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The Acute Effects of Elevated Blood Lipid Levels on Endothelial Function

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THE ACUTE EFFECTS OF ELEVATED BLOOD LIPID LEVELS ON ENDOTHELIAL FUNCTION

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THE ACUTE EFFECTS OF HIGH LIPID LEVELS ON ENDOTHELIAL FUNCTION.

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The association between prolonged elevation of blood lipid levels and coronary artery disease is well established. Research focusing on the acute effects of hyperlipidemia is not as abundant, and often looks only at large-sized arteries. The purpose of our study was to measure the acute effects of elevated blood lipid levels on microvascular endothelial function, non-invasively, by means of laser Doppler flowmetry and the administration of vasoactive challenges.

With IRB approval, six healthy subjects were recruited for a three-session study in which they were given isocaloric meals with variable fat content (non-fat meal = NM; fatty meal = FM; or fatty meal with atorvastatin = FML). Two hours after ingesting the meal, the subjects' microvasculature was examined using laser Doppler flowmetry. Our analysis looked at the ratio of low to high frequency microvascular oscillations (increases if parasympathetically mediated oscillations are inhibited) both at baseline and during a vasoconstrictive challenge; as well as endothelium-independent versus endothelium-dependent vasodilation using topical administration of acetylcholine and nitroglycerin.

We found a greater low to high frequency ratio after the FM compared to the NM both at baseline (mean ratio after FM was 222 +/-109% of that after NM, $p = 0.016$) and during the vasoconstrictive challenge (mean ratio after FM was 321 +/-242% of that after NM, $p = 0.05$), indicating a relative impairment in parasympathetic oscillations after the FM. Additionally, the mean ratio of endothelium-independent to endothelium-dependent vasodilation after the FM was 177.6 +/-98% of that after the NM, $p = 0.036$.

We concluded that elevated blood lipid levels acutely impair endothelial function.

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Introduction

Lipids and Coronary Artery Disease

The relationship between cholesterol and coronary artery disease (CAD) is a well-researched area in medicine today. It has been established through numerous trials that cholesterol is a risk factor for developing CAD through the process of atherosclerosis. Although this is not disputed in current literature, and although the long-term processes taking place in atherosclerosis have been well analyzed and described, the short-term effects of elevated cholesterol, or more broadly, lipoproteins, still need further research. Several experiments in the last decade have tried to analyze the more immediate relationship between blood lipid levels and vascular characteristics (1-15). More specifically, experiments have looked at the following vascular parameters and how they correlate with variable lipid levels in the circulation: endothelium-dependent vasodilation using either administration of acetylcholine (ACh) or Flow-Mediated Dilation; endothelium-independent vasodilation using either systemic or injected nitroglycerin (NTG); or resting and post-ischemic forearm blood flow using venous occlusion strain-gauge plethysmography. The overarching goal has been to shed light on the early stages of vascular pathology that eventually lead to atherosclerosis.

The evidence from these experiments is somewhat conflicting, especially when it comes to impairment in endothelium-independent versus endothelium-dependent vasodilation. This is due, in part, to the fact that eating produces increases in blood flow – it probably is the degree of increase and perhaps the nature of the increase that appear to be altered in the fatty meal (1, 2). Some experiments found that a fat-laden meal (FM) impairs endothelium-dependent vasodilation only (3-6), others found impairment in

endothelium-independent vasodilation only (7), and others found impairment in both processes (8). One review article looking at experiments that utilized Flow-Mediated Dilation to assess vascular characteristics post ingestion of a FM concluded that the results obtained from using this technique differ widely across experiments as a consequence of technical aspects of the measurements, the location and the duration of the occlusion (9).

Our lab has significant experience looking at microvascular reactivity by using topical vasoactive drugs, typically ACh and NTG, and laser Doppler flowmetry. The question came up, therefore, whether the technique used by our lab offers an easier and potentially better way of assessing the effects of high blood lipid levels on the vasculature. It would certainly improve on the ability to understand what happens from the point of view of the *micro*vasculature, since the Flow-Mediated Dilation technique typically looks at the brachial artery as opposed to the capillary bed. Also, one of the advantages of using *topical* vasoactive drugs is that one avoids the issues associated with administering a drug intravenously or intra-arterially, that is, of using invasive tests. Furthermore, because the topical drugs used by our lab only have an effect on a very distinct and relatively small anatomical area of microvasculature, one avoids the systemic changes that are brought about if vasoactive drugs are injected or used in a sublingual manner. It becomes possible, therefore, to measure the local dilatory effect of both endothelium-dependent (ACh) and endothelium-independent (NTG) challenges post ingestion of a FM within a very short time span of each other.

Compensatory Mechanism: Capillary Oscillations

Our lab has previously looked into the oscillatory changes that take place in the human vasculature in response to a vasoconstrictive challenge (16). This research indicates that, whereas finger blood flow decreases in the setting of a vasoconstrictive challenge, forearm and forehead blood flow is to a large extent maintained, and that this might be mediated by an increase in oscillations at the level of the capillary bed. More specifically, we found that the increase in oscillatory behavior in the microvasculature occurred at a frequency level of around 0.105 – 0.195 Hz, which is the characteristic frequency for parasympathetic oscillatory activity. That this observed increase in oscillation is parasympathetically mediated was further strengthened by earlier findings of its disappearance in response to administration of atropine (see Figure 1) (16).

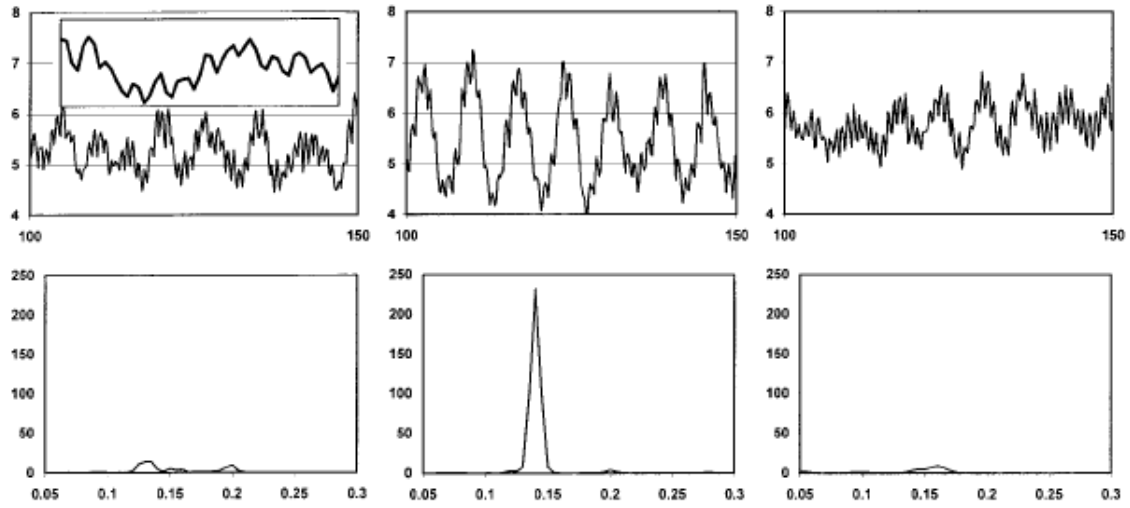

Figure 1: The top row represents forehead blood flow (flux) during baseline (left), phenylephrine administration (middle), and phenylephrine + atropine administration (right) in a typical subject (x-axis = time (50 second segment) and y-axis = flux over a 4-V range).The expanded 10-s segment (from 20 to 30 s during baseline) delineates the pulsations that occurred with each heartbeat. The bottom row relates the oscillatory power as a function of frequency for the segments analyzed (x-axis = oscillatory frequency and y-axis = power (V^2/Hz)). From ref 16.

It appears, therefore, that the oscillations are dependent on the activation of the parasympathetic nervous system with consequent muscarinic receptors stimulation by ACh and that, when this pathway is blocked, the oscillations will be reduced or eliminated. From this we stipulated that if indeed a meal with high lipid content impairs endothelium-dependent vasodilation as the majority of the evidence indicates, and that if this occurs because the normal cascade of events triggered by ACh stimulation is interrupted, we would also see a reduction in the parasympathetically mediated oscillatory power in the setting of elevated blood lipid levels as these oscillations appear to be dependent on an endothelium responding properly to ACh.

Lipitor as Modulator

Previous experiments have looked into ways of improving the impaired state of the endothelium after a FM by giving the subjects medications or supplements for a certain period of time prior to a FM testing session. More specifically, the angiotensin-converting enzyme inhibitor, Quinapril; the angiotensin II type 1 receptor antagonist, Losartan; and the antioxidant Alpha-Tocopherol have been studied. The results indicate an improvement in endothelial function secondary to the administration of all three (17-19).

We wanted to explore whether a similar improvement in endothelial function would be brought about if a single dose of atorvastatin (Lipitor®, Pfeizer) was administered shortly before the FM was ingested. The reason being that in addition to its long-term cholesterol-lowering properties, a statin (e.g. atorvastatin) acutely lowers

inflammatory mediators which are thought to play an important role in the processes that renders the endothelium impaired (20-22)

Design of Experiment

With this knowledge as our starting point, we set out to design an experiment that would utilize the laser Doppler flowmetry technique together with topical administration of ACh and NTG as endothelium-dependent and endothelium-independent vasodilatory challenges, respectively, in order to examine the effects of a FM on microvascular reactivity. Furthermore, we wanted to look at the oscillatory pattern brought about by a systemic vasoconstrictive challenge as shown in prior experiments (16), and how these oscillations would change in response to elevated blood lipid levels after a FM. Lastly, we were interested in what, if any, modulating effect the co-administration of Lipitor with the FM would have on our tests of endothelium dysfunction.

Hypothesis

Hypothesis I

A meal with high fat content will have a greater inhibitory impact on endothelium-dependent vasodilation compared to endothelium-independent vasodilation.

Hypothesis II

The parasympathetically mediated compensatory oscillations seen in the microvasculature in response to a vasoconstrictive challenge will be impaired post ingestion of a meal with high fat content.

Methods

Subjects

We recruited six subjects that each went through three experimental sessions. The subjects were three males and three females between the ages of 22 and 30. They had no known disease of the microvasculature or other major morbidities that would interfere with the study.

Meal Types

For each of the three sessions, the subjects were randomized to receive one of three possible meal-plans: a non-fat meal (NM) consisting of frosted flakes with skimmed milk and orange juice (two subjects suffered from lactose intolerance and hence ate the frosted flakes without the milk, to which we added one extra serving of juice to make up for not drinking the milk); a fat-laden meal (FM) consisting of a hamburger with French fries; and the FM taken with a 20mg dose of Lipitor (FML). The Lipitor was taken one hour prior to ingestion of the meal. We waited for about 75 minutes after the subjects had finished the meal before we started the experiment in the lab thereby allowing for the ingested fat to reach the circulation, a time-lag that is consistent with prior studies.

Measurements

During each experimental session in the lab, we measured blood flow in the forehead, finger and foot, as well as blood pressure (BP) and EKG, while the subjects were exposed to three different challenges: application of cold to the extremities, topical application of ACh and topical application of NTG to the forehead. The following instruments and measurements techniques were used:

EKG: Standard 3 lead EKG set-up was used and recorded during the entire experiment.

Blood pressure: The subjects' BP was recorded prior to starting the experiment on each day, as well as twice during the experiment: once immediately following the removal of the topical ACh and once immediately post the NTG patch removal. As part of the safety protocol, the experiment was to be stopped if the BP (systolic, mean, or diastolic) decreased by more than 20 mmHg on 2 successive readings or by more than 25 mmHg on any given reading.

Forehead Blood Flow: We measured forehead blood flow with two different devices: one was a single laser Doppler probe (Probe Model 301, Perimed, Sweden), the second was an integrating Laser Doppler probe (Probe Model 413, Perimed, Sweden). Both probes are usually attached to the forehead by means of a double stick tape beneath a probe holder into which the probe is subsequently placed.

Research done by our lab as well as multiple other labs has established that one of the challenges to measuring blood flow in the capillary network is caused by spatial heterogeneity (23). That is, one can get greatly different blood flow measurements depending on where the probe is attached even within a fairly confined area on, for

example, the forehead. This makes it harder to accurately analyze the effect of interventions aimed at altering blood flow which are done on different days, as one does not know whether the change in flow seen is secondary to the intervention or just a consequence of looking at a different vascular bed compared to the readings from the control day. This problem is particularly pronounced when using a single probe laser Doppler, but experiments done by our lab also show that the integrating probe is plagued by some of the same issues, albeit if to a lesser extent. In order to get around this problem of spatial heterogeneity, our lab has designed two devices.

The first device aims at finding a reliable way to get back to the same spot anatomically on the forehead on different days of the experiment. To achieve this goal we designed a construction that utilizes the anatomy of the nose and the nose bridge, i.e. something that will not vary from day to day, and by using this as our point of reference anatomically, we mark the areas on the forehead that we would like to measure thereby limiting day to day variability (see Figure 2 and Picture 1)

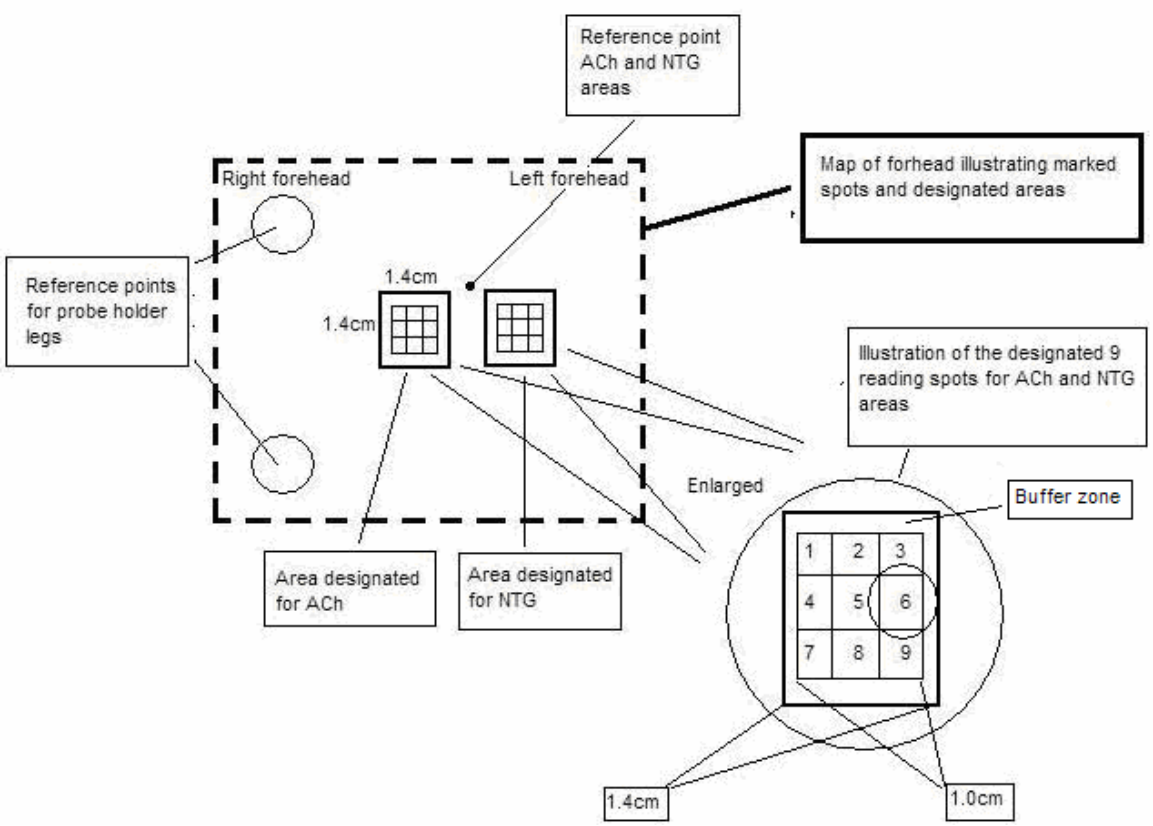

Figure 2: Reference points and designated areas on the forehead. For the enlarged part of the figure, a circle has been put over spot 6 to illustrate the location of the probe when placed to read at spot 6.

The second device was a product of our earlier research on spatial heterogeneity and utilizes a self-designed, movable, probe holder (see Pictures 2-4). This allows us to obtain multiple samples within a small region on the forehead as compared to when using the original probe holder which is fixed to the forehead once attached and can only obtain readings from a single site. This new, movable, probe holder was engineered so that the integrating probe is suspended a couple of millimeters above the forehead with the ability to be moved at fixed intervals in a two dimensional fashion. More specifically, we measure blood flow in a 1cm x 1cm area on the forehead by obtaining 9 different readings in this area, with reading spots distributed at an equal distance apart from one

another in a grid-like pattern (see Figure 2). We have previously shown that the reliability of this method is superior to that of a single probe when it comes to intrasubject consistency. That is, when comparing increase in forehead blood flow post application of topical NTG (delta NTG), there was a statistically significant reduction in day-to-day intrasubject variation with the integrating probe compared to the single probe (the mean ratio of delta NTG between different days with the single probe was 2.0 (range 1.2-4.0), compared to a mean ratio of 1.2 (range 1.1-1.3) with the integrating probe, $p = 0.04$). This reduction in variation was associated with data that enabled documentation of a statistically significant increase in blood flow as a consequence of topical NTG administration (24)

The single probe on the forehead was attached on the left side of the forehead with the original probe holder and recorded blood flow throughout the experiment without being moved or manipulated. We did not mark this area in order to keep it constant on different days. The reason for this was that we were less interested in the absolute value of this reading and more interested in whether the local application of ACh and NTG in the area of the integrating probe would have a global dilatory effect on the forehead.

Finger Blood Flow: We used a single probe (Probe Model 407, Perimed Sweden) that was attached to the left index finger of the subjects by means of a double stick tape. As the length of the experiment was more than one hour, we tried to make sure that the left arm of the subjects was comfortable throughout the experiment and that blood flow was not compromised in any way by sustained pressure at any specific point over that hour.

Foot Blood Flow: We used a single probe (Probe Model 314, Perimed, Sweden) that was attached to the underside of the right big toe in the same manner as the single probe on the finger.

Forehead Oscillatory Activity: We used the blood flow values from the integrating probe when calculating the oscillatory activity in the forehead. The software we used in the analysis of the data enables calculations of oscillatory power by means of Fourier analysis. More specifically, the software is able to graph oscillatory power on the y-axis at different pre-set frequency intervals. In our case, we were interested in the oscillatory powers in the frequency range given by $0.045Hz < X < 0.195Hz$. That is, we wanted to look at the ratio of low frequency oscillatory power representing both parasympathetic and sympathetic oscillatory activity ($0.045Hz < X < 0.105Hz$), to high frequency oscillatory power representing mostly parasympathetically controlled oscillations ($0.105Hz < X < 0.195Hz$). The reason we decided to take out the oscillatory power in the frequency range from 0.00Hz to 0.045Hz was that by doing so we were able to eliminate the effect of any noise introduced by the subjects having to move slightly or any other external stimulus that would appear in the < 0.045 frequency range. Looking at low to high frequency ratio in this manner is the standard way of analyzing oscillatory patterns in the sympathetic and parasympathetic range.

Vasoactive Challenges

The three different challenges that the subjects were exposed to during each experimental session – application of cold to the extremities, topical application of ACh and topical application of NTG to the forehead – were implemented as follows:

Cold Exposure to Extremities: this was done by using zip lock bags filled with cold water at a temperature of 5.0 degrees Celsius that were put onto the subjects' right hand and left foot for a total duration of 1 minute and 40 seconds. The reason for choosing these two sites was that we wanted to avoid direct stimulus to the extremities where blood flow was measured. That is, as described above, we measured finger blood flow in the left index finger and foot blood flow in the right big toe and so we arranged for the cold to be applied in an opposite pattern. We made sure that the temperature of the water in the zip lock bags was identical (T = 5.0 degrees Celsius) across experimental sessions by storing the bags in a refrigerator that kept a constant temperature. We further made sure that the water was not cold to the point that it would elicit a pain response in the subjects, as that would consequently bring about the release of catecholamines as part of a global sympathetic response that might obscure homeostatic responses to temperature-induced vasoconstriction.

Application of ACh to the Forehead: our lab has used topical ACh in prior experiments and the knowledge gained from those experiments was used as the framework for this experiment. As ACh comes in the form of a solid, the first issue was to dissolve it into a solution creating a desirable concentration. We chose the same concentration of ACh that we used in prior experiments: we measured 100mg of ACh which we then mixed with 6ml of sterile water leaving a 7ml solution with a concentration of ACh equal to 14.3 mg/ml. As by our protocol, the maximum dose of ACh we allowed ourselves to use out of safety concerns was 10% of the standard 20mg dose injected into the eye during cataract surgery, i.e. 2mg. This implied that from our newly mixed solution with a concentration equal to 14.3 mg/ml, we could use no more

than 0.14ml in order not to exceed our safety limit. Through a process of trial and error, we established that 2 drops from a 25G needle would equal just under 0.14ml, and we therefore used this as our standard means for generating the 0.14ml aliquot. This dose was then placed on the adhesive side of a piece of transparent tape measuring around 1.4cm x 1.4cm and put onto the forehead in the designated area on the right side of the forehead under the moving probe holder with the integrating probe (see Figure 2). Prior measurements indicated that whereas there was a minimal loss of transmission of the laser Doppler through the tape, this was not significant to the point where we felt the need to correct for it. Furthermore, as we were primarily interested in the variability of the change in blood flow as a function of the fat content of the meal, and since all experimental sessions followed the same protocol, this slight reduction in transmission – and therefore also recorded blood flow – would be more or less constant across sessions.

Application of NTG to the Forehead: the technique used in this part of the experiment was similar to the one used for ACh. However, as opposed to the ACh which we had to mix and then apply to a transparent tape, the NTG was already in the form of a translucent drug patch ready to deliver NTG at a fixed rate of 0.03mg per cm^2 per hour. Again, out of safety concerns we wanted to limit the dose of NTG to 10% of the total dose delivered by the standard-size patch measuring 20cm^2, hence, we cut out a 1.4cm x 1.4cm (= 1.96cm^2) area. This was placed on the designated area on the left side of the forehead under the moving probe holder with the integrating probe (see Figure 2)

It is worth noting that the size of both the tape with ACh and the NTG patch was quite a bit larger than the 1cm x 1cm area from which the 9 measurements with the integrating probe was taken. By creating this buffer zone we minimized the chance of the

integrating probe recording over areas where the two drugs had not been applied. It also simplified the marking process outlined above under Forehead Blood Flow by allowing us to mark a simple dot at the center of the forehead that would serve as the reference point for the northeast and northwest corners of the ACh and NTG patch, respectively (see Figure 2).

Experimental Sessions

In all of the experimental sessions, the subjects were in a supine position on a standard medical examination table. They had a pillow under the head as well as under the left arm and under the knees and/or feet depending on what made them feel comfortable. They were instructed to lay as still as possible without talking. The sequence of events in terms of measurements and interventions for all of the session was as follows:

Step 1: After the equipment outlined above under Measurements was attached and working properly, we recorded 10 min of baseline while the subjects were relaxing on the table.

Step 2: The second step was part of a separate research project that was done alongside my thesis project; hence I will go through it quickly. After the baseline recordings were completed, we presented the subjects with a word-puzzle exercise in which the goal was to induce a state of mental stress and look at what effect this had on blood flow in the different body parts. This segment lasted for about 1 min and required the subjects to look at words on a computer screen as well as verbalize the solutions reached to the puzzles at the completion of the 1 minute exercise.

Step 3: Once the subjects had verbalized their solution to the word puzzle they returned to their baseline state of relaxation without talking, and we recorded another 5 min of baseline prior to initiating Step 4

Step 4: At this point we put the zip lock bags filled with cold water onto the subject's extremities as outlined above. We removed the bags after 1 minute and 40 seconds.

Step 5: In order to facilitate the return to baseline post the cold exposure, we applied zip lock bags of warm water to the same extremities that had previously been cooled. This warming was conducted for about 3 min.

Step 6: Upon removing the warm-water zip lock bags, we let the subject return to baseline for 5 minutes prior to starting Step 7

Step 7: We recorded the baseline values at the 9 spots in the 1cm x 1cm area designated for the application of ACh (see Figure 2). In order to simplify the calculation of the mean for all 9 spots, we made sure that we recorded blood flow for exactly 20 seconds at each spot. This time period is sufficiently large to average out any oscillatory variations caused by the parasympathetic nervous system, as well as short enough to minimize any temporal, systemic variations. In order to effectively use the software provided for data analysis (PowerLab), we stopped the recordings after each 20 second-reading. The reason for this is that the noised introduced when moving the integrating probe interferes significantly with the software's ability to accurately calculate the mean (see Picture 5).

Step 8: After we had recorded the blood flow for all the 9 spots for 20 seconds, we stopped the recording for a short period, attached the tape with ACh onto the forehead

in the designated area, put the integrating probe to spot 1 in the 9-square-grid in the area designated for ACh (see Figure 2), and started to record blood flow again. We waited for 7 minutes for the ACh to take effect prior to initiating Step 9.

Step 9: After the 7 minutes had passed, we repeated the procedure outlined in detail under Step 7, that is, we recorded the blood flow for all of the 9 spots in the ACh grid for 20 seconds to measure post-drug values.

Step 10: At this point we removed the tape with the ACh and wiped the area covered by the tape gently with a Q-tip to remove any remnant ACh. We then put the integrating probe back to spot 1 in the area designated for the NTG (see Figure 2) and started to record the blood flow in this area after baseline conditions were reestablished. One of the advantages of doing this was that it enabled us to compare the absolute value of this spot post ACh application to the absolute value we already had from this spot prior to the application of ACh (spot 1 in the NTG-grid area was the default spot for the integrating probe unless the experiment dictated otherwise). We could therefore easily gauge if there was any dilatory effect beyond the local area at which the drug had been applied. We waited for 5 min for the subjects to return to baseline before moving on to Step 11. We also recorded the subjects' blood pressure right after the ACh had been removed to make sure they did not have any significant systemic effect from the drug.

Step 11: Once 5 minutes had passed we recorded baseline blood flow for all the 9 spots in the 1cm x 1cm area designated for the application of NTG in a identical manner to that outlined under Step 7.

Step 12: We attached the NTG patch to the designated area (see Figure 2), and put the probe to spot 1 in the NTG area, and waited for 7 minutes for the drug to take effect.

Step 13: We recorded blood flow for all of the 9 spots in the NTG grid for 20 seconds to measure post-drug values.

Step 14: We removed the NTG patch and cleaned the forehead of the subject for any remaining drug. We also recorded blood pressure to make sure there was no systemic effect of the drug.

Step 15: We removed all of the equipment attached to the subject and he/she was monitored for a short while prior to being allowed to leave.

Analysis

Based on the literature (3), we anticipated that the vasodilatory response after a FM would be dampened by about 50% when compared to a NM. In order to detect a 50% difference with a power of 0.8 and an alpha level of 0.05, we determined that we needed to include ten subjects in the study. Due in part to time constraints, however, we ended up with only six subjects. We realize that in having too few subjects, we risk that our results will not be as strong in statistical terms as we would have liked.

Comparisons between groups for our interim (6-subject analysis) were performed with paired t-test; upon completion of the study, ANOVA will be employed for testing among all three groups. P-values > 0.05 are expressed as “NS”, whereas actual values are given for $p < 0.05$ to allow for subsequent corrections for multiple comparisons.

Results

Blood Flow (Table 1)

Forehead: Baseline blood flow did not vary significantly for the different meal sessions, although there was a slight tendency for increased flow in the FML session. There was a decrease in forehead blood flow when applying the vasoconstrictive challenge (cold) both for the NM and FML sessions (mean percent decrease in blood flow for NM session: 5.6 +/-5.4%, $p = 0.02$; and FML session: 9.0 +/-11%, $p = 0.03$). For the FM session, there was a similar, but weaker, tendency for decreased blood flow ($p =$ NS)

Finger: Baseline blood flow tended to decrease in the FM session compared to both the NM and FML sessions ($p =$ NS). There was a decrease in blood flow after applying the vasoconstrictive challenge (cold) for both the NM and FM sessions (mean percent decrease in blood flow for NM session: 42 +/-25%, $p = 0.012$; and FM session: 53 +/-13%, $p < 0.01$). A similar, but weaker, tendency of decrease in mean blood flow was seen in the FML session (mean percent decrease in blood flow for FML session: 33% +/-17, $p =$ NS).

Foot: Baseline blood flow showed a tendency for increased flow in both the FM and FML sessions compared to the NM session ($p =$ NS). After the application of the vasoconstrictive challenge (cold), the NM session showed no change in blood flow compared to the FM and FML sessions in which there was a tendency for decreased flow. None of these tendencies, however, had a $p < 0.05$.

	Meal Type	Average Baseline Blood Flow +/-SD	Average Blood Flow Cold +/-SD	Average [(Cold-Baseline)/Baseline] +/-SD
Forehead	NM	0.190 +/-0.037	0.180 +/-0.039	-0.056 +/- 0.054
Forehead	FM	0.193 +/-0.043	0.187 +/-0.044	-0.030 +/-0.112
Forehead	FML	0.221 +/-0.060	0.196 +/-0.044	-0.090 +/-0.110
Finger	NM	1.70 +/-1.42	0.85 +/-0.83	-0.42 +/-0.25
Finger	FM	1.11 +/-0.77	0.59 +/-0.55	-0.53 +/-0.13
Finger	FML	1.84 +/-1.62	1.25 +/-1.05	-0.33 +/-0.17
Foot	NM	0.34 +/-0.12	0.31 +/-0.07	0.004 +/-0.32
Foot	FM	0.59 +/-0.60	0.47 +/-0.41	-0.076 +/-0.37
Foot	FML	0.76 +/-0.83	0.67 +/-0.69	-0.024 +/-0.30

Table 1: Blood flow in volts (mean +/-SD). NM = non-fat meal; FM = fat-laden meal; FML = fat-laden meal + Lipitor.

Endothelium-Dependent versus Independent Vasodilation (Table 2)

Baseline blood flow for both the endothelium-dependent (ACh) and the endothelium-independent (NTG) areas of the forehead was more or less equal across session days, although there was a slight tendency for increased baseline flow in the FML compared to the NM and FM sessions.

The blood flow in the endothelium-dependent area after the application of ACh showed an increase in flow in all meal sessions (delta ACh), but this increase did not vary across the sessions. The blood flow in the endothelium-independent area after the application of NTG also showed a clear increase in flow across the meal sessions (delta

NTG). Furthermore, the delta NTG was relatively greater in the FM and FML sessions compared to the NM session (p = NS).

When comparing the ratio of endothelium-independent to endothelium-dependent vasodilation, mathematically expressed as [(delta NTG/baseline NTG)/(delta ACh/baseline ACh)], we found a relatively greater ratio in the FM compared to the NM session (mean ratio after FM was 177.6 +/-98% of that after NM, p = 0.036). Also, when comparing the FML to the FM session, this ratio was relatively increased in the latter session (p = NS).

Meal Type	NM	FM	FML
Baseline average (ACh area) prior to ACh application +/-SD	0.189 +/-0.056	0.194 +/-0.044	0.228 +/-0.051
Average post ACh +/-SD	0.338 +/-0.095	0.339 +/-0.117	0.380 +/-0.147
(Delta ACh/Baseline ACh) +/-SD	0.878 +/-0.520	0.798 +/-0.582	0.878 +/-0.354
Baseline average (NTG area) prior to NTG application +/-SD	0.192 +/-0.042	0.200 +/-0.040	0.215 +/-0.050
Average post NTG +/-SD	0.343 +/-0.069	0.409 +/-0.127	0.430 +/-0.127
(Delta NTG/Baseline NTG) +/-SD	0.826 +/-0.327	1.044 +/-0.412	0.992 +/-0.337
[(Delta NTG/Baseline NTG)/ (Delta ACh/Baseline ACh)] +/-SD	0.941 +/-0.635	1.310 +/-0.708	1.130 +/-0.937

Table 2: Endothelium-dependent versus endothelium independent vasodilation. Blood flow after each meal expressed in volts (mean +/-SD). NM = non-fat meal; FM = fat-laden meal; FML = fat-laden meal + Lipitor.

Oscillations (Table 3)

As mentioned above, when looking at oscillatory activity in the microvasculature, we are interested in oscillations in two frequency ranges; the first is 0.045Hz – 0.105Hz which represents both sympathetic and parasympathetic activity (low frequency oscillations); and the second is 0.105Hz – 0.195Hz representing primarily parasympathetic activity (high frequency oscillations). There are consequently three different ways one can gauge relative parasympathetic impairment, namely, the absolute power of high frequency oscillations; the ratio of low to high frequency oscillations and the ratio of high to low frequency oscillations. The most commonly used parameter is the ratio of low to high frequency oscillations, hence we focused on this ratio in our analysis. The ratio of low to high frequency oscillations would increase if oscillations in the parasympathetic range decrease (in relative terms), and vice versa.

When looking at baseline oscillations, the mean ratio of low to high frequency oscillations after the FM was 222 +/-109% of that after the NM, suggesting a relative decrease in parasympathetic activity after the FM ($p = 0.016$) A similar trend, i.e. a relative decrease in parasympathetic oscillations, was seen when comparing FML to the NM session ($p = NS$).

When comparing the ratio of low to high frequency oscillations at baseline versus during the application of a vasoconstrictive challenge (cold) within each of the meal sessions, there was a strong tendency towards a decrease in this ratio during the vasoconstrictive challenge in all of the three sessions, suggesting an increase in parasympathetic oscillatory activity during the challenge ($p < 0.03$, $p = NS$, and $p < 0.03$ for NM, FM, and FML, respectively).

Lastly, when comparing the ratio of low to high frequency oscillations during the vasoconstrictive challenge for the NM versus the FM session, there was a clear tendency towards a greater ratio after the FM, suggesting a relative decrease in parasympathetic oscillatory activity after the FM (mean ratio of low to high frequency oscillations after FM was 321 +/-242% of that after NM, $p = 0.05$). If one compares the absolute power in the high frequency range for the NM versus the FM during the vasoconstrictive challenge, there was even stronger evidence of impaired parasympathetic oscillatory activity after the FM. During the vasoconstrictive challenge, the mean high frequency absolute power after the FM was 52.8 +/-24.9% of that after the NM, $p < 0.01$. When comparing the NM to the FML session during the vasoconstrictive challenge, there was a similar, but weaker, tendency for reduced power of parasympathetic oscillations in the FML session (p = NS).

Meal Type	NM	FM	FML
Baseline low frequency oscillations +/-SD	7.6 +/-3.0	13.7 +/-7.9	12.0 +/-6.6
Baseline high frequency oscillations +/-SD	73.3 +/-43.0	49.6 +/-15.7	108.6 +/-161.1
Baseline (low frequency/high frequency) oscillations +/-SD	0.16 +/-0.11	0.31 +/-0.17	0.34 +/-0.34
Post cold low frequency oscillations +/-SD	11.9 +/-13.5	14.9 +/-15.4	13.3 +/-6.8
Post cold high frequency oscillations +/-SD	214.5 +/-98.4	110.8 +/-71.7	197.0 +/-180.3
Post cold (low frequency/high frequency) oscillations +/-SD	0.07 +/-0.07	0.22 +/-0.21	0.31 +/-0.39

Table 3: Oscillatory power in volts^2/Hz after each meal (mean +/-SD). NM = non-fat meal; FM = fat-laden meal; FML = fat-laden meal + Lipitor.

Discussion

Overall Impression

Our data indicate that the ingested FM had an effect on both the vasodilatory characteristics of the endothelium as well as on its ability to initiate parasympathetically controlled oscillatory activity. Likely partly due to the fact that we only studied six subjects, the statistical power of our study was at times a bit weak, but we nonetheless found several strong trends. Moreover, it appeared that the different trends of the experiment were in line with one another. Specifically, it seems clear that elevated blood lipid levels impair proper endothelium function, demonstrated through both dysfunctional vasodilation as well as reduced oscillation.

Endothelium-Dependent versus Independent Vasodilation

The FM prompted a greater relative increase in endothelium-independent vasodilation as compared to endothelium-dependent vasodilation. In other words, the ratio of NTG-induced to ACh-induced vasodilation was greater after the FM than after the NM. This ratio of NTG-induced to ACh-induced vasodilation was also greater in the FML compared to the NM, although this difference was less pronounced, suggesting that Lipitor has some modifying effect on endothelial dysfunction in the setting of acutely elevated blood lipid levels.

It is worth noting that if one looks at vasodilation induced by topical drug applications individually, i.e. delta ACh and delta NTG, in the NM versus FM, neither of them showed a statistically significant pattern. If, however, one looks at the ratio of the two deltas, either by comparing the ratios of the deltas directly, or by comparing the

ratios of [(delta NTG/baseline NTG)/(delta ACh/baseline ACh)] as we decided to do, the results show a statistically significant trend toward a greater ratio after the FM.

Thus, endothelium-dependent vasodilation did not go down to any significant extent; rather it was the endothelium-independent vasodilation that went up to a greater degree after the FM compared to the NM – although this increase was not statistically significant in and of itself. Most of the experiments in the literature have documented a statistically significant decrease in endothelium-dependent vasodilation post a FM, without using the ratio of endothelium-dependent to independent dilation. And none of these experiments have documented a tendency for *increased* endothelium-independent vasodilation to correlate with blood lipid level in the same way that we did.

One key difference in our experiment which might help to explain why we found a somewhat different pattern in terms of vasoreactivity is that we were analyzing capillary blood flow in the forehead whereas most of the other studies have looked at the brachial artery. In addition, we used topical drugs to induce the vasodilatory changes compared to most other studies which have used Flow-mediated Dilation, injected or sublingual vasoactive drugs or venous occlusion strain-gauge plethysmography. Furthermore, it is possible that the amount of ACh used in our experiment was so high that it overcame the dysfunction in the endothelium by simply overpowering it. The latter theory is supported by the findings in the oscillation-part of the experiment which is described below.

Thus, the results from this portion of our study suggest that, after a FM, the augmentation of blood flow which is typical after eating any meal has a greater impact on endothelium-independent blood flow relative to endothelium-dependent blood flow. The

etiology of this difference was not clear from this portion of the study; however, evidence of impaired endothelium-dependent vasodilatory capacity is suggested by the oscillatory response to a vasoconstrictive challenge in the different meal sessions (discussed below).

Oscillations

The results in terms of the compensatory parasympathetically controlled oscillations were more in line with what we had hypothesized prior to the experiment.

First of all, there was a clear trend towards a relative increase in oscillatory activity in the parasympathetic range (measured through the ratio of low to high frequency oscillations) during the vasoconstrictive stimulus within each of the three meal sessions. This trend was significant for both the NM and FML sessions (p-value < 0.03 using paired t-test), and the same tendency was seen in the FM session (p = NS). Such a response is consistent with the homeostatic oscillatory response of the forehead microvasculature reported in response to systemic phenylephrine (16)

Secondly, the data showed a relative decrease in parasympathetic oscillatory power when comparing the NM to the FM sessions. This relative decrease was seen both when comparing baseline oscillatory activity (p = 0.016) as well as when comparing oscillatory activity after the application of the vasoconstrictive challenge (p = 0.05). It was somewhat surprising that comparing the baselines would show a more statistically powerful trend compared to after the vasoconstrictive challenge, as we expected the vasoconstrictive challenge to be the best way to reveal the impaired capacity of the endothelium to oscillate. If, however, we look exclusively at the power of oscillations in the frequency range of 0.105Hz – 0.195Hz, i.e. primarily parasympathetic oscillatory

power, for the NM versus FM sessions post the vasoconstrictive challenge, we get p-value of less than 0.01 (paired t-test), indicating a strong decrease in absolute power in the parasympathetic range post a FM.

As was seen in the vasodilation part of the experiment, when comparing FML to the FM session, there is a clear tendency for less parasympathetic oscillations in the FM session (both baseline oscillations as well as oscillations post a vasoconstrictive challenge). Again it appears as if the Lipitor has some modifying effect on the interference caused by high blood lipid levels on endothelial function.

In the introduction we pointed out that ACh is a key player in the parasympathetically mediated compensatory oscillatory efforts, and that the proof of this was the elimination of oscillations upon administration of atropine. From the conclusions reached in the analysis of endothelium-dependent versus independent vasodilation above, namely that a FM did not appear to reduce endothelium-dependent vasodilation much in absolute terms (although it was reduced relative to endothelium-independent dilation), it was surprising to see that parasympathetic oscillatory power was reduced to the degree that it was. That is, one wonders why in the vasodilation part of the experiment, ACh-mediated processes appeared not to be impaired to any great extent, whereas in the oscillation part of the experiment – which is also mediated by ACh – a significant impairment did appear to take place. One explanation might be that there actually was more of an impairment in endothelial function post a FM than one is led to think from looking at ACh-induced vasodilation in isolation (as pointed out above, our results reached statistical significance only when comparing the *relative* impairment in endothelial-dependent to endothelium-independent dilation). It is possible, as touched

upon already, that the amount of ACh administered topically was sufficiently high so as to partly or almost completely overcome the impaired state of the endothelium by a mere dose effect. In other words, if we had used a lower dose of ACh to induce vasodilation, the impaired response post a FM would have been more pronounced.

Lipitor as Modulator

The evidence obtained from the session where Lipitor was co-administered with the FM did show a clear tendency towards improved endothelial function relative to the FM session without Lipitor. This pattern was consistent both in the vasodilation and the oscillation part of the experiment. However, the statistics comparing FML to the NM session showed that, although moving towards its normal state, the Lipitor did not cause a complete normalization of endothelial behavior. That is, the "health" of the endothelium in the Lipitor session, as gauged through our vasoactive challenges, was almost exclusively in between the normal state and the dysfunctional one seen post the FM.

Limitations of the Study

There were a couple of limitations which are worth mentioning and which should be addressed in any future experiments. First of all, we had a relatively small number of subjects, hence our statistical analysis was not as strong as we would have liked. Secondly, whereas the NTG patch is a pre-made patch which delivers a standard dose of drug per square unit, the ACh we used had to be mixed into a solvent and then put onto the forehead by means of a translucent tape that was attached to the desired region on the forehead. There were two issues with this. First of all, it was difficult to ensure a uniform

distribution of the ACh solution under the tape, or in other words to make sure that an equal amount of drug was administered onto any given area being analyzed. Secondly, because the required drops of ACh solution were placed underneath the tape, the tape consequently lost some of its adhesiveness and for a couple of the subjects the tape started to detach itself from the forehead at the edges, leaving the ACh solution at risk of evaporating as well as removing some of the driving pressure for the ACh to penetrate the skin. Thirdly, in lieu of the discussion on the amount of ACh used to induce endothelium-dependent vasodilation, and how the dose possibly was large enough to overpower any impairment in the endothelium, it might be beneficial to use a lower dose of ACh than we did. Lastly, another problem with using the microvasculature in the forehead is the great spatial and temporal heterogeneity at the level of the capillary in the region, and although we tried to minimize the potential uncertainty introduced by this problem based on design improvements developed through earlier research, it is unlikely that we were able to sidestep the problem completely.

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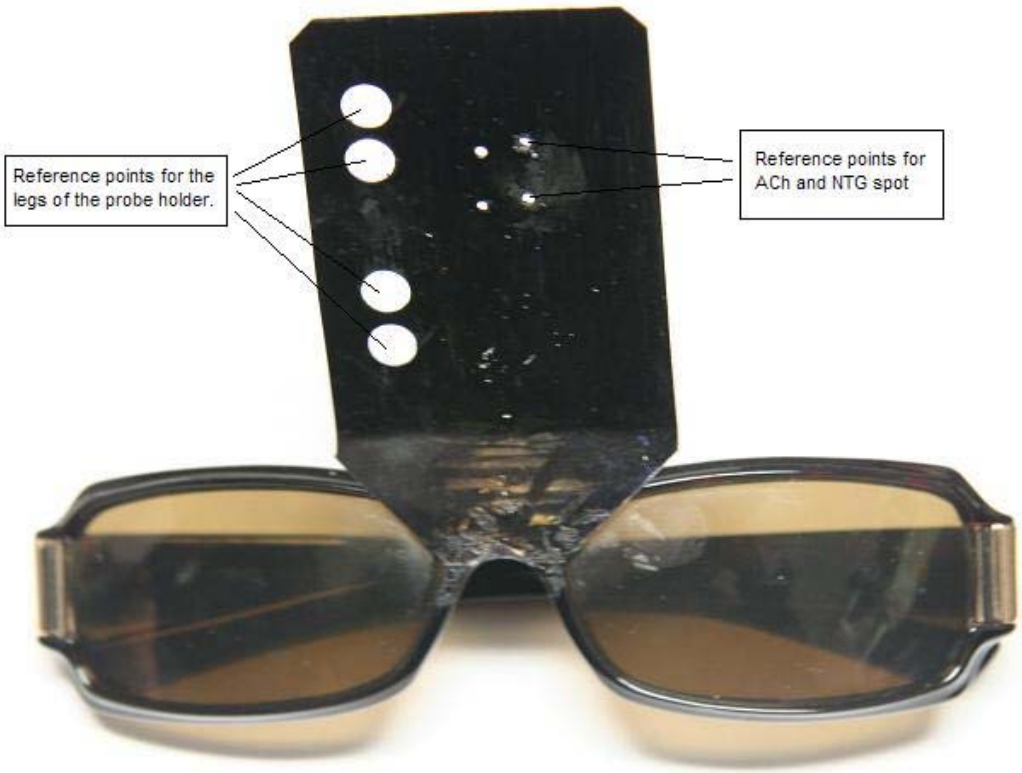
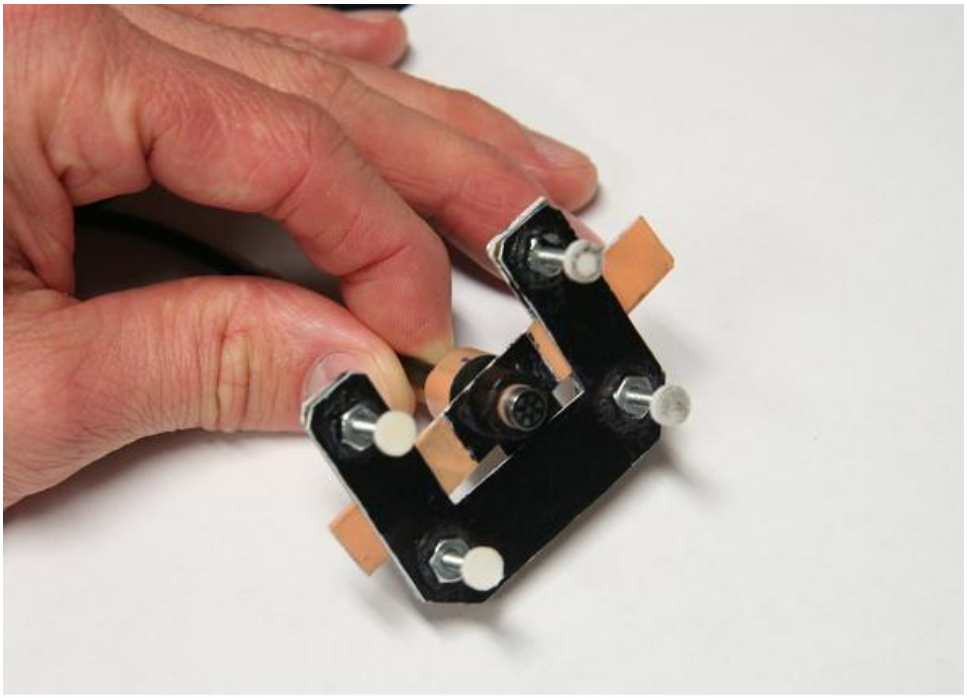
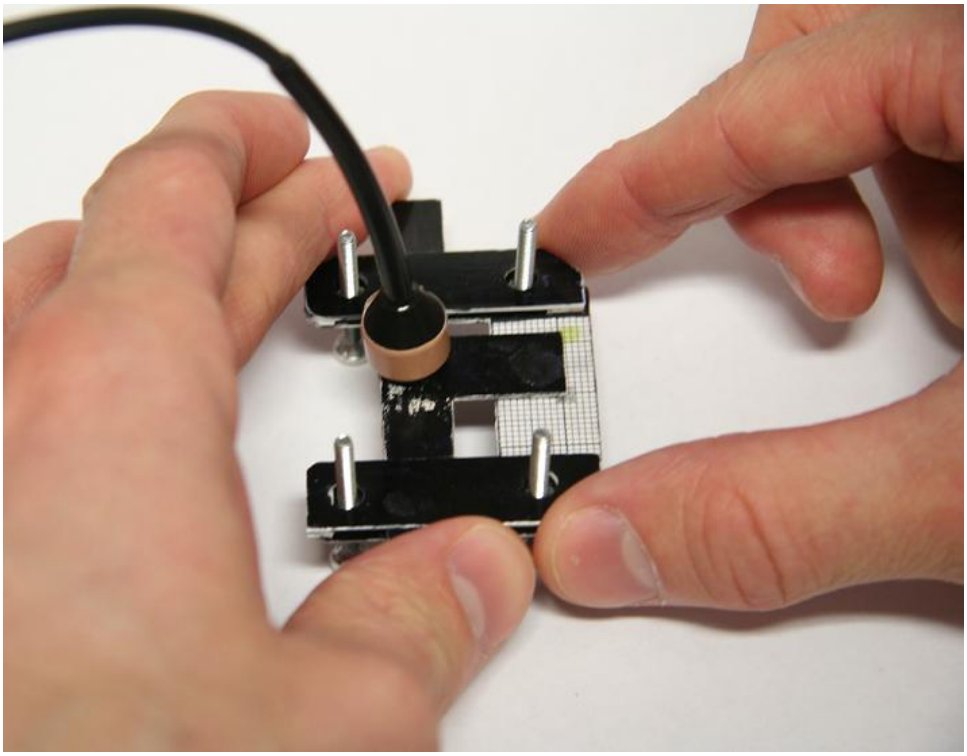
Pictures



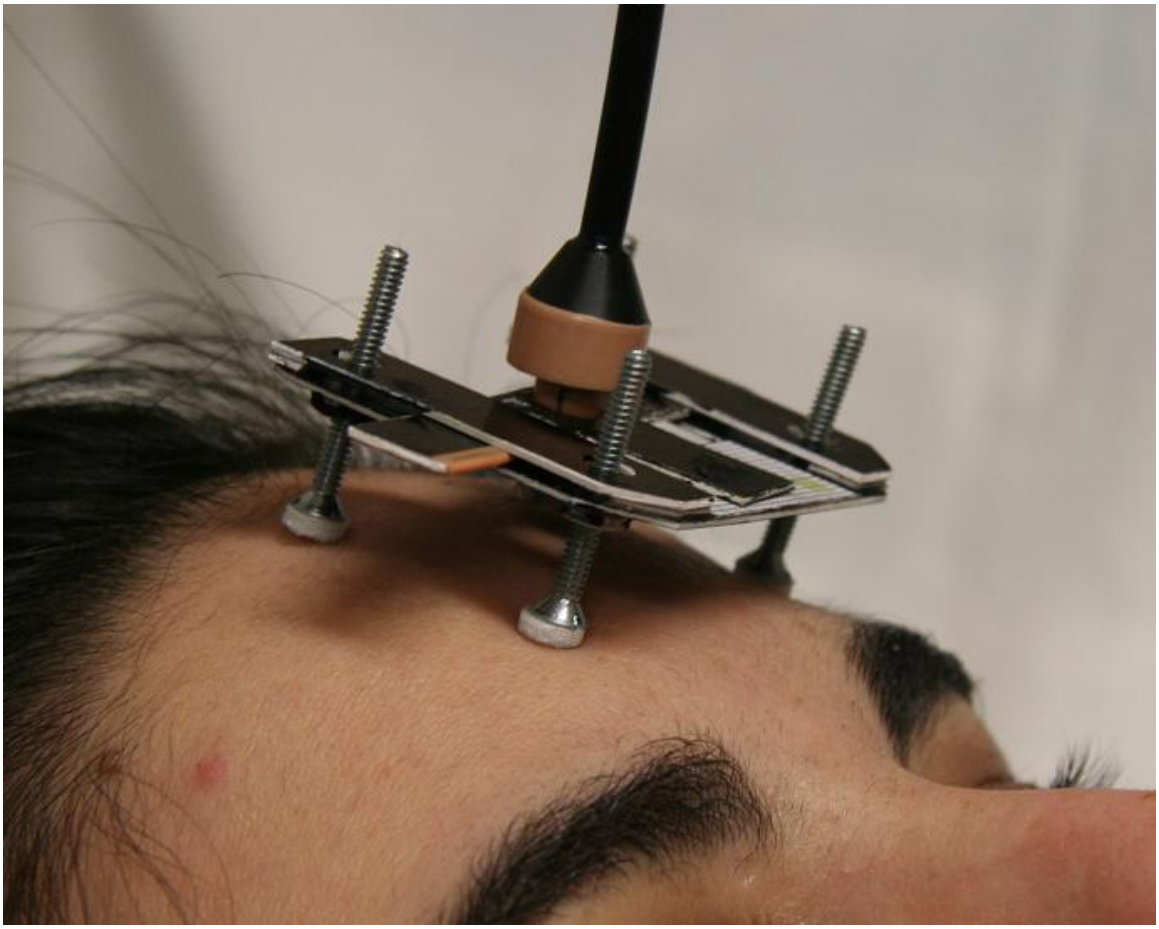
Picture 1: Device used to minimize spatial heterogeneity across experimental session.



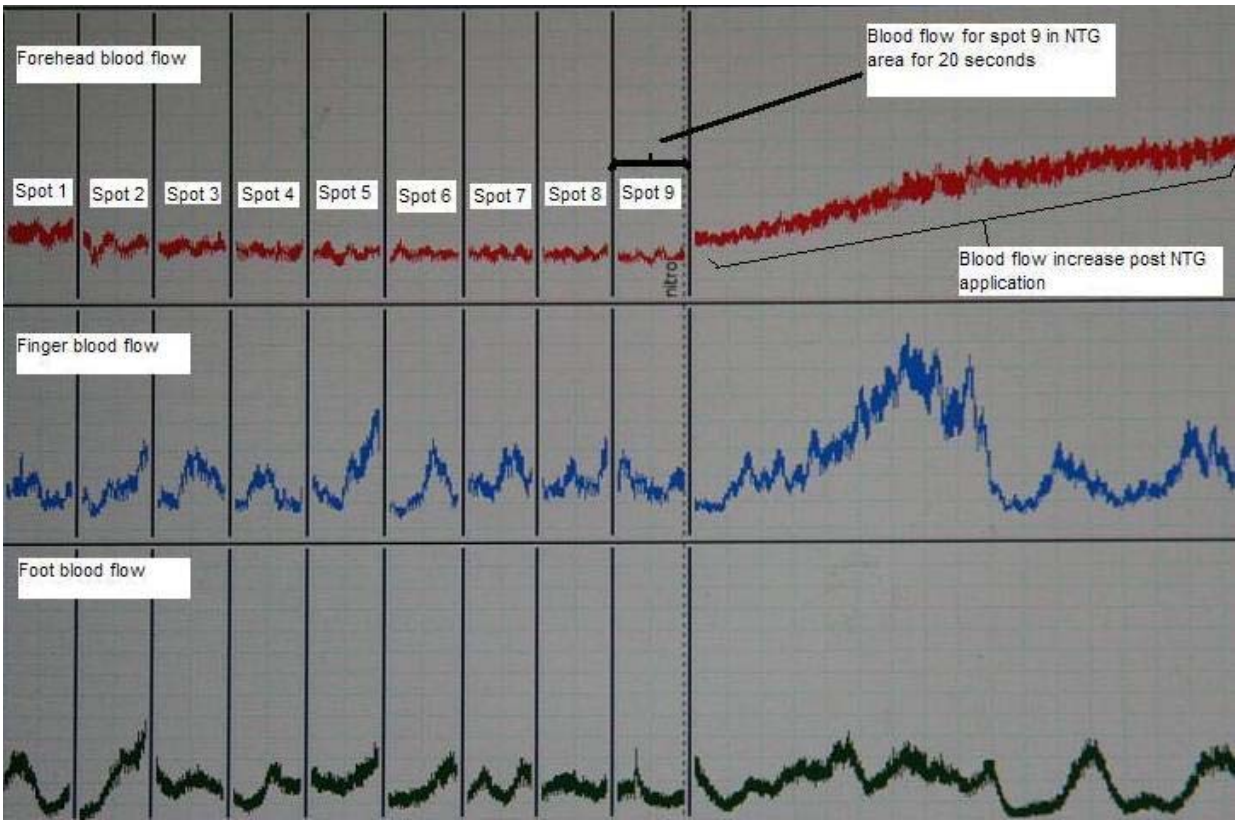
Picture 2: Probe holder – viewed from underside.



Picture 3: Probe holder with probe in place.



Picture 4: Probe holder and probe in place on forehead.



Picture 5: Computer screen during recording of baseline blood flow. The image contains the baseline reading spots in the NTG designated area as well as some minutes post the local application of NTG.