' responses at both active sites were significantly impaired relative to healthy subjects ($p<0.001$ and $p=0.009$, respectively, by Mann-Whitney U Test)

Topical acetylcholine and nitroglycerin induced significant local vasodilatory responses without requiring iontophoresis in both healthy and diabetic subjects. Diminished responses were noted in diabetic patients. This technique may constitute a minimally invasive way to interrogate the microvasculature including its responses in various disorders and the microcirculatory changes induced by therapeutic interventions.

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INTRODUCTION

Knowledge of endothelial cell biology has grown dramatically over the past two decades. Once thought to be simply a static physical barrier between the blood and other tissues, the endothelium is now known to participate in a wide range of physiologic processes. Normal endothelial functions include regulation of blood pressure and flow, production and growth of new vessels, mediation of capillary transport, and alteration of inflammation.

Of particular clinical significance is the role the endothelium plays in the regulation of vascular homeostasis, which allows adequate end-organ perfusion through the continuous control of vessel tone and blood flow and the constitutive inhibition of thrombosis. Endothelial cells modulate vascular smooth muscle tone through the production, secretion, removal, or metabolic degradation of vasoactive substances, such as nitric oxide, prostacyclin, endothelin, and platelet-activating factor. These substances are capable of dilating or constricting vascular beds. In addition to synthesizing and responding to their own vasoactive mediators, endothelial cells may transduce signals from circulating vasodilators and constrictors such as thrombin, bradykinin, ADP and ATP[1].

Endothelial dysfunction has been observed in patients with a wide variety of diseases including diabetes[2, 3], atherosclerosis[4], hypercholesterolemia[5, 6],

homocystinuria[7], and hypertension[8, 9, 10]. Individuals with dysfunctional endothelium show impaired endothelium-dependent vasodilatation or flow mediated dilatation as shown by decreased response to the administration of acetylcholine. Similar dysfunction has been observed in subjects with advanced age[11], exposure to cigarette smoke[12, 13], sedentary life style[14], and those who have undergone organ transplantation[15]. Endothelial dysfunction appears to be a widespread systemic process with correlations existing between impairment in the coronary arteries and in the peripheral circulation[10, 16, 17, 18].

In addition to its association with a variety of pathological conditions, endothelial dysfunction may serve as a predictor of acute vascular conditions. Several studies have suggested a direct connection between endothelial dysfunction and cardiovascular events, such as myocardial infarction or sudden death, in patients with and without coronary disease[19, 20, 21]. Endothelial dysfunction is also associated with the development of transplant vasculopathy. In a study of 73 patients who underwent heart transplantation, the presence of endothelial dysfunction predicted the development of complications such as graft failure or sudden death[22].

Impaired endothelial function is also thought to play a key role in the development of many of the secondary complications of diabetes mellitus, such as retinopathy, accelerated atherosclerosis, microvascular disease, nephropathy,

neuropathy, and impaired wound healing[23, 24]. Persistent exposure to hyperglycemia may lead to endothelial damage through the production of reactive oxygen intermediates. Hyperglycemia also activates protein kinase C and the aldose reductase pathway, resulting in an accumulation of sorbitol and diminished levels of myo-inositol[23, 24]. In addition, hyperglycemia induces the formation of advanced glycation end products (AGEs) that modify structures on erythrocytes. This modification allows erythrocytes to engage a specific receptor, RAGE, (receptor for advanced glycation end products)[25]. AGE-RAGE interactions promote vascular permeability and increased expression of pro-inflammatory cytokines, thrombogenic factors, and cell adherence molecules which can aggravate and accelerate the development of endothelial lesions[26, 27].

When certain secondary complications appear in a diabetic patient, other complications are likely to be present, as well. For example, one study found that in diabetic subjects with systolic blood pressures of at least 145 mm Hg, the incidence of retinal disease was more than twice that in diabetic subjects with systolic pressures of less than 125 mm Hg[28]. Other studies have found a correlation between the presence of retinopathy and nephropathy[29, 30]. Therefore by the time a diabetic patient presents with retinopathy, dysfunctional endothelium is already likely to be present on a widespread basis.

The presence endothelial dysfunction in the early phases of multiple diseases has a number of important clinical implications. It might be assumed that at this early stage that arterial wall damage would be least extensive and most likely to be reversible. There now exists a wide variety of different therapies that improve endothelial function. HMG-CoA reductase inhibitors (statins)[31, 32], diet modification[33], [34], weight loss[35], aerobic exercise[36], aspirin[37], angiotensin converting enzyme (ACE) inhibitors[38, 39], angiotensin II receptor blockers[40, 41, 42], and estrogen therapy[43] have been associated with ameliorated endothelial dysfunction.

If compromised endothelial function could be easily and accurately identified in subjects, therapies could be initiated and monitored, and potential damage could thereby be prevented, retarded, or diminished. Unfortunately, no methods for such screening currently exist. Unlike other organs, the endothelium is not visible on imaging studies, nor is endothelial damage is associated with predictable changes in blood chemistry.

At the present time, several methods are used to assess endothelial dysfunction in the research setting. These techniques include quantitative angiographic measurement of changes in the coronary artery flow in response to intracoronary acetylcholine or serotonin infusions, ultrasound measurement of the brachial artery after hyperemia induction with blood pressure cuff occlusion

and flow-mediated dilatation, and forearm blood flow measurement by venous plethysmography using intraarterial infusion of a cholinergic stimulus. These methods utilize various stimuli to enhance the release of endothelial nitric oxide and cause artery dilatation[44, 45].

Disadvantages of angiography and intra-arterial injection techniques include their invasive natures, which make them inappropriate for studies involving asymptomatic subjects or for use as screening tests. Brachial artery ultrasound, although non-invasive and non-radioactive, is considered to be a relatively difficult technique for technicians to master, and the results and interpretations tend to be inconsistent and difficult to reproduce[46, 47].

Laser Doppler flowmetry (LDF) and iontophoresis have also been used to assess endothelial function in the research setting. LDF is a non-invasive technique that has been used to monitor and study the continuous flow of cutaneous or peripheral blood. It uses the Doppler shift in laser light secondary to moving red blood cells to provide a value (flux) which has been validated as proportional to blood flow. The volume measured is limited to approximately the 1 mm^3 of tissue beneath a surface probe. Since the sampled volume is so small, LDF can indicate localized perfusion without being influenced by surrounding tissues[48]. This methodology has potential of becoming an efficient and useful non-invasive tool for assessing endothelial functioning.

In order for the potential role of LDF in endothelial assessment to be better explored, it would likely benefit from being paired with a different method of drug delivery. Although iontophoresis is less invasive than intra-arterial injections, it has significant limitations. A slight risk of electrical burns is associated with this technique, and the electric current itself has been shown to cause vasodilatation[49]. Even when potential vasodilatation from the current is taken into account when analyzing data, effects vary among different vehicles and drugs[50,51], and tend to confound studies[52].

Given the limitations of current methods of endothelial assessment, a simple, reliable, noninvasive test for identifying endothelial dysfunction would potentially have relevant clinical applicability. Such a test might identify presymptomatic subjects at high risk for atherosclerotic complications and therefore allow initiation of primary preventive treatments. It could also potentially allow characterization of disease states and monitoring the responses to therapies.

PURPOSE AND HYPOTHESIS

Initial unpublished trials by our investigative team identified 100-300% increases in local blood flow when acetylcholine chloride eye drops (Miochol-E,™ Novartis, East Hanover, NJ) were applied to the forehead under an LDF probe. This targeted administration of small amounts of a topical agent seemed to affect local microcirculatory flow at the site of application without significant systemic effects. However, the presence of mannitol in the Miochol preparation may have affected local vascular changes.

These results prompted a decision to explore the effects of a transdermal preparation of pure acetylcholine chloride powder dissolved in water with the hope of inducing similar microvascular dilation without the effects of Miochol. In addition to this endothelium-dependent test of vasodilation, we used a commercially available translucent nitroglycerin patch to test endothelium-independent vasodilation in a similar manner.

We proposed that endothelial dysfunction may be identified and characterized with noninvasive assessment of microcirculatory responses to transdermal administration of acetylcholine and nitroglycerin using laser Doppler flowmetry. It was anticipated that patients with known endothelial dysfunction would show impaired vasodilatation when given these topical

vasoactive agents when compared to healthy volunteers. By comparing the different responses of healthy volunteers to diabetic patients with documented retinopathy, we hoped to describe the various responses and possibly correlate with the presence of disease. With adequate data, not only could such testing potentially allow assessment of disease extent and monitoring of therapeutic interventions, but could also allow earlier identification of endothelial dysfunction in asymptomatic subjects.

We hoped these methods would provide primary results that would encourage future testing, and possibly eventually become the foundation for a future test of endothelial function that would be more comfortable, simple, safe, and reliable than tests previously described.

METHODS

After IRB-approved trials with acetylcholine in multiple diluents with a range of hydrophilicity, we elected to administer transdermal acetylcholine by placing acetylcholine chloride powder dissolved in water on a translucent adherent surface. Then, with IRB approval, 10 healthy volunteers and 10 patients with diabetic retinopathy were recruited for LDF measurements of forehead perfusion at sites of transdermal application of acetycholine, nitroglycerin, and placebo.

For each session, after informed consent was obtained, subjects lay on a bed in a semi-recumbent position in a temperature-controlled room (24±1 degrees Celsius). A three-lead electrocardiogram and a non-invasive brachial artery blood pressure cuff were applied. Local forehead skin oils were decreased by wiping the skin lightly with an alcohol swab, followed by wet and dry gauze. Two minutes later, three Double-Stick Discs (3M Health Care, Neuss, Germany) with an overall diameter of 38.1 mm and a central hole diameter of 8.7-mm were placed on the forehead. These discs served as an adherent base onto which laser Doppler probes were placed as well as to define the 59.4 mm^2 area for drug application.

The acetylcholine solution was freshly prepared for each session by mixing 100-mg of acetylcholine chloride (Spectrum Chemical; New Brunswick, NJ) with 0.6-mL high pressure liquid chromatography (HPLC) grade water to a final volume of 0.7-mL and molar concentration of 786.4 M. Then, 0.02-mL (2.8 mg) of this concentrated acetylcholine solution was spread on a double-stick clear disk (diameter 12.6 mm, area 124.7-mm^2). The dry side of the acetylcholine "patch" was adhered to the bottom of a laser Doppler flowmeter probe (PF 5010 with Probe Model 407, Perimed, Sweden). A placebo drug patch was made in the same way with 0.02-mL of HPLC-grade water instead of acetylcholine solution.

The nitroglycerin patch was obtained by using a standard hole-punch to cut a 6-mm diameter (28.3-mm^2 area) section of a 0.6 mg/hr Minitran™ nitroglycerin patch (3M, Minnesota). The patch is designed for homogeneous delivery of drug at a rate of 0.03-mg/hour per 100-mm^2 area of patch. The small patch area in our study was thought to deliver at a rate of approximately 0.008-mg/hr. The nitroglycerin patch then was placed onto a double stick disc and positioned over a laser Doppler sensor in a similar manner to the acetylcholine patch.

The three laser Doppler probes with patches attached were adhered onto the three double-stick discs on the forehead so as to enable undisturbed monitoring and drug delivery. LDF monitoring was performed continuously at each site until a vasodilatory plateau was maintained for $\geq$3 minutes or for a maximum of 20 minutes. Data were collected at a rate of 1000 Hz using Chart for Windows (ADInstruments, Colorado Springs, Colorado). All LDF sensors were calibrated using a motility standard (Perimed, Sweden) twice during the three-month study period.

The local microvascular effects of transdermal acetylcholine and nitroglycerin were first tested in 10 healthy volunteers (7 males, 3 females; mean age 36.1, range 19 to 56 years) without known vascular disease or diabetes. The effects also were tested in 10 diabetic subjects (3 males, 7 females; mean age 56.5,

range 40 to 73 years) recruited in the Yale Eye Center. Nine of the 10 diabetic subjects had documented microvascular retinal pathology by fluorescein angiography; the other did not undergo angiography. Patients were excluded from the study if they had taken nitrates in the previous 48 hours or if they had already received mydriatic eye drops on the study day. Other than these restrictions, subjects were not asked to alter their diet or medications prior to the study.

An investigator blinded to the status of the subject and to the nature of the study site assessed the laser Doppler tracing at each study site. The pre-study trials had indicated that, within 10 seconds of probe application, a steady baseline interval was consistently obtainable and that a progressive drug-induced rise in flow with both acetylcholine and nitroglycerin was noted to begin as soon as 30 seconds after probe application. This enabled a 20-sec baseline period to be obtained, without the need to remove the probe for subsequent drug application. Each probe remained in place for up to 20 minutes.

The drug-induced increase in flow in the present study was determined by comparing the mean during the lowest 10-second interval during baseline period with the mean during the maximum 10-second interval after a plateau was reached. If no distinct rise in blood flow was evident, then the highest 10-second interval that occurred beyond the first two minutes of readings was used.

Data are presented as mean percent increase in LDF voltage with 95% confidence intervals (CI). Since the primary goal was to test whether the means of delivery and testing introduced herein generate and delineate a consistent vasodilatory response, the primary endpoint was the change in LDF voltage after transdermal application of active drug versus placebo in healthy volunteers. This was accomplished with the Wilcoxon Signed Rank Test (WSRT), using SPSS for Macintosh (SPSS, Inc. Chicago, IL). Previous investigations with iontophoretic delivery of acetylcholine had generated increases in skin blood flow ranging from 160 to 710%, depending on concentration of drug and iontophoretic current[13, 53].

Given these data and our preliminary trials, we anticipated at least a 100% increase in blood flow (with a standard deviation of 50%) at the sites of active patches versus a 20% difference (with a standard deviation of 10%) at sites of placebo application (LDF readings have temporal variation even under baseline conditions, a feature that would be emphasized by our comparison between the highest 10-second interval after drug application with the lowest 10 second interval at baseline). In order to identify the difference between the active and placebo patches with an alpha of 0.025 and a beta of 0.8, we calculated that a sample size of seven subjects would be required. P-values greater than 0.05 are reported as not significant (NS); for values <0.05, the actual value is reported.

Secondary endpoints were also assessed, with the realization that our study design was not tailored to these comparisons. First, the effects of the single dose of acetylcholine were compared to those of the single dose of nitroglycerin in the healthy volunteers, with the differences analyzed using WSRT. Second, a preliminary assessment of the response in the healthy volunteers versus the 10 diabetic patients (who were older with varied medical conditions and medications) were compared using the Mann-Whitney U Test (MWUT).

RESULTS (FIGURES 1-3)

Healthy Volunteers, Acetylcholine

Acetylcholine induced a visible rise in LDF voltage within two minutes of administration. This was evident with respect to mean flow, as well as with respect to the amplitude of the pulsation coincident with each heart beat. This elevation of blood flow persisted throughout the duration of the study session. Readings increased, on average, by 406% (245%-566%) at the acetylcholine sites and 36% (26%-46%) at the placebo sites ($p=0.005$ by WSRT).

Diabetic Patients, Acetylcholine

In the diabetic group, acetylcholine also induced a visible rise in LDF voltage within approximately two minutes of administration. The mean percent

increases in blood flow were 156% (91%-221%) at the acetylcholine sites, compared to 21% (4%-37%) at the placebo sites (p=0.005 by WSRT). While the vasodilatory responses were significantly greater than those observed with placebo, they were less than those in the healthy volunteer group.

Healthy Volunteers, Nitroglycerin

Nitroglycerin administration caused a marked rise in LDF voltage in the healthy volunteer group, similar to that seen with acetylcholine administration. Within two minutes of drug application, blood flow at the treatment sites rose and remained increased throughout the remainder of the study session. The average increase from baseline was 365% (179%-550%) compared to the 36% (26%-46%) increase at the placebo site (p=0.005 by WSRT versus placebo; p = NS versus acetylcholine).

Diabetic Patients, Nitroglycerin

Similar to the response seen with acetylcholine, nitroglycerin-induced vasodilatory responses in diabetics were significantly greater than to placebo, but less than in healthy volunteers. Mean percent increases in blood flow were 116% (79%-153%) at nitroglycerin sites vs. 21% (CI 4-37%) at the placebo site (p=0.005 by WSRT).

Overall, the responses of the diabetic patients to acetylcholine and nitroglycerin did not differ significantly from each other (p=NS by WSRT). The diabetics' responses at each active site were significantly impaired relative to healthy subjects ($p<0.001$ and $p=0.009$, respectively, by MWUT).

DISCUSSION

This investigation yielded several unique findings and implications for further study. First, by using topical acetylcholine and nitroglycerin in conjunction with LDF, it was possible to induce and observe microvascular dilatation non-invasively. Second, distinct differences in degrees of dilatation were noted between healthy volunteers and patients with assumed endothelial dysfunction. This novel method appeared to allow sufficient uptake of vasoactive drugs for stimulation of clear local effects without detectable systemic changes. Complications associated with iontophoretic electric currents were avoided by using this method of administration. While the local vasodilatory effects of transdermal nitroglycerin have long been appreciated and used in the clinical setting, a comparable response to non-iontophoretic transdermal acetylcholine has not been reported[54].

Acetylcholine and nitroglycerin were chosen for this study on the basis of the likelihood that they would produce local vasodilatation through distinct endothelium dependent and independent mechanisms, respectively. It was hypothesized that if this experimental method were to provide the basis for a possible future clinical test of endothelial function, not only would dilatation be noted after application of the vasoactive agents, but different degrees of response should also occur in individuals with varying disease states.

In this study, it was observed that patients with diabetic retinopathy showed significantly less dilatation when given approximately equal amounts of both acetylcholine and nitroglycerin as healthy volunteers. As the diabetic group was presumed to have widespread endothelial dysfunction, it was predicted that their reactions would be significantly less than those of healthy volunteers, particularly to drugs whose effects are mediated via endothelium-dependent mechanisms (e,g., acetylcholine). In order best to explain these findings and explore their implications, it would be helpful to revisit the results and the physiologic basis for the vasodilatation associated with nitroglycerin and acetylcholine.

Nitric oxide (NO) produced from the endothelium is a particularly important component of vascular homeostasis. It is formed from the oxidation of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS)[55, 56] and is

synthesized constitutively by intact endothelium. NO functions to maintain low arterial tone at rest by binding to guanylyl cyclase, thereby increasing intracellular cyclic guanosine monophosphate levels, reducing intracellular calcium, and consequently relaxing smooth muscle cells[57, 58, 59, 60]. The same pathway is involved in the mechanism of action of exogenous nitrovasodilators, such as sodium nitroprusside and nitroglycerin.

Additional endogenous NO release from normal endothelium can be stimulated by increased blood flow[61] and by bradykinin, thrombin, acetylcholine, and a variety of other circulating agents that activate specific endothelial cell membrane receptors[62]. In additional to its vasodilatory effects, NO inhibits platelet adhesion, activation, secretion, and aggregation, and encourages platelet disaggregation. Leukocyte recruitment and adhesion to the endothelium are controlled in part by NO through its inhibition of smooth muscle cell migration and proliferation[63, 64, 65, 66]. The half-life of unbound NO is in the order of seconds, but a prolonged bioactivity may be conferred by its binding and the nitrosylation of albumin or other plasma proteins[67]. Degradation of nitric oxide by heme-mediated oxidation is a major route of nitric oxide inactivation, with the production of nitrite and nitrate as end products[68].

Whereas the generation of NO is endothelium-dependent, the vasodilatory responses elicited by NO are considered to be "endothelium-

independent" since such responses have been thought to be preserved regardless of the presence or absence of intact endothelium. Despite this preservation, however, some patients with endothelial dysfunction have been observed to have a reduced endothelial response to exogenous sources of NO, such as nitroglycerin[69]. This is thought to be indicative concomitant endothelium-independent smooth muscle dysfunction.

The data in this study show that topical administration of nitroglycerin induced an average 365% (179%-550%) increase in blood flow in healthy volunteers and 116% (79%-153%) increase in diabetic patients. These results indicate that significant local vasodilatory responses were elicited in both groups without requiring iontophoresis. Furthermore, the degrees of reaction were distinctly different between the two groups, with diminished responses noted in diabetic patients. These findings are consistent with previous reports of patients with endothelial dysfunction having reduced endothelial responses to nitroglycerin (above), and may indicate the presence of smooth muscle dysfunction as well as endothelial dysfunction in the diabetic population.

Stimulation of normal endothelium by acetylcholine also produces vasorelaxation. This is thought to be achieved through the release of NO as well as another endothelium-derived relaxing factor (EDRF). This EDRF causes hyperpolarization of underlying smooth muscle by increasing potassium ion

conductance and activating the cyclic guanosine monophosphate (cGMP) pathway[70, 71]. However, if the endothelium is removed experimentally, acetylcholine causes a seemingly paradoxical constriction of blood vessel. This is the result of acetylcholine directly stimulating medial smooth muscle cells. A similar reaction also occurs in vivo in vessels with denuded intima or dysfunctional endothelium. The vasodilatory responses elicited by acetylcholine have therefore been regarded as "endothelium-dependent", since they were thought to produce vasodilation only in the presence of intact endothelium.

More recent studies, however, have shown that the classification of acetylcholine as a mediator of endothelium-dependent vasodilation is not absolute in all settings. For example, iontophoretic application of acetylcholine has been shown to induce vasodilation partially through prostanoid mediated pathways that are independent of endothelial cell function[72]. The extent to which topical acetylcholine-induced microvascular dilation may reflect endothelial cell function remains to be delineated, as does the effect of different doses of acetylcholine on individuals' responses.

The data in this study show that topical administration of acetylcholine induced an average 406% (245%-566%) increase in blood flow in healthy volunteers and 156% (91%-221%) increase in diabetic patients. Increases at placebo sites were 36% and 21% for healthy and diabetic groups, respectively. It

was again concluded that transdermal administration of acetylcholine caused local vasodilatory responses in both groups without requiring iontophoresis. Furthermore, similar to the responses to nitroglycerin, the differences in extent of vasoreactivity between the two groups were significant enough to conclude that the presence of endothelial dysfunction will yield diminished responses.

In addition to its non-invasive nature, this novel method has several other advantages. Unlike brachial artery ultrasound, the technique is relatively simple and requires minimal training on the part of the technician. The setup and cleanup time is in the order of minutes, and the equipment itself is portable. The test is also more comfortable for patients than brachial artery ultrasound during reactive hyperemia, as it does not require the five-minute occlusion of arterial flow.

In terms of the reagents used, both acetylcholine and nitroglycerin are rapidly broken down. This makes them well-suited to transdermal application for local microvascular testing without systemic effects. Furthermore, the transdermal patches themselves are amenable to placement in regions of the body for which the more elaborate iontophoresis apparatus may not be suitable or would be deemed undesirable. This could allow for testing of microvascular reactivity in multiple areas of the body.

The results described herein can be thought of as very encouraging, but preliminary. Limitations of the study warrant attention before drawing conclusions and implications. The translucent patch design in this study made it possible to obtain a short baseline reading in the initial period of drug application without moving the probe. The technique, however, might be improved by a delayed-release patch that would allow for a more prolonged baseline measurement. In addition, LDF readings are generally characterized by spatial variation, and it is probable that the described patch technique would be prone to such variation, as well. Spatial variation could possibly be reduced with larger LDF probes which encompass more than the 1-3 arteriolar-capillary networks that typically are interrogated by a typical LDF probe[73].

As previously mentioned, endothelial function is affected by many factors. A decline in endothelial function has been observed with normal aging. The phase of a woman's menstrual cycle also may affect vasoreactivity. It would therefore be advantageous to conduct more studies using this technique where the groups being compared are carefully age-matched and gender-matched. If pre-menopausal female subjects were to be used, it would be best to attempt performing the studies during the same phases of their menstrual cycles.

Another limitation of this study involves many of the diabetic patients having already been exposed to interventions shown to affect endothelial

function. HMG-CoA reductase inhibitors (statins)[,] have been associated with ameliorated endothelial function in both young patients with Type 1 diabetes[74] and elderly patients with Type 2 diabetes, with or without mild hypercholesterolemia[75]. These effects have been observed as early as three days after the initiation of therapy, and appear to be independent of observable changes in lipid levels. Diet modification[,], weight loss, aerobic exercise, aspirin, angiotensin converting enzyme (ACE) inhibitors[38,], angiotensin II receptor blockers[,,], and estrogen therapy have also been shown to improve endothelial function in certain subjects, as have supplementation with vitamins C, E, folate, and 5-methyltetrahydrofolate[76, 77].

As the diabetic patients in the study were recruited from a clinic where they were receiving treatment for retinal disease, most individuals had been on one or several medications known to improve endothelial function. Some had been on such medications for many years. This would have certainly affected the results of this study, yet it would not have been ethical to request or instruct patients to discontinue proven protective medications. Therefore, a study recruiting healthy control subjects and patients with assumed endothelial dysfunction who have not yet started endothelial-ameliorating medications would likely show more dramatic differences in vasoreactivity between groups.

The limitations, though required to note for potential future studies, do not necessarily prevent several important conclusions. One of the primary goals of this investigation was to determine whether transdermal administration of vasoactive drugs would allow sufficient absorption to induce vasoactive changes without systemic effects. The vasodilatation associated with the acetylcholine and nitroglycerin patches compared to placebo patches was indeed adequate to assume directly related changes. A second goal was to compare the amount of dilatation between two groups to assess whether individuals with assumed endothelial dysfunction showed compromised vasoreactivity compared to healthy subjects. Although it is not possible to quantify the specific degrees of dysfunction based on our results, given the substantially diminished responses in the diabetic group, it is reasonable to conclude that this method successfully identified the presence of endothelial dysfunction within that group.

In conclusion, the present data suggest that examining vasoreactivity with transdermal acetylcholine and nitroglycerin may be justified. That endothelial dysfunction is associated with acute complications and is seen in the early phases of multiple diseases implies that it would be valuable to identify and treat such dysfunction. A simple, non-invasive test to screen for presence of endothelial dysfunction could theoretically permit earlier identification of such dysfunction in asymptomatic subjects. It might be assumed that at this early stage, the

arterial wall damage would be least extensive and most likely to be reversible. Theoretically, if therapeutic interventions are initiated before the damaging effects of the individual diseases manifest, it might be possible to prevent or retard potential damage. Even in advanced disease, modification of endothelial dysfunction might decrease the propensity to vasoconstriction, thrombosis, or both, and thereby decrease the risk of acute complications, such as cardiovascular events. Serial testing of endothelial function may also be a useful measure to monitor and guide therapy, once initiated.

With further elaboration, this technique may constitute a useful and minimally invasive way to assess endothelial function. With development and refinement, perhaps such a test could provide information such as the quantifiable extent of dysfunction or areas of particular concern. Although further studies are indicated in order to develop a reliable clinical test that would be appropriate for such applications, this investigation provided evidence that such studies may indeed be worth pursuing.

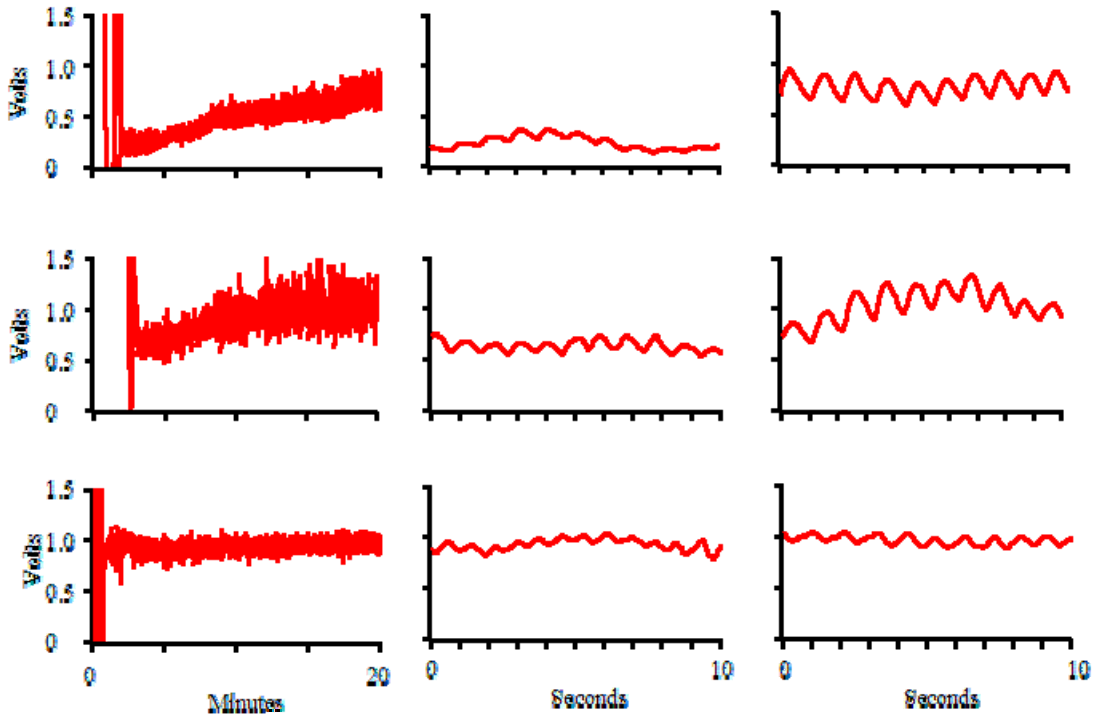

Figure 1: Representative data from a healthy subject illustrating readings at the acetylcholine site (top row), nitroglycerin site (middle row), and placebo site (bottom row). Entire 20 minute study period is shown in the left hand column; 10 seconds at baseline and 10 seconds during the plateau phase are shown in the middle and right hand columns, respectively. Prompt increases in blood flow at the sites of transdermal acetylcholine and transdermal nitroglycerin application are seen. This is manifested not only by an increase in mean flow but also in the amplitude of each pulsation at the cardiac frequency.

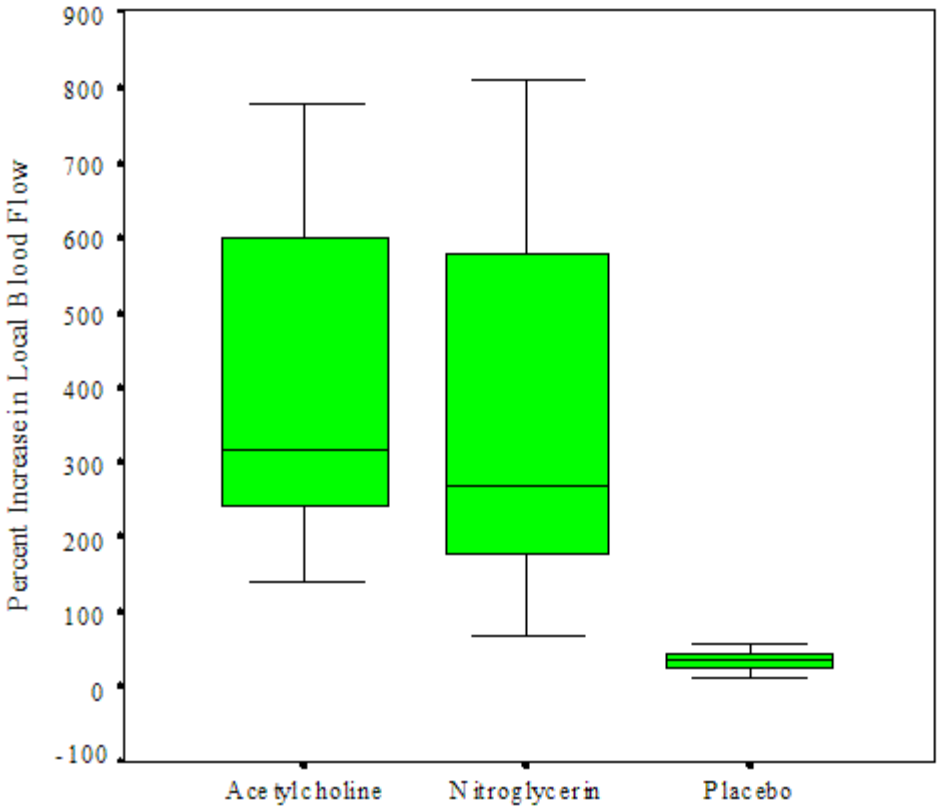

Figure 2: Boxplots of percent change in blood flow in healthy subjects at sites of acetylcholine, nitroglycerin, and placebo patches. The box incorporates the data between the 25th and 75th percentiles; the line within the boxes is the median; the whiskers on either side show the full range of data values to a maximum of 1.5 times the interquartile range (if there had been points beyond such a range, they would have been represented as individual dots and considered outliers). **P=.005** for both drugs versus placebo.

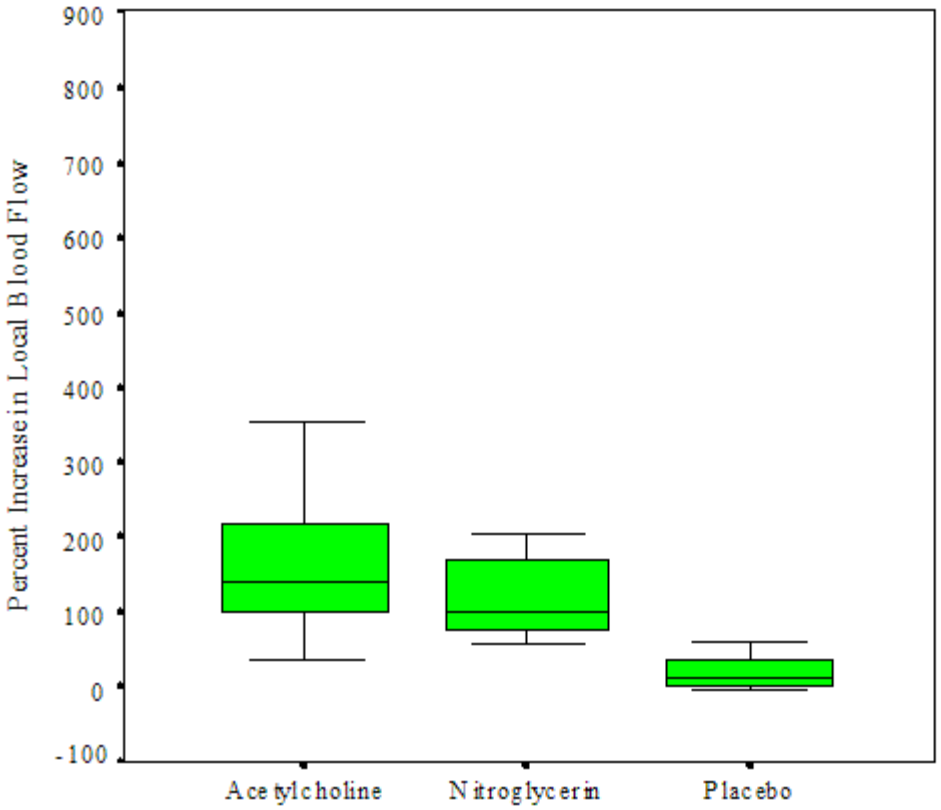

Figure 3: Boxplots of percent change in blood flow in patients with diabetes at sites of acetylcholine, nitroglycerin, and placebo administration (**p=.005** for both drugs versus placebo).

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