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IMPACT OF SALT LOADING ON ENDOTHELIAL DEPENDENT VASODILATION AND ARTERIAL BLOOD PRESSURE

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IMPACT OF SALT LOADING ON ENDOTHELIAL DEPENDENT VASODILATION AND ARTERIAL BLOOD PRESSURE

by

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ABSTRACT

Cardiovascular diseases are the leading cause of premature mortality. Hypertension, or high blood pressure, is the most prevalent risk factor, with high dietary sodium intake being a strong predictor. Some claim that sodium has a pathological role when intake is low or high and a physiological role when sodium intake is moderate, however the population groups these studies were performed in had underlying health conditions so they were not considered to be healthy. Recently, researchers indicated a 2-compartment model of sodium regulation and arterial dilation involving storage in the sugar molecules, glycosaminoglycans (GAGs), that are on the surface of epithelial/endothelial cells to form the glycocalyx. When the glycocalyx is disrupted, sodium enters endothelial cells and reduces their ability to cause dilation. Exercise can increase blood flow/shear stress and oxidative stress, which may cause the glycocalyx to be perturbed. In this study, participants were tested on four different occasions – twice at baseline and twice post-supplementation. Continuous ECG and blood pressure measurements were collected through each testing session. Participants underwent a flow mediated dilation (FMD) protocol to measure endothelial dependent dilation of the brachial artery using Doppler ultrasound as well as a 30-minute exercise bout using a handgrip dynamometer. Following the baseline testing session, participants were instructed to ingest 16 capsules per day of either salt or placebo (sugar), for a seven-day period that began on the day their baseline measurements were gathered. After a week of taking the capsules for a week, participants were tested following the same steps as the baseline session. They then underwent a "wash-out" period for a week and were tested again at the end of that week as a second baseline. Participants were then given the capsules that contained the other corresponding contents for another seven-day period and were then tested again at the end of the seven-day period. The data was analyzed using a within-subject repeated measures analysis of

variance (ANOVA), with the software JASP. We hypothesized that when there is an increase in sodium levels, the endothelial function will be affected especially in vascular systems where the glycocalyx has been modified by increases in blood flow through exercise. We also hypothesized that arterial blood pressure will be increased systemically. We found significant changes in both absolute and relative FMD responses between the baseline and post testing for the salt condition. We did not find any significant differences from baseline to post testing for the placebo. We also did not find any significant differences between the shear rates, baseline brachial artery diameters, and blood flow for both the salt and placebo conditions and testing sessions. Exploratory post-hoc analyses also suggest that absolute dilation was reduced after sodium intake compared to their baseline and placebo conditions. Finally, we found no significant changes in systolic, diastolic, and mean arterial blood pressure in either condition or from baseline to post testing and there was no significant difference in heart rate. The methodology presented here lays a strong foundation for future work and has the potential to be further developed into a larger scale study. If future studies see significant differences in exercise-induced endothelial function post salt-loading and if other physiological mechanisms effect endothelial function and arterial blood pressure, individuals will then be able to adjust their dietary sodium intake to a level which reduces the risk of cardiovascular disease and hypertension.

Thesis Supervisor: Dr Mark E. Rakobowchuk

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1. INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of premature mortality and disease burden globally (Roth et al., 2020; Namara et al., 2018). Hypertension or high blood pressure is the most prevalent risk factor for CVD. One of the strongest predictors of hypertension is the high dietary sodium intake (Tran et al., 2021). The World Health Organization (WHO) recommends that individuals ingest less than 5 grams of dietary salt (2 grams of sodium) per day to lower the risk of developing cardiovascular disease and hypertension (Babcock et al., 2019; World Health Organization, 2022). However, the diet of many individuals in western society exceeds this recommendation, increasing their risk of developing cardiovascular diseases (Olde Engebrink et al., 2015). The major sources of salt are processed foods, ready-made meals, and salt added during food preparation, cooking, and at the table (Wang et al., 2020). This increase in dietary salt leads to an increase in blood pressure, which can lead to the development of cardiovascular diseases (Wang et al., 2020; Grillo et al., 2019; O'Donnell et al., 2015).

1.1 Two-Way Compartment Model for Sodium Regulation

Babcock et al. (2019) discuss the past belief that only the kidneys regulated long-term blood pressure through the regulation of renal sodium excretion. This belief led to researchers such as Mente et al. (2018) to claim that sodium has a pathological role when intake is low or high and a physiological role when sodium intake is moderate. These studies only looked at sodium excretion in urine and they also did not study populations that were considered healthy. Instead, majority of individuals that were tested had underlying health conditions and their kidneys did not function properly. However, Olde Engebrink et al. (2015) and Casale & Crane (2022) identified that sodium is regulated in a 2-compartment model with sodium storage that can occur in the skin interstitium, where glycosaminoglycans (GAGs) bind and inactivate sodium and thus remove it

from renal sodium excretion to help the body regulate sodium levels and therefore, blood pressure. High sodium diets increase the amount of sodium content in the skin, leading to an increased expression in both skin glycosaminoglycan content and XYLT-1 (xylosyltransferase 1), an enzyme that that initiates GAG synthesis. This increase is thought to be the main driving force behind skin sodium accumulation during a high sodium diet (Wenstedt et al, 2018).

1.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are molecules found throughout the body, including skin, joints, blood plasma, and the mucous membrane of various organs (Casale & Crane, 2022). GAGs are large, negatively charged linear polymers consisting of disaccharide unit repeats and are responsible for storing sodium (Casale & Crane, 2022). Specific combinations of these repeating units result in different types of GAGs, such as heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronan, with heparan sulfate GAGs being the most prominent on endothelial cells followed by chondroitin sulfate and hyaluronan GAGs (Olde Engberink et al., 2015).

1.3 The Endothelium Surface Layer

The endothelium surface layer (ESL) is a dynamic layer on the luminal side of the endothelial cells that is in continuous exchange with flowing blood. It comprises a network of glycoproteins, adsorbed plasma proteins, and proteoglycans to which glycosaminoglycans (GAG) chains are attached forming the glycocalyx (Olde Engberink et al., 2015; Wenstedt et al, 2018). The ESL is in direct contact with plasma sodium and therefore, could function as the first sodium buffer before sodium enters the interstitium (Wenstedt et al., 2018). The negative charges of the ESL automatically attract ions of the opposite charge when they are located within an electrolyte solution, such as blood. Because sodium is the most abundant cation in circulating blood, sodium

forms a so-called ion atmosphere around the endothelial cell and ESL. Considering the sodium-binding properties of GAGs that makeup the glycocalyx, it is conceivable that the attracted sodium ions are bound and osmotically inactivated by the glycocalyx in the ESL (Olde Engberink et al., 2015).

An impaired ESL may also facilitate leakage of glycosaminoglycans into the interstitium. Increased leakage of glycosaminoglycans to the skin together with increased skin glycosaminoglycan synthesis may, therefore, serve as the explanation for a sodium-induced increase in tissue glycosaminoglycan content and concomitant sodium accumulation when ESL perturbations are present (Wenstedt et al., 2018).

The ESL also plays an important role in mediating shear-induced nitric oxide (NO) production through mechanotransduction and activation of the enzyme, endothelial nitric oxide synthase (eNOS). Due to its location, the ESL must be considered a possible sensor of fluid mechanical shear stress that can distribute force to other regions of the endothelial cell where transduction to biomolecular signals may occur (Pahakis et al., 2007). Apart from regulating mechanotransduction, the ESL can modulate NO availability by increasing sodium transport into the endothelial cell (Olde Engberink et al., 2015). A study conducted by Pahakis et al. (2007) discovered the epithelial sodium channel (EnNaC) on the endothelial luminal surface. It was shown that this EnNaC regulates endothelial nanomechanics and subsequently affects NO production. By enhancing sodium influx, the EnNaC increases mechanical stiffness of the endothelial cellular cortex (Jeggle et al., 2013; Pahakis et al., 2007). The stiffness of this 50- to 100-nm layer, which mainly consists of actin filaments, subsequently modulates endothelial NO synthase activity and NO production, where an increasing stiffness attenuates NO production (Jeggle et al., 2013; Kusche-Virog et al., 2010). The density of EnNaCs on the endothelial surface

is regulated by aldosterone and plasma sodium concentration. A rise in plasma sodium concentration increases EnNaC density, which in turn, increases sodium uptake, stiffens the endothelial cellular cortex, and subsequently, leads to diminished NO production (Kusche-Virog et al., 2010; Korte et al., 2012). An increase in sodium delivery to the endothelial cell as a result of an increase in sodium intake could, therefore, lead to an increase in vascular tone (Olde Engeberink et al., 2015).

1.4 Nitric Oxide

Shear-induced nitric oxide production is a hallmark of endothelial mechanotransduction that is a significant marker of vascular tone as it is the most powerful physiological regulator of endothelial Nitric Oxide Synthase, leading to rapid rises in NO (Sprague et al., 2010; Pahakis et al., 2007). Inactive eNOS is bound to the protein caveolin. When intracellular levels of Ca^{2+} increase, eNOS detaches from caveolin and is activated and goes on to produce NO (Sandoo et al., 2010). Nitric oxide is a labile, lipid soluble gas that utilizes the enzyme eNOS, which catalyzes the amino acid L-arginine to L-citrulline, with NO as a free radical by-product. These are what make NO (Green et al., 2004; Sprague et al., 2014). It is important to consider that the production of NO from the conversion of L-arginine through the eNOS enzyme can often become compromised, such as in cardiovascular diseases, and the reduced availability of NO in the body limits the ability of the arteries to properly dilate. The body, however, counteracts this effect through the activation of other redundant dilation pathways, such as prostacyclin (PGI_2), which plays a compensatory role in dilation of the vessel when NO is reduced or blocked (Sandoo et al., 2010).

1.5 Reactive Oxygen Species

The maintenance of a healthy endothelial phenotype relies on a delicate balance between NO production and reactive oxygen species (ROS) formation (H_2O_2 and O_2^-), both of which are crucial to the maintenance of cellular redox potential and redox-related cell signalling (Lee et al., 2017). Shear stress exerted by laminar blood flow in the ESL increases NO availability, while reducing ROS production. Shear stress protects endothelial redox homeostasis and counteracts endothelial dysfunction. ROS may uncouple the eNOS-catalyzed reduction of molecular oxygen from the oxidation of L-arginine, resulting in the production of the ROS superoxide anion (O_2^-) instead of the reducing NO. Alternatively, ROS may react with NO directly, reducing its bioavailability (Zhao et al., 2015; Schulz et al., 2011; Förstermann & Münzel, 2006). When increased oxidative stress and endothelial dysfunction are encountered, the expression of eNOS increases to compensate. The demonstration of endothelial dysfunction in the presence of increased expression of eNOS indicates that the capacity of the enzyme to produce NO may be limited, and the concept that eNOS itself can be a superoxide source and thereby contribute to endothelial dysfunction (Schulz et al., 2011; Joyner & Green, 2009). Decreased NO availability, secondary to enhanced NO degradation by ROS can tip the redox balance and cause impaired NO-mediated signalling, an early hallmark of endothelial dysfunction (Lee et al., 2017; VanBavel, 2007). The buildup of these ROS can then lead to a decrease in bioavailability of NO, leading to endothelial dysfunction.

1.6 Effect of Sodium on Nitric Oxide

There is also evidence that differing sodium levels alter endothelial function and NO production (Aldecoa et al., 2020; Olde Engberink et al., 2015). During high sodium intake, the glycocalyx becomes deteriorated or “stiff” due to a lack in heparan sulphate residues and thus exhibiting a reduced sodium buffer capacity. This then interrupts cell signals, such as the

mechanical stimulus which triggers eNOS to produce NO (Oberleithner, 2011). Oxidative stress due to an increase in ROS generation decreases NO bioavailability and sodium induced endothelial dysfunction (Edwards & Farquhar, 2015). This decrease in NO would then increase arterial blood pressure because when there is a decrease in NO bioavailability, the blood vessels become less responsive to the normal signals that regulate blood flow, leading to an increase in vascular resistance and a rise in blood pressure (Hermann et al., 2007; Edwards & Farquhar, 2015).

1.7 Exercise and Endothelial Function

Exercise improves endothelial function through increased in shear stress mediated NO production (Goto et al., 2007). The repeated induction of eNOS activity that occurs during exercise training, might prolong the half-life of NO by reducing its degradation by free radicals or by directly decreasing free radical production (Green et al., 2004). However, exercise also leads to immune activation through increased movement of blood and lymph and catecholamine induced increases in immune cell function (which contains white blood cells). The increased shear and catecholamine responses increase cell motility and more immune cells from lymph nodes and the spleen migrate into the bloodstream (Cerqueira et al., 2020). This increased immune response then leads to elevations of ROS present. The glycocalyx can also be modified after acute perturbations like an increase in blood flow and following this perturbation, it may be contributing to changes in how well blood vessels dilate. A study conducted by Sapp et al. (2019), predicts that a high intensity exercise bout induces acute glycocalyx shedding due to increased amounts of ROS due to an increase in oxidative stress. The endothelial function decreases as a result as the glycocalyx plays a major role to shear-mediated NO production. As the glycocalyx sheds, NO bioavailability decreases along with endothelial function (Sapp et al., 2019; Kröpfl et al., 2021). In our study, the level of exercise will only be moderate, so we may not see immune response induced glycocalyx

shedding. Locally mediated blood flow can also affect the thickness of the glycocalyx. When blood flow is increased, the shear stress from the flowing blood can cause the glycocalyx to become less thick or even to be shed from the endothelial surface, allowing for decreased dilation of the blood vessel (Kröpfl et al., 2021).

1.8 Flow-Mediated Dilation

Flow-mediated dilation (FMD) is used as a non-invasive approach to observe and analyze endothelial function and endothelium-derived NO bioavailability, particularly that of the brachial artery (Thijssen et al., 2011; Green et al., 2011). The FMD technique increases blood flow through an artery to cause smooth muscle relaxation and subsequent dilatation on the principal that the increased blood flow produces shear forces on the endothelium and is transduced using mechanoreceptors and subsequently stimulates endothelial cells to release NO (Sandoo et al., 2010; Harris et al., 2010). Reduced dilatation following an increase in shear forces is representative of impaired NO bioavailability and therefore, FMD is a good surrogate marker of NO bioavailability (Green et al., 2011; 2004). This method involves direct imaging, using a Doppler ultrasound, of large artery dilator responses to shear-stress-induced FMD consequent to a brief period of limb ischaemia, a decrease in blood supply to the tissues in the limb. Assuming the occluding cuff is placed distal to the scanned artery and that the period of ischaemia does not exceed 5 min, the increase in arterial diameter in response to this stimulus is almost exclusively mediated by NO (Green et al., 2004; Thijssen et al., 2013).

When pairing FMD with exercise, multiple studies have discovered that there is a biphasic change in FMD after exercise. This is where there is a decrease in endothelial function and therefore, a decrease in the FMD response when performed immediately after a 30-minute bout of exercise (Dawson et al., 2013). A study conducted by Johnson et al. 2012, found that FMD

decreased immediately following 30 minutes of either moderate or high intensity cycle training. The FMD response then returned to baseline levels by the 60-minute mark post exercise. Nitric oxide bioavailability is decreased and there is a development of oxidative stress during exercise which likely contributes to an immediate decrease in FMD after a single exercise bout (Dawson et al., 2013). When sodium levels are increased, it has been demonstrated to impair endothelial function, as assessed via FMD. When paired with exercise, we predict that the exercise will exacerbate the sodium induced endothelial dysfunction and prevent the artery to recover and return to baseline endothelial function when the final FMD is conducted.

Upon completion of this study, it is expected that when there is an increase in sodium levels, the endothelial function will be affected especially in vascular systems where the glycocalyx has been modified by increases in blood flow. It is also hoped that arterial blood pressure will be increased systemically. We will alter the glycocalyx in an isolated limb to test whether this salt sensitivity happens in young adults. If these results are validated, future studies can assess differing sodium levels in the ESL to help determine when the endothelial function and arterial blood pressure become affected. With this information, individuals will then be able to adjust their dietary sodium intake to a level which reduces the risk of cardiovascular disease and hypertension.

2. MATERIALS AND METHODS

2.1 Ethical Approval

Ethical approval for this study was granted by the Thompson Rivers University Research Ethics Board. All participants gave their written and informed consent, both after the experimental procedure and risks had been explained to them and prior to the start of testing.

2.2 Study Participants

Nine healthy participants including 5 males and 4 females between 21 – 23 years of age were recruited to participate in this study. Mean participant age was 21.7 ± 0.867, height was 179 ± 7.30 cm, and mass was 73 ± 11.3 Kg. Participants were screened prior to testing to ensure they met inclusion criteria. All participants had no blood or platelet/bleeding disorders, and no known cardiac diseases and/or cardiovascular risk factors. None of the participants were hypertensive, smokers, diabetics, or had other known metabolic diseases. The participants were not taking medication, other than the contraceptive pill, or being treated for any disease, did not experience aversion to the sight of blood and/or needles, and were over 19 years of age and under 40 years of age.

Preceding testing, participants were instructed to arrive at the laboratory rested, having refrained from strenuous physical activity, alcohol, marijuana, or non-prescription drug ingestion 24 hours prior to sampling. They were instructed not to cycle or run to and from the laboratory on sampling days, nor partake in any physically demanding work in the hours after the sampling day. Participants were asked to avoid caffeine on the day of sampling before their testing and eating within four hours prior to sampling. The menstrual cycle was also taken into account for female participants. Both baseline days were conducted during the same phase of their menstrual cycle to ensure hormone levels were the same as a change in hormonal levels can affect FMD (Thijssen et al., 2019). Additionally, if the female participants were taking the contraceptive pill and participated in “stacking” the pills, where they did not take the sugar pills and continued to take the contraceptive pill and forgo their period, they followed the same testing schedule as the male participants as they did not experience a phase change in their menstrual cycle.

2.3 Experimental Design

Participant testing consisted of blood pressure, electrocardiogram (ECG), and ultrasonographic measurements **(Figure 1)**. Upon arrival at the lab, participants were placed in a supine position in a temperature controlled (21-24°C) room. Participants were instrumented with a non-invasive continuous blood pressure monitor that output an analog continuous waveform to a BIOPAC data acquisition system (Biopac MP160, Biopac Systems, California, USA). The CNAP (CNAP Monitor 500 NBP100D-1, CN Systems, Graz, Austria) monitor consisted of finger cuffs placed around the left index and middle fingers, one of which was monitored continuously using photoplethysmography. Once an adequate blood pressure signal was obtained, the participant remained in a supine position for ten minutes to ensure baseline sampling of blood pressure and ECG was taken at rest, with minimal sympathetic nervous system activation. Blood pressure and ECG data was obtained by collecting a 2-minute resting average during each session for every participant.

Once the rest period was complete, participants underwent a flow mediated dilation (FMD) protocol to measure endothelial dependent dilation of the brachial artery using Doppler ultrasound. Following the FMD, participants underwent a 30-minute exercise bout using a handgrip dynamometer. A second FMD was conducted immediately after the exercise period was complete. The participant was then given a 30-minute rest period. Once the 30-minute rest period was complete, a third and final FMD was conducted. At the end of testing, participants were given 112, one-gram supplements. The supplements given either contained white, granulated sugar or table salt. Participants were unaware of which supplements they were given as this information was only known to the individuals on the research team. The decision to give participants either salt or placebo supplements was randomly decided using an online generator

(https://wheelofnames.com/). Participants were instructed to ingest 16 capsules per day for a seven-day period that began on the day their baseline measurements were gathered. The salt capsules weighed 1.2270 ± 0.0188g. This amount matched previous work by Babcock et al. (2019), as this enabled 6 g of sodium to be ingested everyday by the participants for a seven-day period. The sugar capsules were given to participants to act as a placebo and weighed 0.9295 ± 0.0125g. All participants returned a week after their baseline measurements were taken having ingested all 112 supplemental capsules. Upon return for post testing, the same procedures described above were repeated. Participants then underwent a "wash-out" period, where they did not ingest any supplements for at least seven days. After the wash-out period, participants returned to the laboratory and the same procedures described above were repeated to gather their second baseline measurement. After testing was complete, participants were given another 112, one-gram supplements that contained the other corresponding contents. They were once again instructed to ingest 16 capsules per day for a seven-day period that began on the same day as their second baseline measurements were gathered. Participants then returned to the laboratory for a final time after the seven-day period and the same procedures described above were repeated **(Figure 2).**

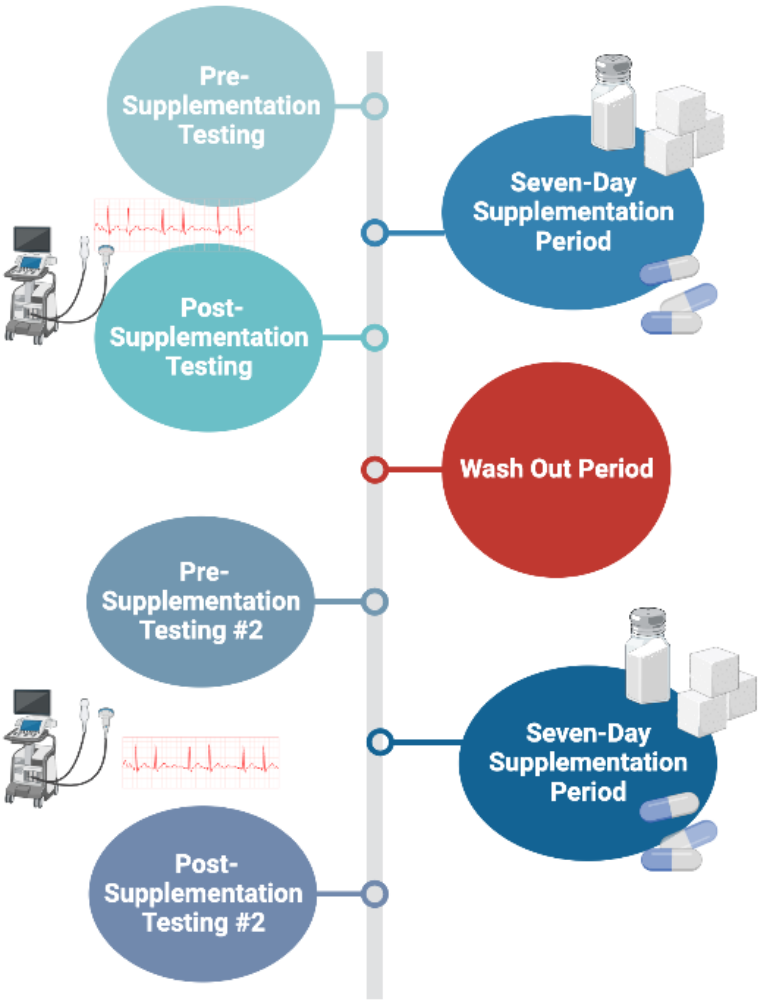

Figure 1. Timeline of experimental procedure.

Figure 2. Timeline of testing session.

2.4 Hand-grip Exercise

The 30-minute hand-grip exercise was performed using a handgrip dynamometer which was attached to a PowerLab unit (PowerLab 26T, ML856, ADInstruments, Sydney, Australia). Each participant had the hand-grip dynamometer (Hand Dynomometer, MLT003/D, ADInstruments, Sydney, Australia) calibrated to their maximum voluntary contraction. The calibration was performed by having the participant squeeze the handgrip dynamometer maximally for three seconds. This maximal handgrip was performed three times each separated by one minute of rest. The output from the dynamometer was recorded using the data acquisition software (LabChart 7. V7.3.8, ADInstruments, Sydney, Australia). After the maximal voluntary contraction was determined, LabChart was calibrated to have the participants maximum grip strength be equivalent to 100%. After calibration participants were instructed to squeeze the handgrip dynamometer at 25% of their maximum voluntary contraction at a rhythmic pace of two-seconds contraction and three-seconds relaxation for a 30-minute period. This protocol was identical to that from Sinoway et al. (1996). Participants were able to easily view their percentage of maximum

voluntary contraction and were given a PowerPoint presentation that instructed them when to contract and when to release the dynamometer.

2.5 Vascular Assessments

2.5.1 Blood Pressure

Blood pressure was recorded during the initial 10-minute rest period and through the duration of the FMD protocol. Each participant's resting blood pressure was determined by averaging their values over 300 seconds midway through the rest period.

2.5.2 Endothelial Function

Endothelial function was assessed using a brachial artery flow mediated dilation (FMD) protocol that measures an endothelium-dependent dilation after a period of occlusion downstream of the assessed artery. A pneumatic cuff around their left forearm was connected to a pressure monitor and an inflation bulb. Doppler ultrasound (Epiq 5G, Philips Health Care, Mississauga, Ontario, Canada) was used to image a longitudinal section of the left brachial artery, with clearly defined intima-media borders. Prior to proceeding with the FMD protocol, a 30 second video was recorded of the left brachial artery at rest. To carry out the FMD protocol, the cuff around the left forearm was inflated to 220 mmHg and held for 5 minutes to stop blood flow into or out of tissues beyond the cuff (i.e., the left forearm and hand). A one and a half-minute video of the left brachial artery was taken, starting 15 seconds before the cuff was deflated (at 4:45 minutes of the 5-minute protocol) and extended one minute and seventeen seconds after the cuff was deflated to capture the vessel's response to blood flow re-establishment. Changes in FMD response were analyzed and compared between the pre and post placebo or salt conditions. All dilation responses for all participants occurred within the measured timeframe.

2.5.3 Absolute and Relative Arterial Diameters

Resting brachial artery digital video clips were analyzed using an image analysis software (Carolab 5.0, Creatis UMR 5220, Université de Lyon, Lyon, France) on a laboratory computer, HP (Intel ® Core ™ i7-8700 CPU @ 3.20GHz). Each video clip was 30-seconds in length and each FMD performed had four video clips associated with it (1 baseline; 3 post cuff release). A region of interest (ROI) was chosen by the investigator and was kept consistent at all time-points within each participant. The investigator sized and positioned the ROI borders to encompass the inner blood vessel walls. A command was then issued that allowed the program to begin tracking the diameter changes throughout the subsequent frames of the digital video clip. The software program created a text file that had all the frame numbers and corresponding diameters.

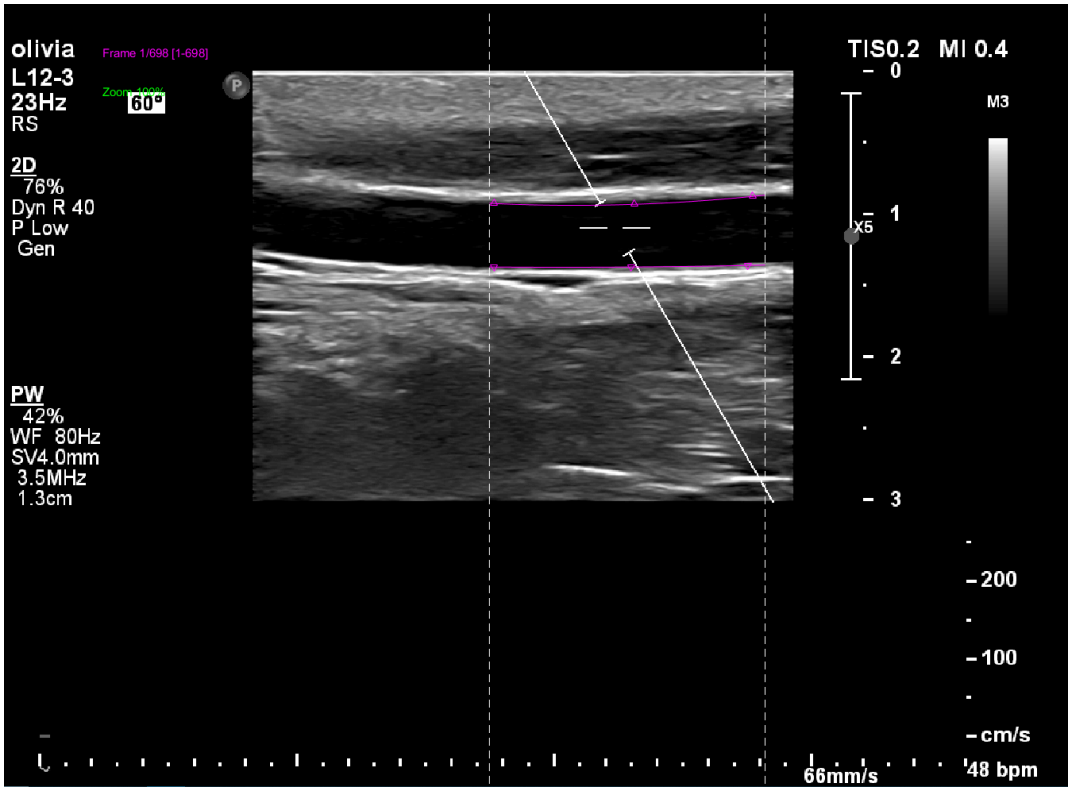

Figure 3. Resting brachial artery image observed on the CaroLab program. Parameters are set for proper measurement of brachial artery diameter.

First, end diastolic diameter (in millimeters) was averaged between 20 and 30 cardiac cycles during rest (FMD_{min}), and was compared to the average of the three consecutive maximum end diastolic diameters upon cuff deflation (FMD_{max}). Subsequently, calculations were carried out to determine the absolute and relative difference between FMD_{min} and FMD_{max}.

Absolute Difference: $FMD_{max} - FMD_{min}$

Relative Difference: $[(FMD_{max} - FMD_{min}) / FMD_{min}] \times 100\%$.

These values were calculated for both the pre and post supplementation conditions. Baseline end diastolic diameters at rest were recorded both pre and post placebo or salt ingestion, as this value may have changed upon ingestion of placebo or salt and needed to be controlled for during the analysis.

2.5.4 Post Occlusion Blood Flow and Shear Rate

Blood velocity recorded following brachial occlusion was used to obtain two quantifications of post occlusion blood velocity. First, beat-by-beat average blood velocity was determined as the area under the curve from R-to-R points on the simultaneously recorded ECG using the Doppler ultrasound. The video clips were then exported as AVI files and were uploaded onto the laboratory computer. A digital software, FloWave.US, based in MATLAB (The MathWorks, 2015a, Natick, USA) was used to analyze the R-to-R points on the video files and determine the blood velocity. Investigators followed the procedure outlined in a study conducted by Coolbaugh et al. (2016), which validated the use of FloWave.US to conduct ultrasound blood flow analysis. The software, FloWave.US, was developed by Coolbaugh et al. (2016) in MATLAB. To use the software, the investigator had to first, create a settings file, using FloWave.US, that

would recognize the R-to-R points in the ultrasound video file and be able to properly analyze them. Once this was created, the investigator was able to use the settings file that was created and upload the individual video clips. For this analysis, only the baseline video and first video after cuff release were needed as this included the hyperemic response. FloWave.US exports the measured data (blood velocity and beat-to-beat data) to a comma-separated variable (CSV) files. This can then be used to calculate blood flow and shear rates.

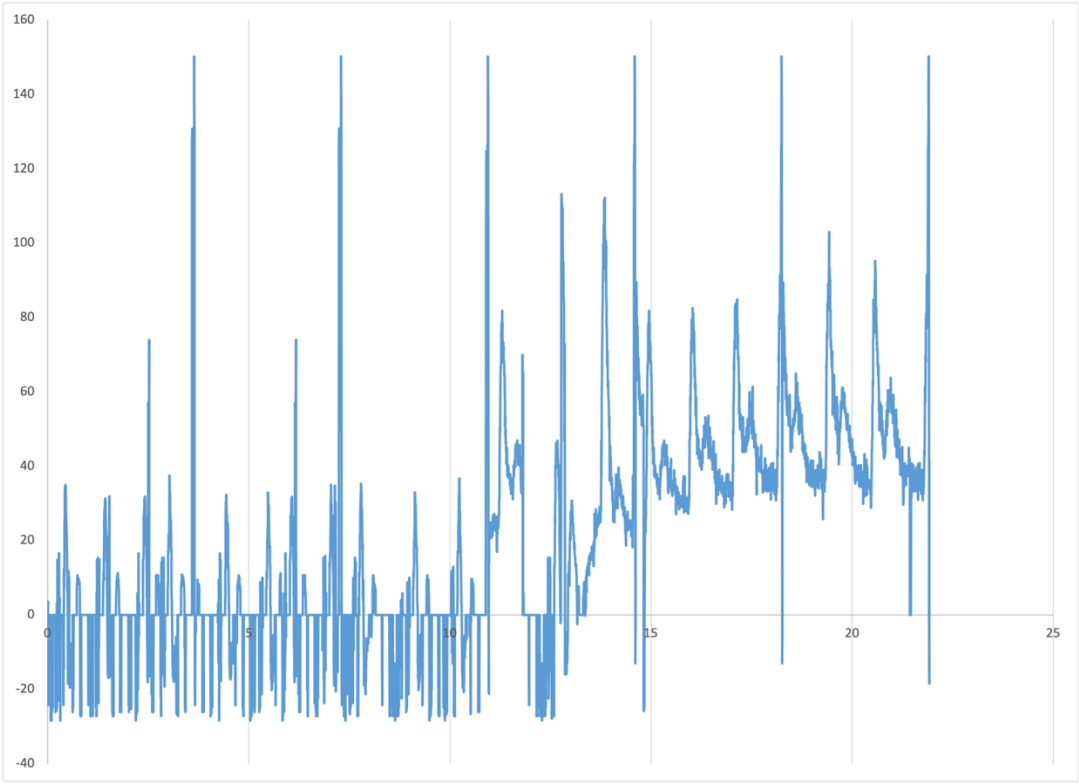

Figure 4. Example of blood velocity and beat-by-beat data calculated and exported using MATLAB.

A fifteen-second average post occlusion blood velocity was defined as the blood velocity fifteen seconds following cuff release, excluding the first beat. The peak beat was defined as the highest single beat average velocity after exclusion of the first beat following cuff release. These values were subsequently used to calculate peak blood flow, fifteen second average blood flow, peak walls shear rate and fifteen second average wall shear rate.

Blood flow was calculated using both the peak beat and fifteen second average methods described above. The following equation was used to calculate these values at all time points for all brachial endothelial-dependent dilation tests:

$FBF_{peak} = \pi(d/2)^2 \times BV_{peak} \times 60s$

Where, FBF is forearm blood flow
d is resting brachial diameter
BV is blood velocity

$FBF_{15s} = \pi(d/2)^2 \times BV_{15s} \times 60s$

Where, FBF is forearm blood flow
d is resting brachial diameter
BV is blood velocity

Shear rates were calculated both as peak beat and fifteen second average shear rate based on the blood velocity measurements described above. The following equations were used:

$$Shear_{peak} = \frac{4 \times BV_{peak}}{d_{mean}}$$

Where, BV is blood velocity
d_{mean} is the average heart cycle diameter

$$Shear_{15s} = \frac{4 \times BV_{15s}}{d_{mean}}$$

Where, BV is blood velocity
d_{mean} is the average heart cycle diameter

2.6 Statistics

All variables were analyzed by a within-subject repeated measures analysis of variance (ANOVA), using the software JASP (JASP Version 0.17.1 Intel). When a significant main effect was noted, Post Hoc tests (Holm) were used for subsequent analysis. Significance for all analysis was set at $p \leq 0.05$. All values are presented as mean $\pm$ standard deviation.

3. RESULTS

Figure 1 illustrates the impact of salt on a) absolute and b) relative FMD. There were significant main effects for condition and session indicating salt induced endothelial dysfunction. We also noted a trend for a session by condition interaction and completed exploratory post-hoc tests. They indicated a trend (p=0.066) for reduced dilation after salt intake compared to all other conditions and time points (p<0.05, see appendix A).

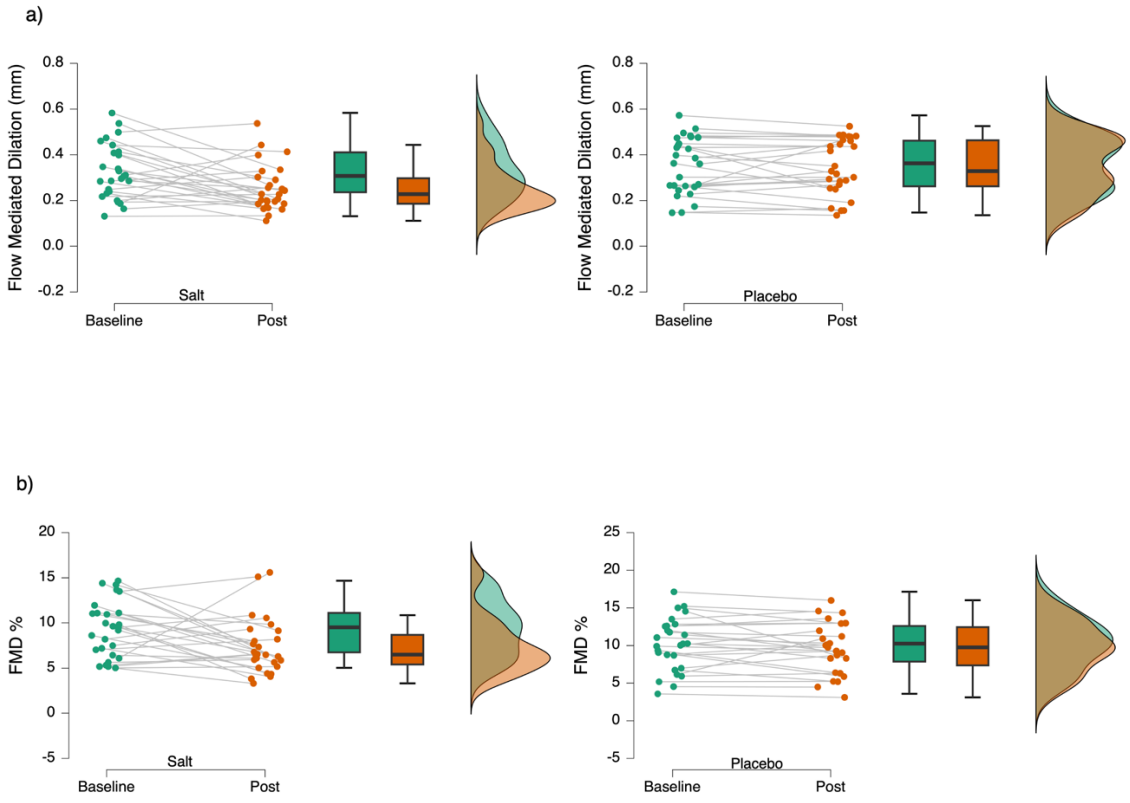

Figure 5. The impact of salt loading on a) absolute and b) relative FMD (n=9). There were significant main effects for the salt condition from baseline to post testing sessions ($p < 0.05$). There were no significant differences for the placebo condition from baseline to post testing sessions ($p > 0.05$). Plots indicate the median and quartiles as well as distributions.

Table 1 shows the means for the baseline brachial artery diameters, average shear stress, average blood flow, peak shear stress, and peak blood flow for both the placebo and salt conditions,

the baseline and post sessions, as well as the three different time points for flow mediated dilation protocols. There were no significant differences for any of these factors between sessions, conditions, or time periods ($p > 0.05$). All values are reported as mean ± standard deviation.

Table 1. Hemodynamic Responses for placebo and salt conditions, baseline and post sessions, and three different flow mediated dilation protocols (n=9). All values are reported as mean ± SD.

	Before Salt			After Salt		
	Pre	**Post-Exercise**	**30 min Post Exercise**	**Pre**	**Post-Exercise**	**30 min Post Exercise**
Minimum Diameter (mm)	3.5 ± 0.60	3.6 ± 0.64	3.6 ± 0.71	3.6 ± 0.68	3.6 ± 0.69	3.6 ± 0.81
15s Average blood flow (ml/min)	57 ± 13	56 ± 11	54 ± 11	59 ± 9	60 ± 13	55 ± 7
Peak blood flow (ml/min)	85 ± 14	82 ± 13	79 ± 13	83 ± 16	85 ± 15	84 ± 14
15s Average Shear Rate (s-1)	67 ± 20	64 ± 11	60 ± 12	67 ± 14	72 ± 13	67 ± 14
Peak Shear Rate (s-1)	99 ± 18	93 ± 12	88 ± 12	95 ± 16	97 ± 17	97 ± 20

	Before Placebo			After Placebo		
	Pre	**Post-Exercise**	**30 min Post Exercise**	**Pre**	**Post-Exercise**	**30 min Post Exercise**
Minimum Diameter (mm)	3.6 ± 0.71	3.6 ± 0.69	3.5 ± 0.68	3.6 ± 0.89	3.6 ± 0.73	3.6 ± 0.78
15s Average blood flow (ml/min)	58 ± 10	63 ± 10	58 ± 9	54 ± 12	62 ± 9	52 ± 12
Peak blood flow (ml/min)	84 ± 9	81 ± 14	82 ± 13	82 ± 19	84 ± 19	78 ± 15
15s Average Shear Rate (s-1)	69 ± 18	68 ± 20	64 ± 12	62 ± 15	70 ± 14	58 ± 14
Peak Shear Rate (s-1)	98 ± 20	92 ± 20	95 ± 20	92 ± 21	93 ± 16	87 ± 16

Table 2 shows the means for systolic and diastolic blood pressure and heart rate for baseline and post testing sessions for both the salt and placebo condition. There were no significant differences for any of these factors between sessions or conditions ($p > 0.05$). All values are reported as mean ± standard deviation.

Table 2. Mean arterial blood pressure and heart rate measurements for baseline and post sessions and salt and placebo conditions (n=9). All values are reported as mean ± SD.

	Before Salt	**After Salt**
Systolic Blood Pressure (mmHg)	131 ± 8.7	135 ± 11
Diastolic Blood Pressure (mmHg)	77 ± 5.8	75 ± 13
Heart Rate (bpm)	71 ± 16	72 ± 18

	Before Placebo	**After Placebo**
Systolic Blood Pressure (mmHg)	133 ± 10	127 ± 22
Diastolic Blood Pressure (mmHg)	75 ± 8.7	72 ± 7.2
Heart Rate (bpm)	74 ± 17	72 15

4. DISCUSSION

This study assessed the effects of sodium on endothelial function and arterial blood pressure as well as whether exercise caused glycocalyx perturbation, causing endothelial dysfunction. We also assessed if endothelial dysfunction caused by salt-loading was exacerbated by exercise.

4.1 Effects of Sodium on Endothelial Function

To analyze the effects of sodium on endothelial function, we performed flow-mediated dilation (FMD) protocols before and after supplementation for both salt and placebo conditions. We found significant changes in both absolute and relative FMD responses between the baseline and post testing for the salt condition. We did not find any significant differences from baseline to post testing for the placebo. We also did not find any significant differences between the shear rates, baseline brachial artery diameters, and blood flow for both the salt and placebo conditions and testing sessions. Exploratory post-hoc analyses also suggest that absolute dilation was reduced after sodium intake compared to their baseline and placebo conditions.

An increase in sodium intake is thought to decrease NO bioavailability, causing the blood vessel to become less responsive to the normal signals that regulate blood flow, leading to an increase in vascular resistance and causing sodium-induced endothelial dysfunction (Hermann et al., 2007; Edwards & Farquhar, 2015; Oberleithner, 2011). In accordance with the findings from previous work, we hypothesized that an increase in sodium levels will cause endothelial dysfunction and cause arterial blood pressure to increase.

There are several possible factors to consider that can impact NO bioavailability and the degree of vascular response, in regard to the glycocalyx, when sodium levels are increased. First, the reduction in mechanotransduction signals in the glycocalyx which reduces NO availability leading to decreased endothelial function. Second, the effect of reactive oxygen species (ROS) on

NO bioavailability due to acute exercise and increased salt. Lastly, the effects of the sympathetic nervous system on endothelial function when there is an increase in sodium.

4.1.1 Reduction in Mechanotransduction Signals

The endothelial surface layer (ESL) is found on the luminal side of the endothelium and contains the glycocalyx (Wenstedt et al., 2018). It acts as the first sodium buffer through the osmotic inactivation of sodium molecules in the blood and preventing them from entering the skin. This layer also plays a very important role by acting as a mechanotransducer that converts a biomechanical signal, shear stress, into a biochemical signal which is the production of NO by the activation of the enzyme, endothelial nitric oxide synthase (eNOS) (Wenstedt et al., 2018; Pahakis et al., 2007). Oberleithner (2011) found that epithelial sodium channels (EnNaC) in the ESL are involved in the regulation of nanomechanics and subsequent eNOS activation and NO bioavailability. Kusche-Virog et al. (2010) found that when sodium levels are increased, there is an increase in density of EnNaC, which allows for more sodium ions to enter the ESL and endothelium, causing endothelial stiffness. This influx of sodium ions causes a disruption in mechanotransduction signals, causing eNOS to not become activated and limiting NO bioavailability (Oberleithner, 2011). The reduction in the phosphorylation of eNOS, leading to reduced NO can cause sodium-induced endothelial dysfunction.

In our study, participants ingested supplements that increased their plasma sodium levels. Through FMD testing, we were able to determine that they experienced a significant change in both their absolute and relative FMD responses from baseline to post under sodium conditions. It is possible that the increased sodium levels in our participants caused an influx of sodium ions in the ESL, disrupting mechanotransduction signals and therefore, preventing the activation of eNOS

which caused a reduction in NO bioavailability. This reduction then resulted in decreased FMD responses in the post-supplement testing, aligning with the findings from previous studies.

4.1.2 Effect of Acute Exercise on ROS

Oxidative stress is a physiological pathway that involves reactive oxygen species (ROS) which have important roles in the body under normal conditions. Reactive oxygen species include active molecular oxygen such as hydroxyl (HO.) and superoxide ($O_2^{\bullet -}$) radicals as well as hydrogen peroxide (H_2O_2) (Yavari et al., 2015; Edwards & Farquhar, 2015). Exercise training is accompanied with the induction of oxidative stress via overproduction of ROS, which will damage tissue, resulting in the decline of the body's working ability (He et al., 2016). High levels of ROS can cause damage to biomacromolecules in cells, such as lipids, proteins, and nucleic acids, leading to cell senescence, even death (Wang et al., 2021). Acute exercise can promote the excessive production of ROS, which causes an imbalance in the oxidation-antioxidant homeostasis in cells (He et al., 2016; Wang et al., 2021). Alhayaza et al. (2020) found that increased ROS can uncouple the eNOS-catalyzed reduction of molecular oxygen from the oxidation of L-arginine, resulting in the production of the ROS superoxide anion (O_2^-) instead of the reducing NO. Decreased NO availability due to the uncoupling of eNOS by ROS can tip the redox balance and cause impaired NO-mediated signalling, causing endothelial dysfunction (Lee et al., 2017; VanBavel, 2007). A study conducted by Sapp et al. (2019) found that acute glycocalyx shedding occurred due to an increase in ROS after exercise, leading to decreased NO bioavailability and decreased endothelial function. Sena et al. (2013) found that the major ROS produced in response to increased sodium levels is superoxide anion (O_2^-), which quickly combines with NO to produce peroxynitrite ($ONOO^-$) decreasing NO bioavailability. O_2^- and $ONOO^-$ can then oxidize and uncouple eNOS and generate more O_2^- rather than NO, leading to sodium-induced endothelial

dysfunction (Patik et al., 2021). At present, exercise after placebo appeared to impair endothelial function immediately afterwards however, this was not significant nor sustained at 30 minutes after exercise. We also determined that salt induced endothelial dysfunction was not exacerbated by exercise and may be ameliorated, but the small sample size limits interpretation so, further testing with a larger sample size is required. When looking at the results for absolute and relative change in FMD responses between baseline and post salt condition we do see a significant difference. The buildup of ROS due to increased sodium levels could be a potential mechanism to explain the different in FMD responses, but further research must be conducted to determine the cause of the change in response.

4.1.3 Effect of Increased Salt on ROS and Inflammation

Li et al. (2018), conducted a study to investigate the effects of increased salt on ROS. They found that increased salt intake leads to increased levels of O_2^- and H_2O_2, causing reduced endothelial function. Lenda et al. (2000), conducted a similar study and found that a high-salt diet leads to increased generation of reactive oxygen species, and this increased oxidative state may be responsible for decreased endothelium-dependent responses associated with high salt intake.

Vinaiphat et al. (2023), found that increased salt intake for 12 weeks causes endothelial inflammation and a buildup of macrophages, leading to decreased endothelial function. A study conducted by Krajina et al. (2022), found that an increase in dietary salt causing an increase in ROS, leads to an increase immune response (high levels of IL-17, T lymphocytes, and macrophages), all of which work together to cause endothelial dysfunction. When looking at the results in our study for absolute and relative change in FMD responses between baseline and post salt condition we do see a significant difference. The buildup of ROS due to increased sodium levels and increased immune response could be a potential mechanism to explain the different in

FMD responses, but further research must be conducted to determine the cause of the change in response.

4.1.4 Effects of the Sympathetic Nervous System on Endothelial Function

Sympathetic nervous system activity has a pivotal role in the pathophysiological abnormalities underlying the development of hypertension, and sympathetic activation attenuates the skin blood flow by causing microvascular vasoconstriction (Oyama & Node, 2014). Vasodilatation and vasoconstriction coordinate the control of blood flow in the peripheral resistance arteriole network, and reactive hyperemia causes vasodilatation in the resistant arterioles independent of sympathetic nervous activation. Gamboa et al. (2012), found that NO can act as an inhibitor for the sympathetic tone. When there is an increase in sodium levels, the activation of eNOS is limited, and therefore, limits the bioavailability for NO. When NO levels are low, this could then allow the sympathetic nervous system to become activated and act as vasoconstrictors, reducing endothelial function. A different study, conducted by Brian et al. (2018), found that increased in sodium levels cause an increase in MSNA and blood pressure, leading to endothelial dysfunction. The significant difference seen from baseline to post salt conditions for both absolute and relative FMD responses could be explained through the mechanism of SNA activation due to increased sodium levels. Further research is required to determine the if SNA activation is a mechanism leading to the change in the FMD response.

4.2 Effects of Sodium on Arterial Blood Pressure

To analyze the effects of increased sodium on arterial blood pressure, we took blood pressure measurements before and after supplementation for both salt and placebo conditions as assessed by finger photoplethysmography. We found no significant changes in systolic, diastolic,

and mean arterial blood pressure in either condition or from baseline to post testing. We also found no significant difference in heart rate.

Although we did not see any changes in arterial blood pressure, this was not the case in other studies. A study conducted by Campese et al. (1996), observed the effects of increased sodium intake on the endogenous vasodilator, atrial natriuretic peptide (ANP), as it may be acutely affected following a salt load. ANP is produced mainly in the cardiac atria and is released into the circulation in response to volume expansion and increased atrial distention. It has potent natriuretic, diuretic, vasodilator, sympatholytic, and renin- and aldosterone-suppressing activities, all of which tend to lower blood pressure (Oparil, 1995). Campese et al. (1996) found there to be a significant decrease in ANP in salt-sensitive individuals, which could cause elevated arterial blood pressure as the renin-angiotensin-aldosterone pathway is activated, causing vasoconstriction through the activation of the sympathetic nervous system. Dickinson et al. (2014) observed the effects of high-sodium intake on normotensive (normal blood pressure) individuals. They were able to determine there was no significant difference in ANP as well as arterial blood pressure before and after salt-loading. In our study, we saw no significant difference in arterial blood pressure before and after salt-loading and this finding aligns with the results found in the study by Dickinson et al. (2014).

A study conducted by He et al. (2001), found that in normotensive individuals, there was no change in arterial blood pressure before and after salt-loading. They investigated the renin-angiotensin-aldosterone system and its effects on blood pressure. The renin-angiotensin-aldosterone system (RAAS) is a crucial mediator of cardiac, vascular, and renal physiology through the regulation of vascular tone and salt and water homeostasis (Fountain et al., 2023). The RAAS is highly sensitive to changes in sodium levels in the body. When sodium levels are low, it

triggers the release of renin from the kidneys, which is the first step in the RAAS cascade. Renin acts on angiotensinogen, a protein produced by the liver and released into the bloodstream, to generate angiotensin I. Angiotensin I is then converted into angiotensin II by ACE. Angiotensin II is a potent vasoconstrictor and stimulates the release of aldosterone, which acts on the kidneys to increase the reabsorption of sodium, leading to increased blood volume and blood pressure (Fountain et al., 2023). Increased sodium intake can lead to decreased renin secretion, decreased production of angiotensin II, decreased aldosterone secretion, and increased ANP release. These changes in the RAAS can result in decreased vasoconstriction, increased sodium and water excretion, and overall decreased blood pressure (Farquhar et al., 2015). Dong (2018) observed the relationship between the RAAS and sympathetic nerve activity. They found that they both play a role in the maintenance of hypertension in individuals, and that the RAAS may have a stimulatory role on the sympathetic nerve activity. The mechanism by which this occurs however, is not fully understood and the RAAS pathway could only affect the sympathetic nerve activity to a certain level. The findings in the study by He et al. (2001), are consistent with the findings in our study, and the suppression of the RAAS and SNS would need to be investigated further to determine if they are involved in the maintenance of arterial blood pressure before and after salt-loading.

4.3 Conclusion and Future Research

With the diet of western society exceeding the recommended sodium intake, the risk of developing hypertension which can lead to cardiovascular disease is at an all-time high. The regulation of sodium is also being identified through a 2-way compartment model, with the endothelial surface layer and the glycocalyx, binding and inactivating sodium to help regulate sodium levels in the body, and therefore, blood pressure. In this study, we found significant differences in both absolute and relative FMD responses between the baseline and post testing for

the salt condition. We did not find any significant differences for the placebo testing in regard to changes in FMD. Furthermore, we did not find any significant differences for shear rate or blood flow for both the salt and placebo conditions. The methodology presented here lays a strong foundation for future work and has the potential to be further developed into a larger scale study.

In future studies, to observe if the sympathetic nervous system is activated, MSNA measures can be recorded and analyzed to observe if that is part of the response due to increased salt loading. The RAA system and ANP levels can also be monitored during testing to determine if these two mechanisms affect arterial blood pressure and endothelial function. Also, to observe if exercise does cause the glycocalyx to become perturbed, future researchers can collect blood samples from participants before and after exercise. They can then analyze the samples for components of the glycocalyx post-exercise. A more intensive exercise bout can be performed in conjunction with blood sample collection to determine if an immune response is activated and if that can cause decreased endothelial function. In addition, a larger study population could provide further insight to the effects of salt-loading on endothelial function and arterial blood pressure. If future studies see significant differences in exercise-induced endothelial function post salt-loading and if other mechanisms, such as the RAA and MSNA effect endothelial function and arterial blood pressure, individuals will then be able to adjust their dietary sodium intake to a level which reduces the risk of cardiovascular disease and hypertension.

4.4 Limitations

We acknowledge some limitations in the present study. Firstly, we had a small sample size, which may be a possible reason as to why we did not see a significant difference in some of the measures conducted in this study. Secondly, we did not measure sympathetic nerve activity, and this may play a role during the FMD protocol. A study conducted by Tomiyama et al. (2014),

observed if the autonomic, more specifically the sympathetic, nervous system was induced during a FMD protocol, which could then blunt the results of the FMD. They were able to record the sympathetic output through the direct recording of the muscle sympathetic nerve activity (MSNA). They found that sympathetic nerve activation could affect the results of endothelial function tests performed under various situations.

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6. APPENDIX A

Table 3. Results of within subject repeated measures ANOVA of baseline brachial artery diameters (n=9). There were no significant differences between the placebo and salt condition, the baseline and post sessions, and between the three different flow-mediated dilation protocols performed ($p > 0.05$).

Within Subjects Effects

Cases	Sum of Squares	df	Mean Square	F	p
Session	0.061	1	0.061	0.967	0.354
Residuals	0.506	8	0.063		
Condition	0.041	1	0.041	0.804	0.396
Residuals	0.411	8	0.051		
Time	0.031	2	0.016	0.653	0.534
Residuals	0.381	16	0.024		
Session ✻ Condition	0.025	1	0.025	0.827	0.390
Residuals	0.240	8	0.030		
Session ✻ Time	0.023	2	0.011	0.686	0.518
Residuals	0.267	16	0.017		
Condition ✻ Time	0.039	2	0.020	1.018	0.384
Residuals	0.307	16	0.019		
Session ✻ Condition ✻ Time	0.022	2	0.011	0.500	0.615
Residuals	0.346	16	0.022		

Note. **Type III Sum of Squares**

Table 4. Results of within subject repeated measures ANOVA of peak shear rates (n=9). There were no significant differences between the placebo and salt condition, the baseline and post sessions, and between the three different flow-mediated dilation protocols performed ($p > 0.05$).

Within Subjects Effects

Cases	Sum of Squares	df	Mean Square	F	p
Session	29.215	1	29.215	0.095	0.766
Residuals	2468.519	8	308.565		
Condition	101.904	1	101.904	0.323	0.585
Residuals	2524.304	8	315.538		
Time	284.642	2	142.321	0.786	0.473
Residuals	2897.916	16	181.120		
Session ✻ Condition	316.253	1	316.253	1.369	0.276
Residuals	1847.930	8	230.991		
Session ✻ Time	282.812	2	141.406	0.904	0.425
Residuals	2503.106	16	156.444		
Condition ✻ Time	6.555 [a]	2 [a]	3.277 [a]	0.016 [a]	0.984 [a]
Residuals	3321.607	16	207.600		

Session ✻ Condition ✻ Time	312.701	2	156.351	1.933	0.177
Residuals	1293.895	16	80.868		

Note. **Type III Sum of Squares**

[a] **Mauchly's test of sphericity indicates that the assumption of sphericity is violated (p < .05).**

Table 5. Results of within subject repeated measures ANOVA of average shear rates (n=9). There were no significant differences between the placebo and salt condition, the baseline and post sessions, and between the three different flow-mediated dilation protocols performed (p > 0.05).

Within Subjects Effects

Cases	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**
Session	10.259	1	10.259	0.085	0.778
Residuals	965.028	8	120.628		
Condition	44.893	1	44.893	0.314	0.590
Residuals	1142.700	8	142.838		
Time	702.297	2	351.148	2.093	0.156
Residuals	2684.337	16	167.771		
Session ✻ Condition	515.061	1	515.061	2.772	0.134
Residuals	1486.367	8	185.796		
Session ✻ Time	321.627	2	160.813	1.358	0.285
Residuals	1894.495	16	118.406		
Condition ✻ Time	91.959 [a]	2 [a]	45.979 [a]	0.411 [a]	0.670 [a]
Residuals	1788.612	16	111.788		
Session ✻ Condition ✻ Time	55.621	2	27.811	0.384	0.687
Residuals	1159.444	16	72.465		

Note. **Type III Sum of Squares**

[a] **Mauchly's test of sphericity indicates that the assumption of sphericity is violated (p < .05).**

Table 6. Results of within subject repeated measures ANOVA of peak blood flow (n=9). There were no significant differences between the placebo and salt condition, the baseline and post sessions, and between the three different flow-mediated dilation protocols performed (p > 0.05).

Within Subjects Effects

Cases	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**
Session	7.183	1	7.183	0.047	0.833
Residuals	1216.468	8	152.058		
Condition	36.323	1	36.323	0.179	0.683
Residuals	1622.370	8	202.796		
Time	167.067 [a]	2 [a]	83.534 [a]	0.607 [a]	0.557 [a]
Residuals	2201.790	16	137.612		
Session ✻ Condition	58.830	1	58.830	0.407	0.541

Residuals	1155.450	8	144.431		
Session ✻ Time	98.579	2	49.289	0.696	0.513
Residuals	1132.980	16	70.811		
Condition ✻ Time	0.507	2	0.253	0.002	0.998
Residuals	2222.932	16	138.933		
Session ✻ Condition ✻ Time	124.706	2	62.353	1.020	0.383
Residuals	977.744	16	61.109		

Note. **Type III Sum of Squares**

[a] Mauchly's test of sphericity indicates that the assumption of sphericity is violated (p < .05).

Table 7. Results of within subject repeated measures ANOVA of average blood flow (n=9). There were no significant differences between the placebo and salt condition, the baseline and post sessions, and between the three different flow-mediated dilation protocols performed (p > 0.05).

Within Subjects Effects

Cases	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**
Session	47.903	1	47.903	0.677	0.434
Residuals	565.902	8	70.738		
Condition	19.967	1	19.967	0.225	0.648
Residuals	708.848	8	88.606		
Time	569.766	2	284.883	1.860	0.188
Residuals	2450.382	16	153.149		
Session ✻ Condition	239.994	1	239.994	1.905	0.205
Residuals	1008.007	8	126.001		
Session ✻ Time	159.553	2	79.776	1.137	0.345
Residuals	1122.678	16	70.167		
Condition ✻ Time	67.217	2	33.609	0.479	0.628
Residuals	1123.416	16	70.213		
Session ✻ Condition ✻ Time	10.405	2	5.203	0.102	0.904
Residuals	819.525	16	51.220		

Note. **Type III Sum of Squares**

Table 8. Results of within subject repeated measures ANOVA of absolute change in FMD (n=9). There were significant differences between session and condition (p < 0.005; p < 0.006).

Within Subjects Effects

Cases	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**
Session	0.045	1	0.045	15.215	0.005
Residuals	0.024	8	0.003		
Condition	0.098	1	0.098	13.471	0.006
Residuals	0.058	8	0.007		
Time	0.041	2	0.020	2.240	0.139
Residuals	0.146	16	0.009		

Session ✻ Condition	0.030	1	0.030	4.400	0.069
Residuals	0.055	8	0.007		
Session ✻ Time	0.002	2	8.637×10^{-4}	0.107	0.899
Residuals	0.129	16	0.008		
Condition ✻ Time	0.049	2	0.025	1.233	0.318
Residuals	0.319	16	0.020		
Session ✻ Condition ✻ Time	0.007	2	0.003	0.765	0.482
Residuals	0.069	16	0.004		

Note. **Type III Sum of Squares**

Table 9. Post Hoc comparisons of Absolute change in FMD for session and condition. Exploratory post hoc comparisons were performed for the Session * Condition effect.

Post Hoc Comparisons - Session

		Mean Difference	SE	t	p_{tukey}	p_{holm}
Baseline	Post	0.041	0.010	3.901	.	0.005

Note. **Results are averaged over the levels of: Condition, Time**

Note. **Tukey corrected p-values are not appropriate for repeated measures post-hoc tests (Maxwell, 1980; Field, 2012).**

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	p_{holm}
Salt	Placebo	-0.060	0.016	-3.670	.	0.006

Note. **Results are averaged over the levels of: Session, Time**

Note. **Tukey corrected p-values are not appropriate for repeated measures post-hoc tests (Maxwell, 1980; Field, 2012).**

Post Hoc Comparisons - Session ✻ Condition

		Mean Difference	SE	t	p_{tukey}	p_{holm}
Baseline, Salt	Post, Salt	0.074	0.019	3.889	0.008	0.007
	Baseline, Placebo	-0.027	0.023	-1.167	0.655	0.781
	Post, Placebo	-0.020	0.019	-1.005	0.749	0.781
Post, Salt	Baseline, Placebo	-0.101	0.019	-5.190	< .001	< .001
	Post, Placebo	-0.094	0.023	-4.093	0.004	0.004
Baseline, Placebo	Post, Placebo	0.007	0.019	0.376	0.981	0.781

Note. **P-value adjusted for comparing a family of 6**

Note. **Results are averaged over the levels of: Time**

Table 10. Results of within subject repeated measures ANOVA of relative change in FMD (n=9). There were significant differences between session and condition ($p < 0.004$; $p < 0.022$).

Within Subjects Effects

Cases	Sum of Squares	df	Mean Square	F	p
Session	43.033	1	43.033	15.776	0.004
Residuals	21.822	8	2.728		
Condition	68.943	1	68.943	8.012	0.022
Residuals	68.842	8	8.605		
Time	40.228	2	20.114	3.060	0.075
Residuals	105.165	16	6.573		
Session ✻ Condition	15.039	1	15.039	1.622	0.239
Residuals	74.196	8	9.274		
Session ✻ Time	1.092 [a]	2 [a]	0.546 [a]	0.101 [a]	0.905 [a]
Residuals	86.522	16	5.408		
Condition ✻ Time	25.322	2	12.661	0.773	0.478
Residuals	261.954	16	16.372		
Session ✻ Condition ✻ Time	3.999	2	2.000	0.428	0.659
Residuals	74.751	16	4.672		

Note. **Type III Sum of Squares**

[a] **Mauchly's test of sphericity indicates that the assumption of sphericity is violated ($p < .05$).**

Table 11. Post Hoc comparisons of relative change in FMD for session and condition.

Post Hoc Comparisons - Session

		Mean Difference	SE	t	p_{tukey}	p_{holm}
Baseline	Post	1.262	0.318	3.972	.	0.004

Note. **Results are averaged over the levels of: Condition, Time**

Note. **Tukey corrected p-values are not appropriate for repeated measures post-hoc tests (Maxwell, 1980; Field, 2012).**

Post Hoc Comparisons - Condition

		Mean Difference	SE	t	p_{tukey}	p_{holm}
Salt	Placebo	-1.598	0.565	-2.830	.	0.022

Note. **Results are averaged over the levels of: Session, Time**

Note. **Tukey corrected p-values are not appropriate for repeated measures post-hoc tests (Maxwell, 1980; Field, 2012).**