THE EFFECTS OF MELATONIN ON VASCULAR FUNCTION, OXIDATIVE STRESS AND BLOOD PRESSURE REACTIVITY DURING A HIGH SODIUM DIET

by

Macarena Ramos Gonzalez

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Physiology

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Approved: ____
John J. Jeka, Ph.D.
Chair of the Department of Kinesiology and Applied Physiology

Approved: ____
Kathleen Matt, Ph.D.
Dean of the College of Health Sciences

Approved: ____
Louis F. Rossi, Ph.D.
Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: ____

Shannon L. Lennon, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: ____

William B. Farquhar, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: ____

Melissa A.H. Witman, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: ____

Danielle Kirkman, Ph.D.
Member of dissertation committee

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Growing up, my parents were both primary school teachers and provided the right learning environment. My mom would question me about what I learned in school and my dad, a historian, would teach me about older civilizations and cultures. I wanted to be an archaeologist to discover tombs, burial sites, and our past secrets. However, physical activity and exercise got in the way of that career path. Besides studying for class, I was very active and involved in sports. I discovered tennis and while in practice one day, this high schooler mentioned he was moving away to study Exercise Science in college. I knew that that was going to be the right place for me: exercise and science together.

While in college, I first walked into a research laboratory and was introduced to the scientific method in the biomechanics field. Shortly after, I took Dr. Aranda-Ramirez's- currently President of the University of Granada- human physiology course and fell in love with it. I had the opportunity to help in a cardiovascular research group and I discovered a new career path by accident.

My options in Europe were limited so I decided to move across the Atlantic Ocean to study Exercise Physiology. Dr. Carl Ade at the University of Oklahoma taught me the first things there were to know about human research. I will never forget consenting the first subject for my thesis sitting in his office chair. Your ambition and work ethic are contagious, your desire to help people admirable.

The University of Delaware and Dr. Shannon Lennon trusted me with my doctorate. Dr. Lennon, I want to thank you for not giving up on me. Besides being an exceptional academic mentor with all the big responsibilities that entails, you are a better person who treated me respectfully, understood my point of view, my personal struggles and made me feel heard. To my committee members, Dr. Witman, Dr. Farquhar and Dr. Kirkman, thank you for your honesty, your feedback, and your help during these 5 years. To my lab mates, the old ones, and new ones. Kat and Alexis, without you I would probably be still in the Wet Lab trying to figure out a piece of equipment. You taught me everything I know now, pushed me to become a leader and an independent thinker. You encouraged me to grow, and I have, because of you. Mike, Kate and Andrea, thank you for giving me something very valuable of yours: time. Thank you for extending your hand without hesitation when I needed you and for cheering me up when I was feeling down. To my work husband and friend, Ted, who has been a constant force pushing me forward in and out of work. To my friends back home in Spain, who may be far but whose support is still going strong after 20 years. To my group of Spaniards in the U.S., you helped me forget about the strenuous life of a PhD student with laughter. To my big sister and fellow boomer-sooner, Charity. To my two favorite undergrads, Dan and Paige, whose data analyzing skills shaped this work. To the incredible Liza, who always got out of her way to get me literally anything I needed from cookies to pipette tips. To Wendy, our beloved nurse. To my dog, Hera, my most loyal friend and companion.

Mom and dad, this PhD is for you. Mom, wherever you are, I hope you are proud, I miss you and I love you. Dad, you let me fly so high that my dreams became a reality. To my godparents, Pakumba and Ana, I am sure we will have many more achievements to celebrate soon. Finally, to my grandma, whom I lost during my PhD, thank you for believing in me and making me the strong woman I am.

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ABSTRACT

High sodium diets (HSD) can reduce the ability of the arteries to properly dilate (i.e., vascular dysfunction), partially due to increases in reactive oxygen species (ROS). HSD can also cause exaggerated increases in blood pressure (BP) during different physiological perturbations (BP reactivity). Vascular dysfunction and increased BP reactivity are directly related to future atherosclerosis and hypertension (HTN) development, respectively, and thus, to CVD mortality. Melatonin, a hormone synthesized mainly at night from the pineal gland, ameliorates ROS in healthy and clinical populations, enhances mechanisms underlying proper vascular function in in vitro studies and improves in vivo endothelial function. Limited literature indicates that melatonin inhibits the sympathetic system responses and increases parasympathetic system responses. **OBJECTIVES**: 1) Determine the effects of melatonin on vascular function after 10 days of HSD. 2) Determine the effects of melatonin on BP responses after 10 days of HSD. 3) Determine the effects of melatonin on ROS levels after 10 days of HSD. **HYPOTHESES**: 1) Melatonin supplementation will increase vascular function on HSD compared to HSD alone. 2) Melatonin supplementation will decrease BP reactivity during isometric handgrip exercise (IHG) and post-exercise ischemia (PEI), and cold pressor test (CPT) on HSD compared to HSD alone. 3) Melatonin supplementation will decrease ROS levels on HSD compared to HSD alone. **METHODS**: Healthy, non-hypertensive and non-obese young adults were recruited. Participants underwent a randomized cross-over study including a 10-day HSD (6,900mg sodium/d) and supplemented with 10 mg of melatonin (HSD+MEL) and a 10-day HSD (6,900 mg sodium/d) and supplemented with placebo (lactose) (HSD+PL). 24-h BP was measured with an ambulatory monitor on day 8 of each intervention. Vascular function was assessed at both the conduit artery level via brachial artery flow-mediated dilation (FMD) and at the microvascular level via near infrared spectroscopy during a vascular occlusion test (NIRS-VOT). BP reactivity was evaluated by examining beat-to-beat BP reactivity to IHG and PEI and CPT. ROS levels were determined as superoxide (O_2^-) concentration measured with electron paramagnetic resonance. **RESULTS**: Twenty-four participants (13M/11W, 27.0 ± 2.8 yrs., body mass index 23.8 ± 1.9 kg/m^2, BP: 111/67 ± 8/7 mm Hg) participated in this study. There were no differences in 24-h mean arterial pressure (MAP) at the end of each intervention (HSD+PL: 83 ± 6 mm Hg; HSD+MEL: 82 ± 5 mm Hg; p=0.35). Melatonin supplementation decreased nighttime systolic BP (HSD+PL: 106 ± 12 mm Hg; HSD+MEL: 101 ± 9 mm Hg; p=0.02) and increased nighttime systolic dipping (HSD+PL: 13.3 ± 7.4 %; HSD+MEL: 16.2 ± 6.8 %; p=0.04) on the HSD compared to a HSD alone. Brachial artery FMD% (HSD+PL: 7.4 ± 3.7 %; HSD+MEL: 6.9 ± 3.6 %; p=0.50) and tissue oxygenation index (TSI) recovery slope (HSD+PL: 0.2 ± 0.06 %/s; HSD+MEL: 0.2 ± 0.08 %/s; p=0.75) were similar at the end of each intervention. Free radical molarity (HSD+PL: 7.8 x10^{-5} ± 4.1 x10^{-5} mol/L; HSD+MEL: 8.7 x10^{-5} ± 5.1 x10^{-5} mol/L; p=0.51) or free radical number (HSD+PL: 8.0 x10^{15} ± 4.4 x10^{15}; HSD+MEL: 9.0 x10^{15} ± 4.9 x10^{15}; p=0.47) were not different at the end of each intervention. BP responses to physiological perturbations was assessed. MAP delta change (HSD+PL: 27 ± 13 mm Hg; HSD+MEL: 26 ± 9 mm Hg; p=0.78) and MAP percent change to IHG (HSD+PL: 32 ± 16 %; HSD+MEL: 31 ± 11 %; p=0.80) were similar at the end of each intervention. MAP delta change

(HSD+PL: 22 ± 10 mm Hg; HSD+MEL: 22 ± 9 mm Hg; $p=0.96$) and MAP percent change to PEI (HSD+PL: 26 ± 12 %; HSD+MEL: 27 ± 12 %; $p=0.98$) were also similar at the end of each intervention. Lastly, MAP delta change (HSD+PL: 23 ± 14 mm Hg; HSD+MEL: 22 ± 15 mm Hg; $p=0.60$) and MAP percent change to CPT (HSD+PL: 27 ± 17 %; HSD+MEL: 25 ± 17 %; $p=0.47$) were similar at the end of each intervention. **CONCLUSION**: Melatonin supplementation under a HSD did not improve vascular function, decrease free radicals levels or decrease BP responses to IHG, PEI or CPT under a HSD alone in this cohort of young healthy normotensive adults. Melatonin supplementation under a HSD decreased nighttime systolic BP and increased nighttime systolic dipping compared to a HSD alone.

Chapter 1

REVIEW OF LITERATURE

1.1 Introduction

The physiological need for sodium is estimated at 200-500 mg a day but Americans greatly exceed this amount [1]. The latest edition of the Dietary Reference Intakes (DRI) recommends ≤ 2300 mg of sodium a day [2] while the American Heart Association (AHA) recommends lowering sodium to 1500 mg/day for African-Americans, people >50 years of age, and those with hypertension, diabetes mellitus, or chronic kidney disease [3]. The most recent National Health and Nutrition Examination Survey (NHANES), examining data from 1999 to 2016 in the United States (US), shows an increase in sodium consumption throughout the years and a poor adherence to the DRI guidelines [4]. This report concluded that the mean sodium consumption between 2015-2016 was 3232 mg/day and remained elevated in all segments of the adult population [4].

The latest report from the AHA titled Heart Disease and Stroke Statistics - 2021 Update highlights cardiovascular disease (CVD) as the number one cause of mortality in the U.S. affecting most racial and ethnic groups [5]. Health behaviors identified as risk factors for CVD include smoking, physical inactivity, poor nutrition, high blood pressure (BP) and high cholesterol [5]. High sodium intake is one of the leading dietary risk factors and alone is accountable for 9.5% of the total cardiometabolic deaths [5]. Various meta-analyses and systematic reviews highlight the relation between high habitual sodium intake and increased incidence of CVD outcomes and mortality [1,6]. AHA even issued a presidential advisory in 2011 about the importance of reducing sodium intake to prevent CVD [7].

While the main consequence of a high sodium intake has been historically related to increases in BP, this is by far not the only consequence [8,9]. Excess sodium intake also has BP-independent effects. This includes target organ damage including the blood vessels, heart, kidneys and areas of the brain controlling autonomic outflow, as demonstrated in pre-clinical and clinical studies [8,9]. Specifically, in regard to blood vessel health, both acute and chronic increases in sodium intake have shown impairments in vascular endothelial function [8,9]. This is significant as endothelial dysfunction is associated with the development of atherosclerosis and subsequent cardiovascular events, and appears before angiographic evidence of disease [10]. Sodium also increases brain sympathetic outflow and BP responses to various physiological stimuli, which leads to the development of CVD [8,9].

Education programs and interventions working towards populations consuming the recommended sodium level have been implemented but their effectiveness is inconsistent [11]. Thus, novel approaches are needed to reduce the deleterious effects of sodium. A potential strategy to combat the above-mentioned negative effects of sodium may come from a pineal gland hormone called melatonin. Therefore, this review of literature focuses on the effects of dietary sodium on the vasculature and the autonomic nervous system and the potential role of melatonin to offset these deleterious effects.

1.2 Cardiovascular system: The importance of the Endothelium and Nitric Oxide (NO)

The vascular endothelium is composed of a monolayer of endothelial cells (EC) lining the luminal surface of the blood vessels. The vascular endothelium is an active paracrine, endocrine and autocrine organ that is involved in the control of thrombosis and thrombolysis, the inhibition of platelet and leukocyte cell adhesion, and the regulation of vascular tone and blood vessel growth [12–14]. Thus, the endothelium plays a significant role in vascular homeostasis. Factors such as smoking, aging, obesity, hypercholesterolemia, hypertension, hyperglycemia, sedentary lifestyle, poor nutrition, and a family history of premature atherosclerotic disease are associated with negative alterations in endothelial physiology [15]. This is known as endothelial dysfunction and is characterized by reduced vasodilation, proinflammatory, proliferative, prothrombic and procoagulant states [13,14]. This includes a reduction in the bioavailability and/or production of vasodilators, including prostacyclin, endothelial cell growth factors, interleukins, plasminogen inhibitors and nitric oxide (NO), and/or an increase in endothelium-derived contracting factors [12,13]. NO is perhaps the most important vasodilator and a reduction in NO-bioavailability can lead to endothelial dysfunction [12]. Endothelial dysfunction represents a key early step in the development of atherosclerosis, leading to plaque formation and atherosclerosis derived complications [13]. It is associated with most forms of CVD, i.e. hypertension, coronary artery disease, heart failure, and peripheral vascular disease, amongst others [14,16] and is independently associated with cardiovascular events [17].

Endothelial function can be assessed in the coronary and peripheral circulations [13]. In the coronary circulation, non-invasive tests include Doppler echocardiography, positron emission tomography, intracoronary Doppler techniques, and phase-contrast magnetic resonance imaging. Coronary angiography to quantify changes in diameter due to endothelium-dependent vasodilators (e.g., acetylcholine, ACh) is considered the gold standard, but it is invasive and requires medical expertise and special equipment. In the peripheral circulation, assessment of vessels such as the femoral, popliteal, and brachial artery by high-resolution ultrasound are non-invasive and widely used in research. Specifically, evaluation of the brachial artery by this technique is known as brachial artery flow-mediated dilation (FMD). This technique consists of the induction of vascular occlusion in the arm (via supra-systolic cuff inflation), which will create a region of ischemia distal to the point of inflation. In the absence of blood flow, the metabolic byproducts of cellular respiration accumulate promoting an increase in vascular conductance and allowing a robust increase in blood flow (reactive hyperemia) upon the abolition of the "upstream" occlusion [12]. This reactive hyperemia increases laminar shear forces on the blood vessel wall, which is detected by the mechanoreceptors in the ECs, leading to an increase in endothelial nitric oxide synthase (eNOS) activity which catalyzes the conversion of L-arginine to NO in the presence of the cofactor tetrahydrobiopterin (BH_4). NO is then able to diffuse into the tunica media, activating soluble guanylate cyclase, which converts guanosine triphosphate into cyclic guanosine monophosphate (cGMP) to eventually induce relaxation of the smooth muscle and subsequently vasodilation (**Figure 1.1**) [12,18]. This vasodilatory response is compared to the baseline diameter and expressed as a percentage of the baseline diameter (% FMD) [12].

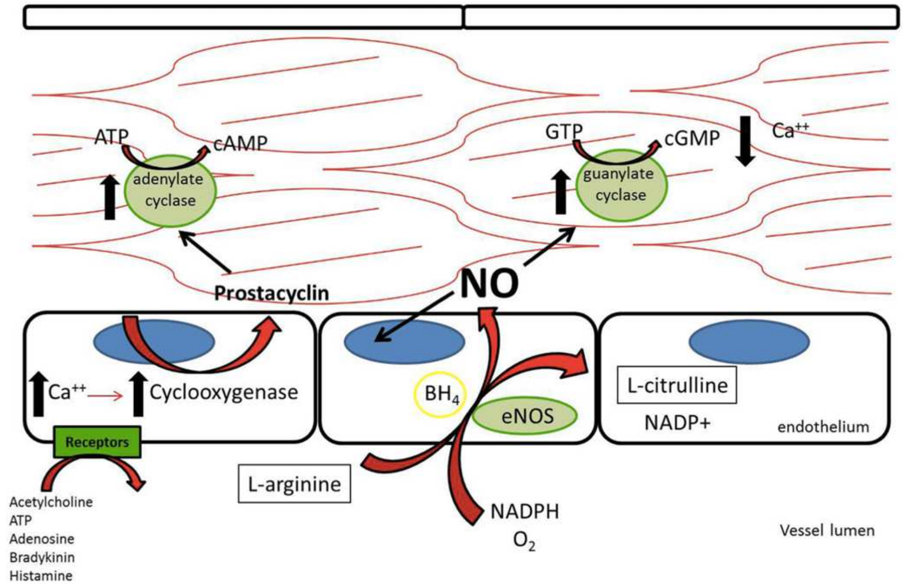

Figure 1.1 NO pathway generated from L-arginine by eNOS. ATP: adenosine triphosphate; BH_4: tetrahydrobiopterin; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; eNOs: endothelial nitric oxide synthase; GTP: guanosine triphosphate; NADP+: nicotinamide adenine dinucleotide phosphate; NADPH: nicotinamide adenine dinucleotide phosphate hydrogen; NO: nitric oxide. Source: Madigan & Zuckerbraun [18]

A decline in NO bioavailability will impact the vessel's response to the ischemic period. The causes for this decline have been identified: decreased expression of eNOS, a lack of substrate or cofactors for eNOS, alterations of cellular signaling leading to inactivation of eNOS and accelerated NO degradation by reactive oxygen species (ROS) [19]. Indeed, reduction of NO bioavailability is a primary cause for the onset of endothelial dysfunction [16]. Assessment of FMD can be predictive of CVD and all-cause mortality and is well reported in the literature [20–22].

The relevance of NO goes beyond its vasodilatory role. NO inhibits leukocyte adhesion to the vessel wall and inhibits platelet aggregation and adhesion, protecting smooth muscle cells from exposure to platelet-derived growth factors. These steps are involved in the onset and progression of atherogenesis making NO the most important line of defense against atherogenesis in the vasculature [23].

FMD is commonly performed on the brachial artery, which is a conduit artery; therefore, it provides information about the macrovasculature. However, CVD is associated not only with vascular dysfunction at the macrovascular level but also at the microvascular level [24]. Microvascular dysfunction, impairment of the resistance arteries, is linked to increased death, myocardial infarction, or stroke independently of major CVD risk factors [25]. Microvascular dysfunction is associated with a wide range of cardiometabolic diseases, e.g. diabetes, hypertension and obesity [26,27]. Furthermore, assessing the health of the microvasculature is important because microvascular dysfunction may precede macrovascular dysfunction [28].

In this regard, near infrared spectroscopy (NIRS) is a non-invasive tool that can measure microvascular reactivity and function [29]. This technique determines the concentration and oxygenation status of chromophores in muscle tissues using a modification of the Beer-Lambert Law. Near infrared light (700-900 nm) can penetrate several millimeters into muscle tissues where chromophores, such as hemoglobin (Hb), myoglobin (Mb) and cytochrome oxidase (cyt_{ox}) are found. Chromophores absorb infrared light depending on their oxygenation status. The primary chromophores measured in NIRS are Hb and Mb as the concentration of cyt_{ox}

in skeletal muscle is less than 5%. In regards to the relative contribution of Mb and Hb to the total NIRS signal, the debate is still ongoing as the spectral absorbance of these two chromophores is almost indistinguishable [30,31]. NIRS quantifies oxygenated Hb and Mb, deoxygenated Hb and Mb, and total oxygenated and deoxygenated Hb and Mb. It also determines tissue saturation index (TSI), which is the oxygenated Hb and Mb out of the total Hb and Mb expressed as a percentage.

NIRS combined with the vascular occlusion test (VOT), or post occlusive reactive hyperemia (PORH), is known as the NIRS-VOT and is a useful tool to measure microvascular function (**Figure 1.2**). NIRS-VOT evaluates microvascular reactivity by measuring the steepness of the upslope of the TSI after a period of approximately 5 minutes of supra-systolic occlusion. When the cuff is inflated, no blood is circulating into the limb, and a robust decrease occurs in the TSI (known as slope 1). Slope 1 is an estimate of the O_2 consumption rate of the muscle tissue and is influenced by the adequacy of the microcirculation to provide enough O_2 to the tissue [29]. Similar to the FMD technique, the lack of O_2 and nutrients during ischemia causes a reactive hyperemia which leads to local vasodilation upon cuff release. TSI will start to increase due to a rise in oxygenated Hb and Mb and a washout of their deoxygenated counterparts, until it returns to baseline values. The steepness of this upslope represents the TSI reperfusion slope or rate (slope 2) and reflects the resaturation rate of Hb and Mb, TSI relies on the integrity and functionality of the vascular endothelium [29]. Reactive hyperemia is quantified as the area under the curve above baseline after the cuff is released and reflects microvascular function. The extent and magnitude of reactive hyperemia depends on the ability of the microcirculation to respond to the ischemic insult [29,32]. Furthermore, the TSI reperfusion slope has been positively correlated with FMD in both the brachial and popliteal arteries of healthy individuals [32,33]. Vasodilation at the microcirculation level upon vascular reperfusion determines the reperfusion slope, which is said to represent the shear rate necessary to cause the vasodilatory stimulus that FMD is based upon [32].

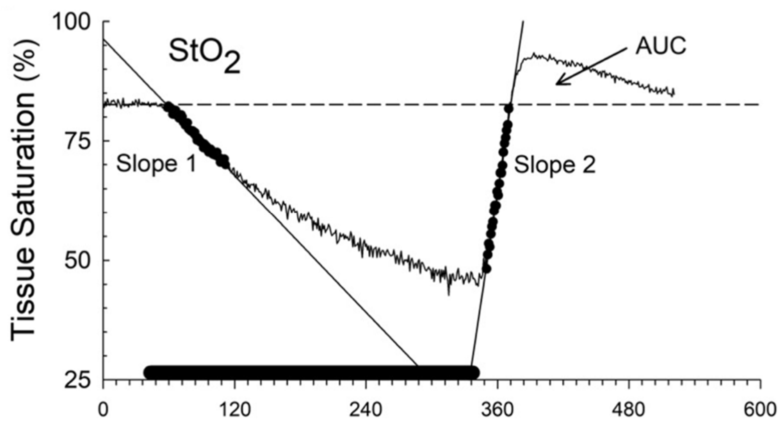

Figure 1.2 TSI response assessed by NIRS during VOT. AUC: area under the curve; StO_2: tissue oxygen saturation; TSI: tissue saturation index; VOT: vascular occlusion test. Source: Barstow [30]

1.2.1.1 Role of ROS in CVD

ROS involve both oxygen free radicals, such as superoxide (O_2^-), hydroxyl radicals ($OH^\bullet$), and peroxyl radicals (OH_{2-}), as well as non-radicals, such as hydrogen peroxide (H_2O_2), hypochlorous acid, and ozone [15]. O_2^- is the first to be generated through the particle reduction of molecular oxygen to O_2^-, causing the formation of the other reactive species [16]. In mammalian cells the main sites of ROS production are the electron transport chain in the mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase (XO) and eNOS [15,34]. Basal levels of ROS are required for diverse cellular functions, including signal transduction pathways, host defense, genomic stability, vascular tone, EC and smooth muscle cell growth, proliferation, migration, apoptosis, and angiogenesis [15,34]. Under normal physiological conditions, oxidative balance is tightly regulated via the equilibrium of pro- and antioxidant systems located in different cellular compartments. Antioxidant systems that protect cells from the formation and damage induced by ROS include enzymatic compounds (e.g., superoxide dismutase (SOD), catalase (CAT), thioredoxins peroxiredoxin and glutathione peroxidase (GPx) as well as non-enzymatic compounds (e.g., tocopherol/vitamin E, beta-carotene, ascorbate, glutathione (GSH), and nicotinamide) [15,35].

The functions and actions of these antioxidants are diverse. SOD catalyzes the dismutation of O_2^- to H_2O_2 and molecular oxygen (O_2). SOD requires a metal cofactor for its activity because it is a metalloenzyme. Cu/Zn-SOD is predominant in eukaryotes organisms, mainly located in the cytosol but also in peroxisomes. CAT catalyzes the reduction of H_2O_2 to water and O_2, finishing the detoxification started by SOD; it uses iron or manganese as a cofactor and is located mainly in peroxisomes. GSH-Px breakdowns H_2O_2 to water and O_2, and lipid peroxides to their corresponding alcohol in mammalian cell mitochondria and sometimes in the cytosol. At least eight GPx enzymes have been identified (GPx1–GPx8). In particular, GPx1 has been specifically implicated in CVD since its deficiency induces vascular oxidative stress and endothelial dysfunction [36].

Excessive levels of prooxidants, or a diminished level of antioxidants can cause a shift in the redox state, known as oxidative stress. Oxidative stress is associated with vascular disease via direct and irreversible damage to macromolecules and disruption of redox dependent signaling processes in the vascular wall. One of the disrupted signaling processes is the NO signaling cascade. O_2^- rapidly scavenges NO to produce peroxynitrite ($ONOO^-$), which is cytotoxic and causes damage to proteins, lipids, and DNA. This reaction is much faster than the dismutation of O_2^- by SOD. Thus, an increase in the O_2^- production rate, could lead to an increased production of $ONOO^-$. In this case, NO is depleted, $ONOO^-$ oxidizes BH_4, leading to uncoupling of eNOS, and O_2 reduction occurs regardless of NO production, turning eNOS into a pro-oxidant enzyme [34]. $ONOO^-$ itself promotes protein nitration and contributes to dysfunction and death of endothelial cells [16]. In conclusion, this imbalance of antioxidants and free radicals has significant negative consequences for our cells and combating agents are warranted.

1.2.2 The Autonomic Nervous System and CV Control

The nervous system consists of two branches: the central nervous system (CNS) (brain and spinal cord) and peripheral nervous system (PNS). The PNS consists of the autonomic (ANS) and the somatic nervous system. The two subdivisions of the ANS are the sympathetic and the parasympathetic nervous systems. Both the sympathetic and parasympathetic branches play an indispensable role in the regulation of the cardiovascular system at rest [37]. The parasympathetic nervous system decreases heart rate (HR), while the sympathetic nervous system continuously alters vascular resistance and BP on a beat-to-beat basis [38].

Several tests involving physiological perturbations and mental challenges have been used to evaluate cardiovascular responses (cardiovascular reactivity) in healthy individuals and clinical populations. One of them is exercise. Three different mechanisms are responsible for the cardiovascular responses during exercise: central command, the exercise pressor reflex (EPR) and the arterial baroreflex (**Figure 1.3**). Adequate function and integration of these feedforward and feedback reflex mechanisms are indispensable to maintain homeostasis during exercise [39]. Central command is a feed-forward mechanism consisting of descending neural impulses originating in higher brain centers in the motor cortex that function both to cause an increase in sympathetic outflow, HR and BP proportional to the intensity of exercise, and also to recruit motor units, and excite medullary and spinal neuronal circuits [40–42]. Central command is associated with cognitive processes related to effort or perceived exertion rather than actual force production [40]. Central command is related to the individual's perception of effort and since neural activity from the cortical region where central command originates is difficult to measure, Borg's rating of perceived exertion (RPE) scale is used to measure the magnitude of central command. However, no experimental study has measured the relation between RPE and central command directly [40]. The arterial baroreflex, composed of afferent fibers originating from the carotid sinus and aortic arch and traveling to the brain stem, controls BP on a beat-to-beat manner via adjustments to HR, stroke volume and total peripheral resistance (TPR). The baroreflex is reset to a higher point to allow increases in arterial pressure during exercise [42,43]. Central command and the baroreflex increase HR and BP proportional to the intensity of exercise [42].

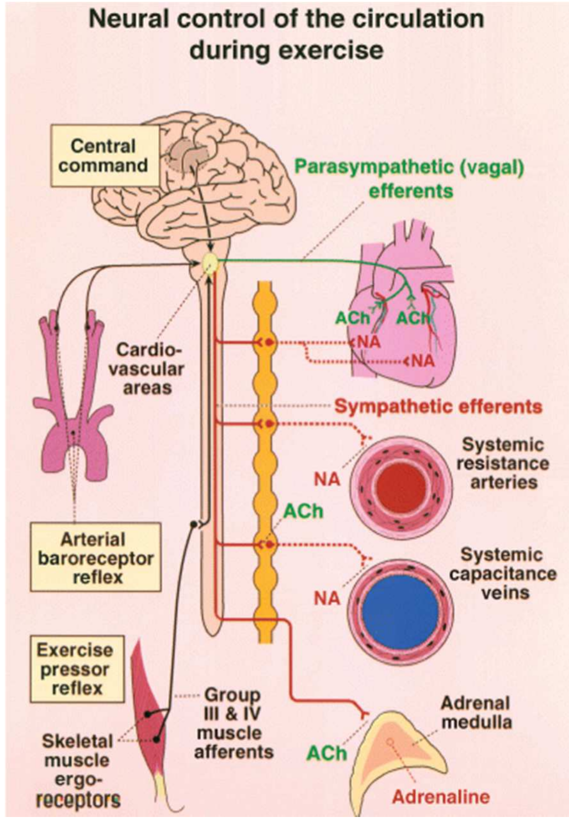

Figure 1.3 Neural cardiovascular control during exercise. ACh: acetylcholine; NA: noradrenaline. Source: Smith et al. [42]

The EPR is responsible for the cardiovascular responses of exercise: increases in BP and HR occur by increasing sympathetic nerve activity and reducing parasympathetic activity (**Figure 1.4**) [42–45]. It is a feedback reflex consisting of the afferent nerve fibers from the skeletal muscle that travel via the lumbar dorsal horn of the spinal cord to the cardiovascular centers in the medulla oblongata [41,44]. The cardiovascular centers involve include the nucleus tractus solitarius (NTS), rostral ventrolateral medulla (RVLM), and the caudal ventrolateral medulla (CVLM) [46]. Two types of afferent nerve fibers have been identified in evoking the EPR: groups III and IV. Group III myelinated afferent fibers are predominantly mechanically sensitive, therefore they are activated by pressure and stretch; group IV unmyelinated afferent fibers are metabolically sensitive and are activated by metabolic byproduct accumulation (e.g. hydrogen ions, potassium, lactic acid, bradykinin, arachidonic acid, analogues of adenosine triphosphate, adenosine, deprotonated phosphate and prostaglandins) [41,42,47,48].

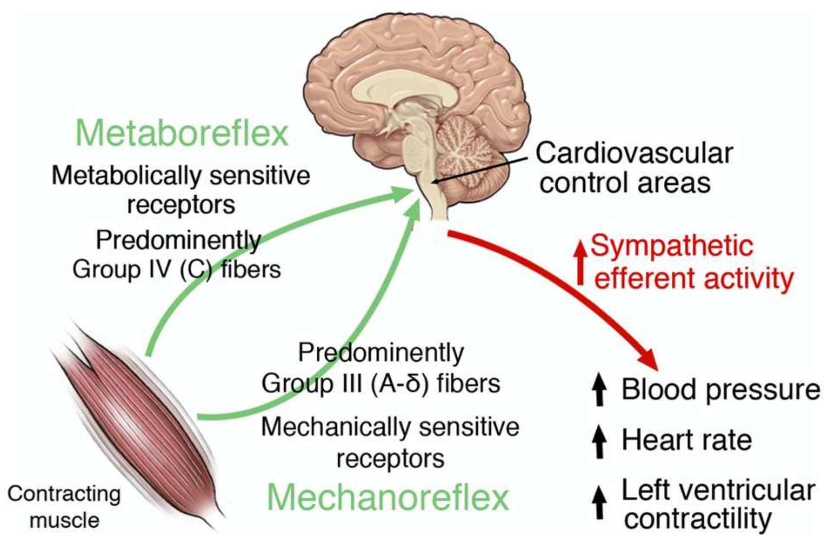

Figure 1.4 Exercise pressor reflex (EPR). Afferent (green) and efferent (red) mechanisms. Source: Mitchell [46].

Isometric handgrip exercise (IHG) is a sympathoexcitatory stimulus used to evaluate autonomic function (and EPR). This test elicits parallel increases in HR and BP, and muscle sympathetic nervous activity (MSNA) that are both part of central command and the EPR. The magnitude of these cardiovascular responses depend on contraction intensity and duration, and the muscle mass involved [49,50]. Whether the increase in BP can be attributed to an increase in cardiac output (Q) or a decrease in total peripheral conductance or both is still under debate [51]. The metaboreflex component of the EPR can be isolated during IHG by causing ischemia via vascular occlusion with a cuff after the ischemic contraction is stopped; this is called post-exercise ischemia (PEI) or post-exercise circulatory arrest [42]. Normotensive adults that exhibit exaggerated BP responses to exercise are more likely to develop hypertension [52,53]. More specifically, IHG is an easy, quick and viable test to potentially unmask future development of hypertension [54,55]. BP reactivity during IHG is increased in prehypertensive adults compared to normotensive adults, in normotensive adults with a family history of hypertension compared to normotensive adults with no family history of hypertension and in mild-hypertensive adults compared to normotensive adults [54–56].

Similarly, the cold pressor test (CPT) is used to evaluate ANS function and BP reactivity. It was first introduced in 1936 by Hines in order to evaluate the reactivity of BP as an index of vasomotor tone [57]. This test consists of immersing a participant's hand in a mixture of ice and water (1 – 4 °C). Sensorial endings of Krausse corpuscles and Ruffini organs detect the low temperature and afferent fibers travel through the spinal cord forming the spinolateral tract. This tract travels to the medulla, pons, and medium cerebrum. At the level of the medulla, the signal reaches the pressor area, i.e., latero-rostral area, which causes the discharge of sympathetic efferent signals leading to an increase in HR and stroke volume, thus, an increase in Q in the first 60 seconds (**Figure 1.5**) [58,59]. During the second minute of the test, increased efferent sympathetic vasoconstrictor nerve activity involving the release of norepinephrine (NEpi), which acts on the α and β adrenergic receptors located at the postsynaptic level, induces arteriolar vasoconstriction and increased TPR, increasing BP [58,59]. HR can either increase or remain unchanged [60]. A BP cuff is placed on the opposite arm and BP readings are taken every 30 seconds until the end of the test. The maximum BP obtained during this test, which usually occurs within thirty seconds, is an index of the response, and referred to as BP reactivity. After 2 minutes of immersion, the hand is removed, and BP comes back to normal within 2 minutes in normotensive participants [57,61,62]. Other biochemical changes responsible for the increased BP during this test include dopamine β hydroxylase activity, dopamine release, neuropeptide γ (NP γ) release, plasma endothelins and prostaglandin synthesis and histamine release, while the role of plasma renin activity and plasma aldosterone is not entirely clear [58]. Factors such as age, sex, race, baseline BP and physical fitness level mediate the BP response to the CPT, with increased activity of the sympathetic nervous system considered one of the major factors that can influence this response [63]. The CPT is considered a significant prognostic tool as an independent relation exists between excessive reactivity or exaggerated increases in BP during the test and incidence of future hypertension [64–68]. During both IHG and CPT, the Valsalva maneuver must be avoided since it causes a reduction in venous return leading to a drop in BP and pulse pressure, which can cause misinterpretation of the results [69].

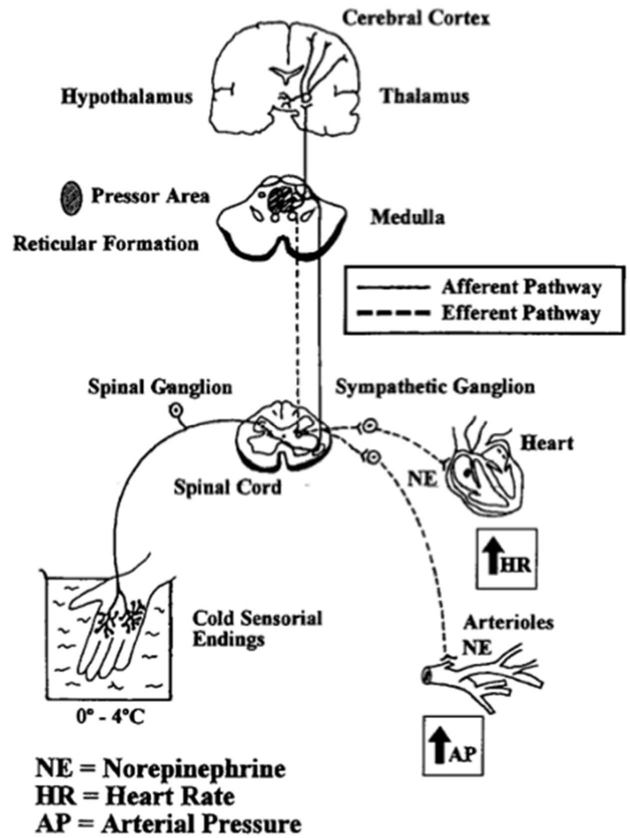

Figure 1.5 Neural pathways of the Cold Pressor Test. Source: Velasco et al. [58]

1.3 Sodium and endothelial function

Sodium is an essential micronutrient required for plasma volume maintenance, acid-base balance, transmission of nerve impulses and normal cell function [1]. While physiological sodium need is estimated at 250 mg a day and the dietary reference intake is ≤ 2300 mg of sodium a day, the last report from NHANES highlights that the mean sodium consumption between 2015-2016 was ~3200 mg/day in the US [4,70]. Furthermore, the impact of a high sodium diet in the development of CVD is well-known and is a known risk factor for CVD [1,6,71]. While the relation between high sodium intake and increased BP has been well established there are BP independent effects as well [8,9]. Pre-clinical and clinical studies highlight organ damage from a high sodium intake that can target blood vessels, the heart, kidneys and areas of the brain controlling autonomic outflow [8,9]. Lenda et al., [72] studied the effect of 4-5 weeks of both low and high sodium diets in Sprague-Dawley rats. Spinotrapezius muscle arteriole responses to ACh were impaired in the high salt group but returned to normal when oxidant scavengers (SOD, Tempol and CAT) were infused. They also observed an increase in arteriolar and venular wall oxidant activity, which were reduced with oxidant scavengers. Furthermore, using the same study intervention, Lenda et al., [73] observed that inhibition of NADPH oxidase and XO, considered the main enzymatic sources of ROS in the peripheral vasculature, reduced arteriolar wall oxidant activity to normal levels in the microvessels of the spinotrapezius muscle of rats fed high salt; however, arteriolar responses to ACh were not restored. In another study, Lenda and Boegehold [74] observed that Cu/Zn SOD activity was reduced in rats fed high salt, which was associated with loss of arteriolar NO activity in microvessels of the spinotrapezius muscle. These results are consistent with a study by Kitiyakara et al. In this study, male Sprague-Dawley rats fed high salt for 7 days had increased urinary 8-isoprostane and malondialdehyde (MDA) excretion, increased renal cortical NADH and NADPH, increased renal cortical mRNA expression of $gp91^{phox}$ and $p47^{phox}$ (components of NADPH oxidase), and decreased expression of Cu/Zn SOD and mitochondrial SOD [75]. In the same fashion, Zhu et al., [76] examined the effect of a low or high salt diet in Sprague-Dawley rats for 3 days. The aortic rings of those fed high-salt showed impaired endothelium-dependent relaxation (induced by methacholine), reduced release of NO and increased O_2^- production. Nurkiewicz et al., [77] confirmed

Lenda et al. results using a murine model. The spinotrapezius muscle of mice fed high salt experienced a decrease in ACh induced arteriolar dilation and an increase in oxidant activity (especially O_2^-) in the arterial wall compared to mice fed a normal salt diet for 4 weeks. High salt fed mice recovered normal arteriolar responsiveness to ACh and reduced oxidant activity upon exposure to Tempol and CAT. Lastly, Zhu et al., [78] showed how methacholine-induced NO production was eliminated in aortic endothelial cells from high salt fed rats but Tempol restored NO values, augmented vessel relaxation and increased methacholine-induced Ca^{2+} responses.

Human studies also highlight vascular dysfunction induced by sodium. Dupont et al., [79] demonstrated that a 7-day high sodium diet caused decreases in FMD compared to a 7-day low sodium diet in healthy normotensive young adults, independent of changes in BP. These findings were replicated by Matthews et al., [80] in a young healthy normotensive cohort who were salt-sensitive, i.e. those who experience an increase of > 5 mm Hg in mean arterial pressure (MAP) from the low sodium to high sodium diet. Greaney et al. [81] observed that cutaneous vascular conductance (CVC), measured with laser doppler flowmetry and indicative of microvascular function, was diminished in the plateau phase (highly dependent on NO) in healthy young normotensive adults undergoing a 7-day high sodium diet compared to a 7-day low sodium diet. Collectively, these studies highlight the BP independent effects of sodium on the vasculature. One potential mechanism by which high sodium diets may cause endothelial dysfunction is oxidative stress [8,9,28]. Greaney et al., [81] demonstrated that the decline in CVC could be mitigated by ascorbic acid (a non-specific ROS scavenger), which restored the NO contribution in the plateau phase. To further investigate the source of ROS, Ramick et al., [82] observed that infusion of apocynin (inhibitor of NADPH oxidase or NOX) and Tempol (O_2^- scavenger) restored the CVC plateau (but no difference in NO contribution) in the high sodium group, which was initially reduced compared to the low sodium group. To summarize, excessive salt consumption leads to decreased vascular function and NO bioavailability and production, which is partly mediated by increased ROS and decrease in antioxidant defense.

High salt intake leads to a reduced NO bioavailability via several pathways (**Figure 1.6).** One potential pathway is increased O_2^- production and suppression of angiotensin II (Ang II). The latter leads to decreased SOD expression and therefore, reduced scavenging activity. As mentioned previously, in addition to scavenging NO, ROS may oxidize BH_4, a critical cofactor for eNOS. This leads to a reduced NO synthesis and increased generation of O_2^-. [10] In conclusion, a high sodium intake increases free radicals and decreases antioxidant enzyme levels leading to oxidative stress which ultimately impairs endothelial function.

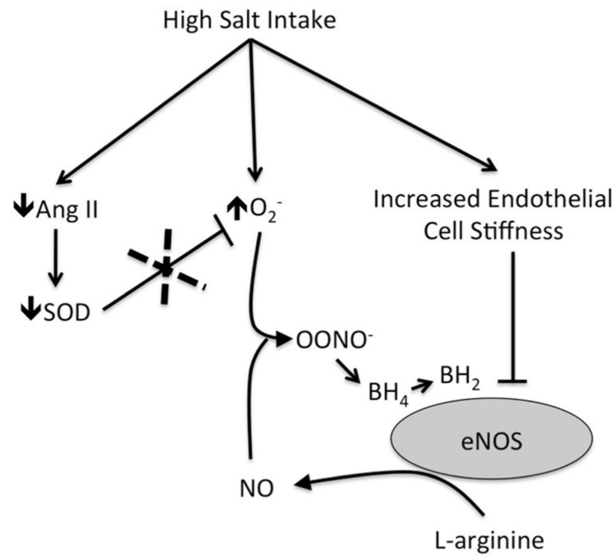

Figure 1.6 Proposed mechanisms by which high salt intake leads to reduced NO bioavailability. Ang II: angiotensin II; BH_2: boron dihydride; BH_4: tetrahydrobiopterin; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; O_2^- : superoxide; ONOO: peroxynitrite; SOD: superoxide dismutase. Source: Edwards & Farquhar [10]

1.4 Sodium and the Autonomic Nervous System

An association between dietary sodium and BP reactivity has been reported. In an animal study, Yamauchi et al., [83] showed that adult normotensive rats fed high salt for 2 to 3 weeks experienced a greater increase in MAP and HR compared to those fed normal chow despite producing the same amount of force (caused by electrodes attached to the peripheral cut ends of the L4 and L5 ventral roots). Adams et al., [84] showed that 14 days of a high sodium diet in rats increased sympathetic or BP responses to sympathoexcitatory stimuli compared to rats fed normal chow. Animal studies highlight that increasing salt consumption augments HR and BP. Human studies build further support for this relation.

In humans, Ditto et al., [85] were first to report that after 2 weeks of dietary sodium loading, healthy normotensive males experienced an increased diastolic BP reactivity to the CPT, although no impact was observed in systolic BP. Similarly, healthy Chinese participants aged 18-60 years old who underwent 7 days of a high sodium diet experienced increases in BP reactivity to the CPT [86]. Exaggerated BP responses have also been reported in the EPR. Ambrosioni et al., [87] reported that 6 weeks of moderate sodium intake restriction reduced BP reactivity to both static handgrip and dynamic bicycle exercise in young borderline hypertensive adults (defined as a participant in supine position who exhibits on three different measures at least one diastolic BP above and one below 90 mm Hg). Brian et al., [88] investigated the effect of elevated plasma osmolality and serum sodium (achieved via acute infusion of hypertonic saline) on sympathetic outflow and BP responses to IHG followed by PEI. These healthy normotensive young adults experienced an exaggerated increase in MSNA and BP reactivity during this physiological perturbation after the infusion compared to baseline. Caldwell et al., [89] observed an increase in MAP during static handgrip exercise in healthy young normotensive participants consuming 15 g of salt in pill format for 7 days compared to placebo pills. Recently, Babcock et al., [90] reported increases in BP reactivity during submaximal aerobic exercise following 10 days of high sodium diet in normotensive young healthy adults, which was negatively correlated with FMD, compared to those that have been taking a placebo for 10 days. This relation has also been depicted in a cross-sectional study of Japanese adults which highlighted a correlation between increased dietary sodium/potassium ratio (assessed as a self-administered food frequency questionnaire) and exhibited exaggerated BP responses to a submaximal exercise test. Hyperresponders reported a higher sodium intake compared to adults whose BP response to exercise was considered normal [91]. Therefore, studies suggest that chronic high dietary sodium consumption exaggerates BP responses to exercise.

Two main mechanisms are thought to be responsible for the increase in BP during physiological perturbations as dietary sodium intake alters neural pathways that control the CV system (**Figure 1.7**) [92]. First, high dietary sodium is thought to alter the excitability and increase gain of the central sympathetic neurons [93]. High sodium intake enhances the sensitivity of the neurons in the RVLM, which is responsible for integration and regulation of sympathetic vasomotor tone [94,95]. RVLM sympathoexcitatory and sympathoinhibitory responses to the local injection of neurotransmitters including L-glutamate and GABA were increased in rats undergoing a high sodium diet [84,96,97]. The second mechanism is related to sodium sensing in the brain. Elevation of sodium chloride in cerebrospinal fluid caused increases in lumbar sympathetic nerve activity and mean BP [98]. Increased extracellular sodium and plasma osmolality, which is sensed by neurons in the circumventricular organs in the brain including the organum vasculosum of the lamina terminalis (OVLT) and subfornical organ, increases sympathetic activity arising from the brainstem [99]. Indeed, intracerebrovascular hypertonic saline infusion in rats was shown to increase discharge from the OVLT causing increases in sympathetic activity and BP [100]. The OVLT has neural connections to the paraventricular nucleus (PVN) of the hypothalamus, which projects to the RVLM, therefore regulating sympathetic nervous activity outflow [101,102]. In healthy young normotensive adults, increases in serum sodium and plasma given by a week of high sodium diet increased cardiovagal baroreflex sensitivity [92]. In conclusion, the brain plays a large role in the control of sympathetic responses to high sodium consumption.

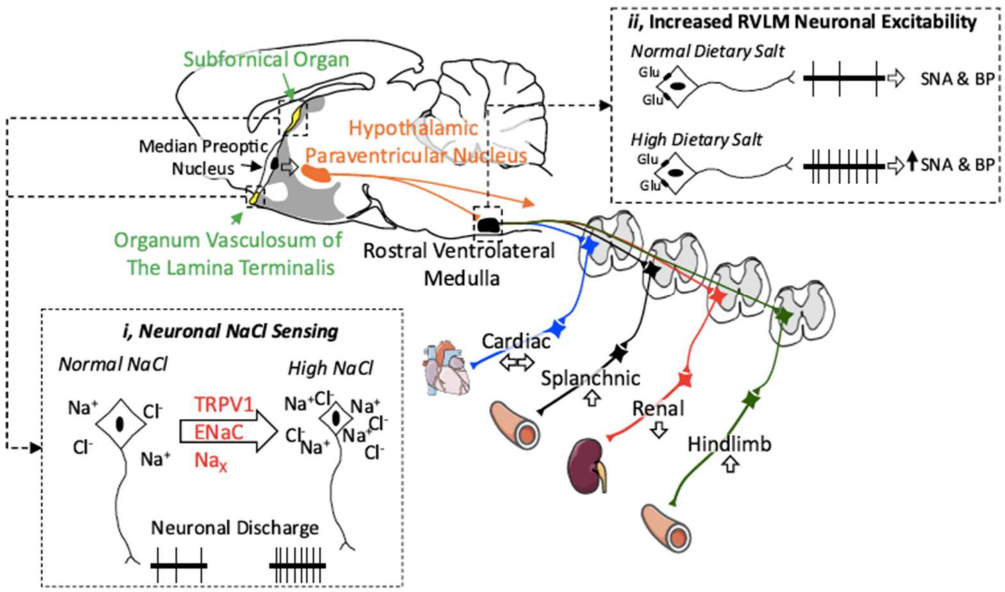

Figure 1.7 Dietary salt alters autonomic function by excitation of NaCl-sensing neurons in the lamina terminalis or increasing gain/excitability of bulbospinal neurons in the rostral ventrolateral medulla. BP: blood pressure; ENaC: epithelial sodium channel; RVLM: rostral ventrolateral medulla; SNA: sympathetic nerve activity; TRPV1: transient receptor potential cation channel subfamily V member 1. Source: DeLalio et al. [103]

1.5 Melatonin overview

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized in the mammalian pineal gland in a circadian fashion, reaching maximum production at nighttime. During the day, photosensitive retinal ganglion cells (ipRGCs) detect visible light, i.e., wavelengths in the range of 460-480 nm, inhibiting the synthesis of the hormone. At night, the absence of this visible light is detected by the ipRGCs, which send a message via the retinohypothalamic tract, embedded in the optic nerve, to the central biological clock (known as the suprachiasmatic nucleus or SCN) in the hypothalamus. The SCN acts as a relay center that sends a neural signal to the pineal gland via the central and peripheral sympathetic nervous systems. This neural pathway involves synapses in the PVN of the hypothalamus, the intermediolateral cell column at thoracic cord levels 1 and 2, and the superior cervical ganglia (SCG), finishing at the pineal gland. This neural pathway culminates with the release of NEpi on the pineal gland. NEpi then binds to pineal α-1 and β-1 adrenoreceptors, which activate indispensable enzymes for the pineal gland cells (i.e. pinealocytes) to synthesize melatonin from the essential amino acid, tryptophan [104–106]. If tryptophan intake is severely reduced, the synthesis of melatonin will be diminished considerably [107]. The biosynthetic pathway (**Figure 1.8**) involves hydroxylation of tryptophan to 5-hydroxytryptophan via tryptophan hydroxylase (TPH), then conversion to serotonin or 5-hydroxytryptamine via aromatic amino acid decarboxylase. Serotonin is then to converted to N-acetylserotonin via serotonin N-acetyltransferase (SNAT, formerly known as arylalkylamine N-acetyltransferase or AANAT) and lastly to melatonin via N-acetylserotonin O-methyltransferase (ASMT). An alternative pathway to synthesize melatonin is the conversion of serotonin to 5-methoxytryptamine via ASMT and the later to melatonin via SNAT. SNAT is believed to be the rate-limiting enzyme of melatonin production and is activated by noradrenergic inputs from the SCG [108–110]. The synthesized melatonin is not stored but released to the cerebrospinal fluid and the blood stream, reaching all biological fluids. In the blood stream, melatonin circulates in both free form and bound to albumin [104–106]. Serum physiological levels of melatonin vary from 5 to 200 pg/mL, according to the time of day and do decrease with age [105,111]. Melatonin displays a half-life in the blood of 20 to 40 minutes, with 90% cleared during a sole passage through the liver, where it is lastly transformed into 6-sulfatoxymelatonin (aMT6s), the main metabolite excreted in the urine [112]. aMT6s correlates with plasma melatonin levels [113,114]. Alternative pathways of degradation involve the production of 5-methoxyindole acetic acid or 5-methoxytryptophol, N1-acetyl-N2-formyl-5-methoxykynuramine and N-acetyl-5-methoxykynuramine, which act as free radical scavengers [109,115].

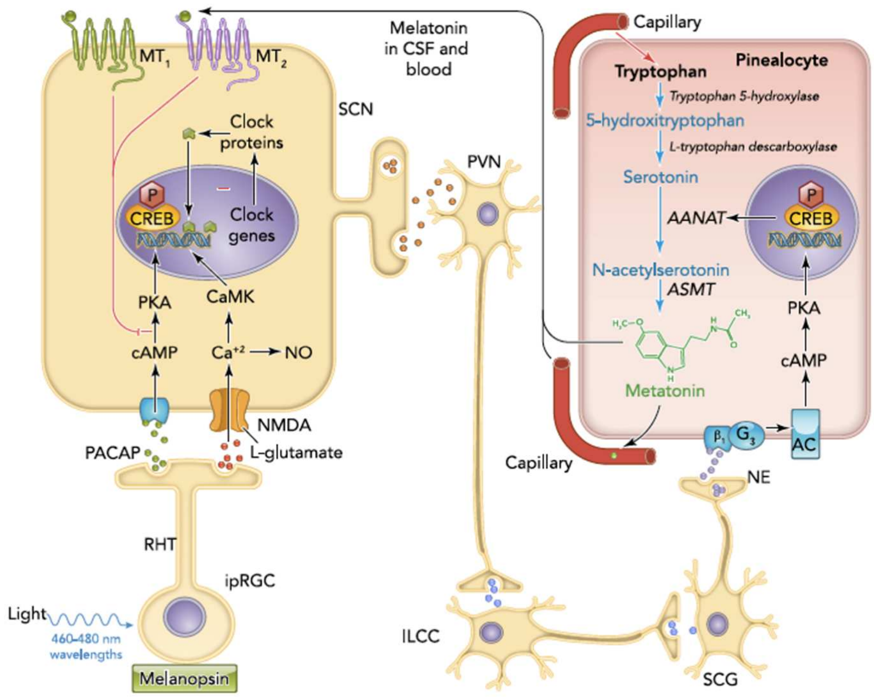

Figure 1.8 Melatonin synthesis pathway in the pineal gland. AC: adenylate cyclase; AANAT: arylalkylamine N-acetyltransferase; ASMT: acetylserotonin methyl transferase; CaMK: calcium/calmodulin protein kinase; cAMP: cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; CSF: cerebrospinal fluid; ILCC: intermediolateral cell column; ipRG: intrinsically photoreceptive retinal ganglion cells; NE: norepinephrine; NMDA: N-methyl-D-aspartate receptor; NO, nitric oxide; PACAP: pituitary adenylate cyclase-activating peptide; PKA: protein kinase A; PVN: paraventricular nucleus; RHT: retinohypothalamic tract; SCG: superior cervical ganglia; SCN: suprachiasmatic nucleus. Source: Reiter et al. [104]

Almost all organs and tissues have been identified as extrapineal sites of melatonin production, including the brain, retina, airway epithelium, skin, gastrointestinal tract, liver, kidney, thyroid, pancreas, thymus, spleen, immune system cells, carotid body, reproductive tract and endothelial cells. However, extrapineal production sites do not follow a circadian rhythm, do not release important amounts into the circulatory system and their production depends on the tissue reaching micromolar concentrations in some instances [106]. In these organs, melatonin acts as an antioxidant, autocoid or paracoid maintaining cellular homeostasis [104,106,116]. It is believed that pineal produced melatonin has more of a chronobiotic effect and extrapineal melatonin is more involved in protecting the cell in which it is produced against oxidative damage. This is supported by the fact that the antioxidant effects of melatonin require higher concentrations than pineal derived melatonin, similar to extrapineal melatonin concentrations [106]. It is important to note that blood melatonin levels are not representative of the concentration of melatonin in a determined organ or tissue [104].

Besides its relation with the circadian rhythm, within the last 20 years, numerous physiological properties have been attributed to melatonin: antioxidant and scavenger, antihypertensive, antilipidemic, anti-cancer, immunomodulatory, anti-inflammatory, anti-apoptotic, sympatholytic, and sleep promoter (**Figure 1.9**) [104,111,116]. Therefore, melatonin has implications in the progression of numerous diseases. Melatonin exerts its effects via receptor dependent or receptor independent mechanisms, discussed in the next section.

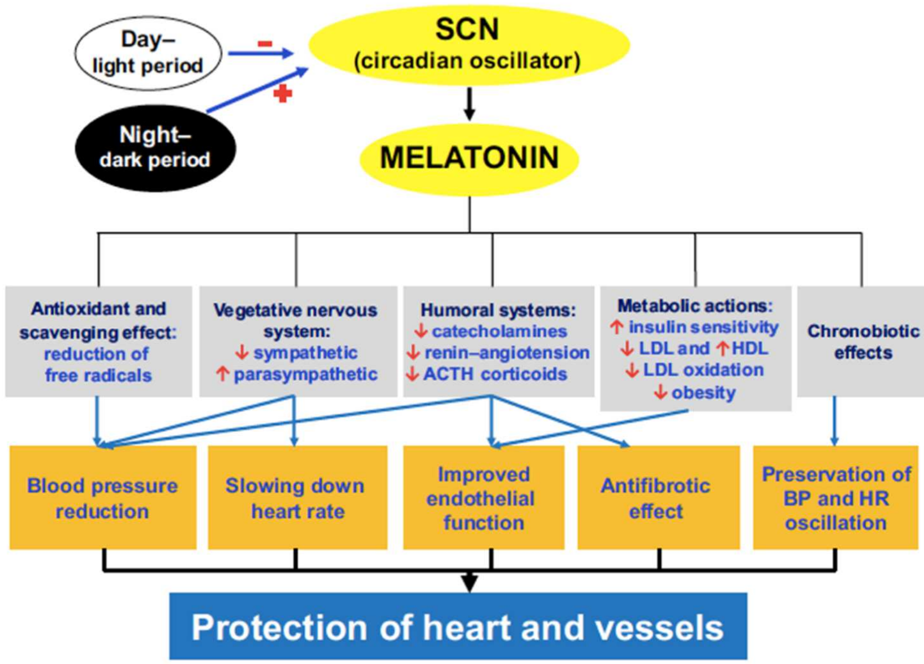

Figure 1.9 Benefits of melatonin on the heart and blood vessels. ACTH: adrenocorticotropic hormone; BP: blood pressure; HDL: high-density lipoprotein; HR; heart rate; LDL: low-density lipoprotein; SCN: suprachiasmatic nucleus. Source: Simko et al. [117]

1.5.1 Melatonin's mechanisms of action

In mammals, the effects of melatonin occur through different pathways: 1) binding to melatonin receptors in the plasma membrane, 2) binding to intracellular proteins, 3) binding to nuclear receptors of the orphan family, and 4) an antioxidant effect (**Figure 1.10**) [118,119]. Melatonin receptors are located throughout different body tissues and organs, which indicates its systemic effects [118]. Three well-known melatonin membrane receptors and one nuclear receptor have been identified in humans to date: MT_1, MT_2, MT_3 and ROR orphan receptors, respectively [119]. MT_1 and MT_2 are membrane receptors that belong to the superfamily of G protein- coupled receptors (GPCRs) and are mainly coupled to $G\alpha_i$ proteins [106,120,121]. $G\alpha_i$ activation leads to inhibition of adenylate cyclase, reduction of cytosolic cAMP levels and inhibition of the protein kinase A signaling pathway [116]. Although the intrinsic affinity of melatonin receptors for different G proteins has not been established completely [117]. MT_1 is also associated with $G\alpha_q$-coupled phospholipase-C activation resulting in increased cytosolic Ca^{2+} or G-coupled activation of the Kir 3 K-channels [106,122–124]. MT_1 receptors are expressed in the brain, cardiovascular system (including peripheral blood vessels, aorta and heart), immune system, testes, ovary, skin, liver, kidney, adrenal cortex, placenta, breast, retina, pancreas and spleen [109]. MT_2 couples with Gq-protein mediated phosphatidylinositol-4,5-bisphosphate hydrolysis [106,122–124]. MT_2 has been found in the immune system, SCN, retina, pituitary gland, blood vessels, testes, kidney, gastrointestinal tract, mammary glands, adipose tissue and skin [109]. MT_2 also modulates guanylyl cyclase and subsequently cGMP production [125]. A cytosolic MT_3 receptor was initially identified but later known to be the cytosolic enzyme quinone reductase 2, which inhibits the electron transfer reactions of quinones and therefore prevents oxidative stress, but has low affinity for melatonin [115,121,126]. The highest levels of MT_3 are present in the liver and kidneys while modest levels are found in the heart and the brain [109]. Melatonin also interacts with several cytosolic proteins (i.e., calmodulin, calreticulin and tubulin), involved in regulation of the cytoskeleton and control of nuclear receptors. Furthermore, melatonin interacts with the Ca^{2+}-calmodulin complex, which regulates intracellular Ca^{2+} levels. Its biological effects depend on the type of cells where the interaction takes place. In smooth muscle cells, melatonin's effect on the Ca^{2+}-calmodulin complex decreases the level of Ca^{2+} leading to relaxation, while in endothelial cells, the decrease in Ca^{2+} levels inhibits eNOS, triggering vasoconstriction [127]. Lastly, ROR orphan receptors include three subtypes (α, β, γ) but only one variant of subtype α and γ relate to melatonin effects [106].

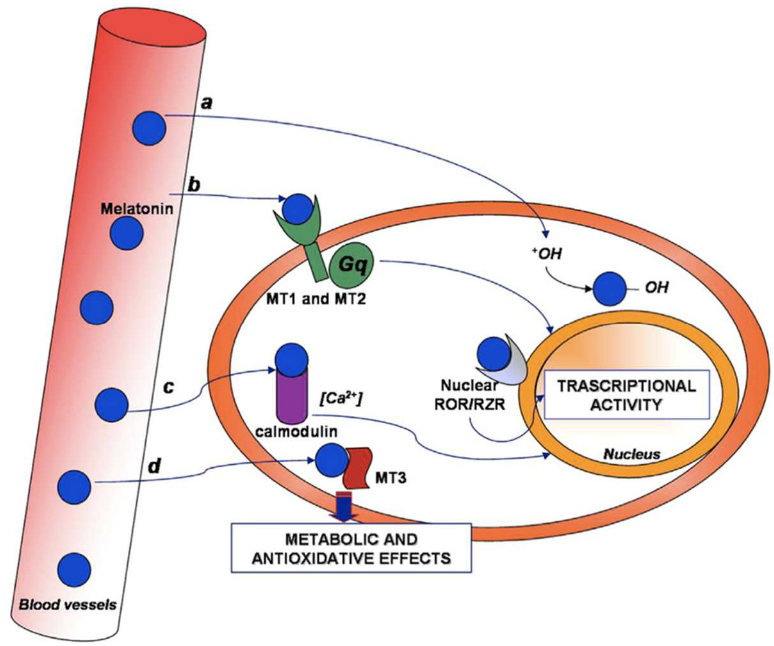

Figure 1.10 Melatonin's different pathways to enter the cell. Ca^{2+}: calcium; •OH: hydroxyl radical; MT1: melatonin receptor 1; MT2: melatonin receptor 2; MT3: melatonin receptor 3. Source: Favero et al. [109]

Regarding the cardiovascular system, the roles of MT_1 and MT_2 vascular receptors needs to be further evaluated as to their vasodilatory and vasoconstriction functions. Animal studies show contradictory results although there is a consensus connecting MT_1 with vasoconstriction and MT_2 with vasodilation [119,128]. The vasodilatory effects are thought to be endothelium-dependent and due to increased NO, and/or inhibition of noradrenergic effects [129]. However, in most experimental models, activation of both MT_1 and MT_2 receptors is associated with cyclic AMP decrease and phosphatidylinositol-4,5-biphosphate hydrolysis, causing vasoconstriction [124]. The activation of MT_2 receptors on the smooth muscle of porcine coronary arteries inhibits NO-induced increases in cGMP and the ability of the smooth muscle to relax in response to NO [130]. Activation of MT_2 located on endothelial cells could increase Ca^{2+} in the cytosol and therefore stimulate NO, guanylate cyclase and cGMP in vascular smooth muscle cells, overcoming vasoconstriction and leading to vasodilation [124,125]. These pathways are illustrated in **Figure 1.11**. Thus, the biological effects of MT_1 and MT_2 need to be further evaluated in humans, but they may vary depending on the particular blood vessel, dose of melatonin and the presence of either MT_1 and MT_2 or both [106,115].

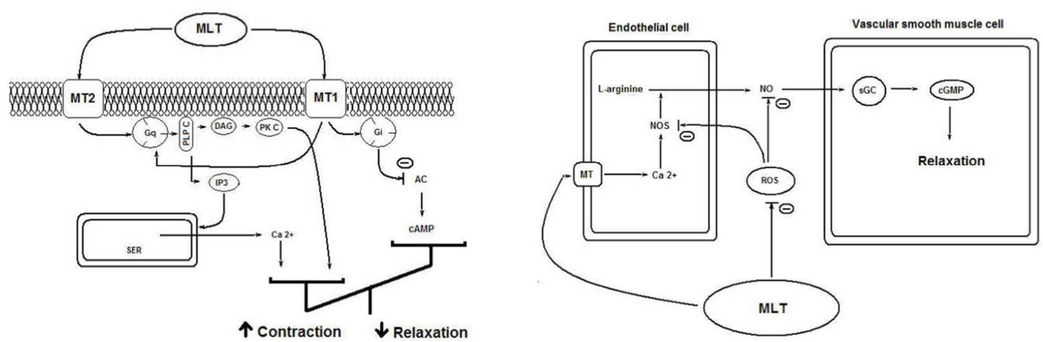

Figure 1.11 Potential pathways mediating melatonin-induced vasoconstriction (left) and vasodilation (right) in vitro. cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; DAG: diacylglycerol; IP3: inositol-1,4,5- trisphosphate; MLT: melatonin; MT1: melatonin receptor 1; MT2: melatonin receptor 2; NOS: NO-synthase; PLP C: phospholipase C; PK C: protein kinase C; ROS: reactive oxygen species; sCG: soluble guanylate cyclase; SER: smooth endoplasmatic reticulum. Source: Paulis & Simko [131]

1.5.2 Melatonin and CVD

Nocturnal synthesis, circulating levels of melatonin, and its urinary metabolite (6-sultatoxymelatonin or aMT6s) are reduced in different CVD populations, such as coronary heart disease, hypertension and heart failure [132–137]. Furthermore, the incidence for cardiac events, including myocardial infarction, sudden cardiac death and arrhythmias is higher in the early morning when circulating melatonin levels are significantly lower. Different pathophysiological mechanisms such as a BP and HR surge, decreased endothelial dilatory capacity of arteries, enhanced sympathetic activity, decreased cardiac electrical stability and increased platelet aggregation, coincide with this time of the day [105,135,138–141].

1.5.2.1 Melatonin and BP

The association between melatonin and BP was first reported when pinealectomy (removal of the pineal gland) performed in rats was shown to induce hypertension [142,143]. Since then, the therapeutic effect of melatonin on BP has been widely reported in animal and human studies. A study using the spontaneous hypertensive rat (SHR) experienced a decrease in BP after 6 weeks of melatonin compared to the SHR group that received a placebo. Additionally, reductions in renal O_2^- and a marker of lipid peroxidation, MDA were seen [144]. Pechanova et al., [145] also found decreases in BP, increases in left ventricular NOS activity and decreases in oxidative load in SHR provided melatonin for 6 weeks compared to untreated SHR.

In humans, normotensive or hypertensive- treated adult women who received 3 mg of melatonin before bed for 3 weeks experienced declines in nocturnal systolic BP, diastolic BP and MAP compared to placebo [146]. Adult untreated hypertensive men also experienced a decline in nocturnal systolic and diastolic BP and MAP after taking 2.5 mg of melatonin daily 1 h before sleep for 3 weeks; they also examined acute affects after supplementation for 24 h and observed no effects [147]. Arangino et al., [148] reported decreases in systolic BP, diastolic BP and MAP in young healthy normotensive men after 90 minutes following 1 mg of melatonin administration compared to placebo. Lastly, 2 mg of melatonin for 4 weeks reduced nocturnal systolic and diastolic BP in adults with nocturnal hypertension compared to the placebo group [149].

The mechanisms involved in melatonin-induced hypotension may not be related to a direct effect of MT receptors since they are linked to Gi- and Gq-receptor activation. Activation of Gi- and Gq- receptors leads to decreased levels of cAMP, increased levels of cytosolic Ca^{2+} and inhibition of vasodilation and/or cause vasoconstriction, respectively [125]. Melatonin appears to cause two different responses in vascular smooth muscle cells depending on its concentration: lower concentrations potentiates contractions and higher concentrations attenuate contractions [150]. Hence, the underlying mechanisms are complex (e.g. MT_2 receptor activation in endothelial cells causes increases in NO production) and multifactorial (i.e. antioxidant and central autonomic regulation mechanisms) [125]. Laflamme et al., [151] demonstrated that the hypotensive effect of melatonin was associated with the attenuation of basal sympathoadrenal tone and inhibition of α1 adrenergic mediated stimulation of the vascular phosphoinositide pathway activity. Others hypothesize that the reductions in BP are due to amplification of the circadian output of the SCN and/or its clock genes in the cardiovascular system [147].

In conclusion, different mechanisms of action are responsible for the decrease in BP induced by melatonin, which in turn offers vascular protection (**Figure 1.12**). These include indirect regulation of BP via the central nervous system and catecholamine secretion modulation, antioxidant and anti-inflammatory effects, relaxation of smooth muscle in blood vessels, NO production and Ca^{2+} signaling [116,152,153]. The following sections will be dedicated to the known effects of melatonin on the topics of interest of this dissertation: sympathetic nervous system, BP, vascular function, and oxidative stress.

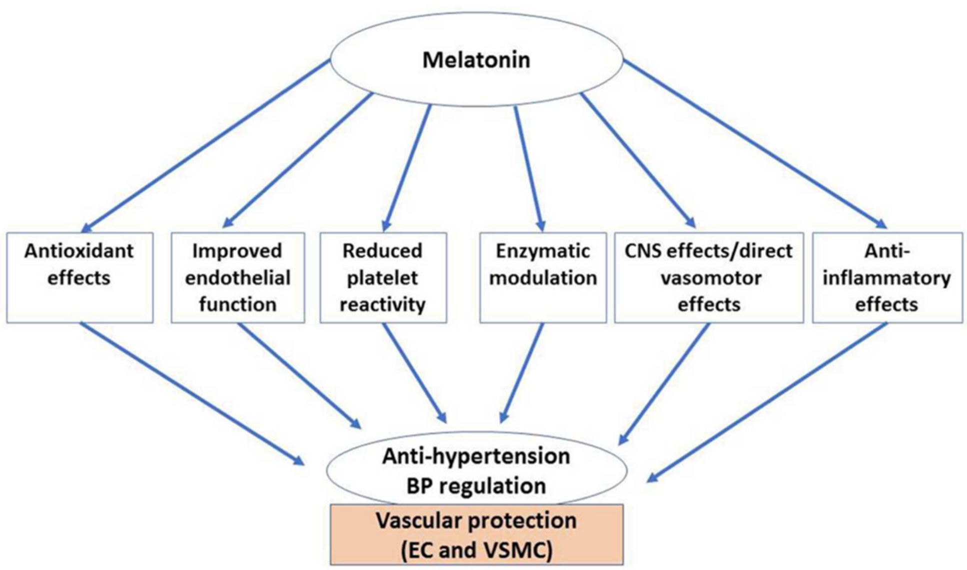

Figure 1.12 Benefits of melatonin on the vasculature. EC, endothelial cells; CNS, central nervous system; CSMC, vascular smooth muscle cells. Source: Jiki et al. [116]

1.5.3 Melatonin and the Autonomic Nervous System

As stated in a previous section, the production of melatonin in the pineal gland is influenced by the sympathetic nervous system. Axons of the PVN neurons project to the cervical intermediolateral cell column. These cells are preganglionic sympathetic neurons, and they control vascular tone and arterial BP. Sympathetic signals from the intermediolateral cell column project to the superior cervical ganglia and the pineal gland releasing NEpi which binds to pineal β-1 and α-1 adrenoreceptors activating ANAAT resulting in melatonin production. The axons of the PVN neurons are inhibited during the day by γ-aminobutyric acid (GABA)-ergic innervation from the SCN, allowing for circadian oscillations [115,117,124] .

Melatonin is believed to cause an inhibition of sympathetic output through several different pathways (**Figure 1.13**). First, melatonin stimulates (GABA)-ergic signaling inhibiting the PVN by the SCN. NO formation potentiates (GABA)-ergic effects in the PVN and given the potential of melatonin to enhance NO bioavailability, melatonin may indirectly augment the inhibition of the PVN. Second, melatonin may epigenetically modify area postrema neurons, which are related to BP regulation and inhibit the activity of the RVLM through the caudal ventrolateral medulla [124]. Melatonin has been hypothesized to represent a protective mechanism against excessive sympathetic excitation [124]. Besides the interaction with the aforementioned brain structures, melatonin may also attenuate sympathetic tone via its antioxidant properties [115]. Another important area of the brain related to regulation of BP in normotensive animals is the anterior hypothalamic area (AHA) which has been postulated to be a site of action of melatonin [154]. Microinjections of melatonin into the AHA caused a fall in BP in normotensive rats and stress-induced hypertensive rats [155]. This response was mainly mediated by MT_1 and MT_2 receptors in the AHA, which decreased the release of the excitatory amino acid glutamate and increased the release of the inhibitory amino acids taurine and GABA in the RVLM [156].

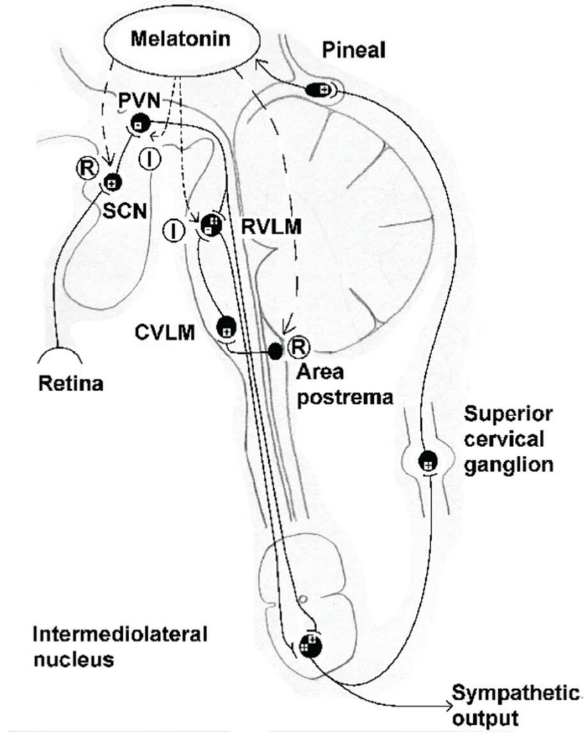

Figure 1.13 Melatonin's modulation of sympathetic tone. CVLM: caudal ventrolateral medulla; PVN: paraventricular nucleus; RVLM: rostral ventrolateral medulla; SCN: suprachiasmatic nucleus. Source: Paulis & Simko [131]

Animal studies have shown an association between the sympathetic nervous system and melatonin supplementation. In the SHR model, an acute melatonin intervention caused a time-dependent decrease in BP without a reduction in HR. This also corresponded with reductions in plasma Epi and NEpi [151]. Similar results have been found in pinealectomized adult male Wistar rats that lack the natural endogenous melatonin production. These rats were administered interleukin-1-β, which causes an increase of Epi and NEpi levels, whose levels were attenuated by intravenous infusion of melatonin [157]. These studies indicate that melatonin inhibits the central pathway of sympatho-adrenomedullary outflow in rats. Furthermore, intravenous administration of melatonin in rats caused a dose-response reduction in BP and HR[158]. Lastly, male SHR provided with melatonin for 4 weeks exhibited decreases in BP and HR, improved bradycardic and tachycardic baroreflex responses. These changes coincided with improvements in erythrocyte antioxidant reserve (SOD and GPx) [159]. In conclusion, animal work suggests that melatonin inhibits the sympathetic nervous system and/or stimulates the parasympathetic nervous system.

Human studies indicate that melatonin modulates the branches of the CNS in the same way as seen in the animal literature. In healthy young men given 1 mg of melatonin, carotid-femoral pulse wave velocity (PWV) reductions were observed after 60 minutes accompanied by reductions in systolic BP and MAP. PWV is an indicator of arterial elasticity, being higher in hardened vessels and lower in distensible, more compliant vessels [160]. Healthy young women, studied in the early follicular phase of their menstrual cycle, were given a capsule containing either 1 mg of melatonin or placebo. Ninety minutes later, internal carotid artery pulsatility index (PI) was observed along with decreases in systolic BP, diastolic BP and MAP and circulating NEpi values. PI is an indication of vasomotor tone and resistance to flow [161]. Similar results were found in healthy young men [148].

The influence of melatonin has also been evaluated on heart rate variability (HRV), which is caused by changes in input to the sinus node of the ANS. Sixty minutes after the administration of 2 mg of melatonin in healthy young males, indexes of vagal activity were increased along with sympathovagal balance. This was accompanied by decreases in systolic BP, NEpi and dopamine [162].

Direct measures of sympathetic nervous system responses have been evaluated in humans consuming melatonin. Three mg of oral melatonin attenuated MSNA responses, a direct index of sympathetic activity in humans, to orthostatic stress induced by lower body negative pressure at both -10 and -40 mmHg in healthy young participants. However, the authors did not observe the same results to the IHG and the CPT; but this was an acute intervention and the isometric handgrip contraction was performed at 30% of their maximal voluntary contraction (MVC) [163]. Similar results were observed during head-down rotation [164]. Healthy males who took 3 mg of melatonin prior to body heating to 38 °C, experienced an attenuated rise in internal temperature and a lower sensitivity to internal temperature changes. This suggested a shifting of skin blood flow control, which is dependent on the sensitivity of the cutaneous vasodilator system [165] . Three mg of oral melatonin also attenuated the increased skin sympathetic nerve activity, HR and MAP responses induced by mental stress (mental arithmetic) in young healthy participants [166]. These studies indicate that melatonin can attenuate reflex changes in sympathetic outflow to both skin and muscle in response to physiological and psychological perturbations. This suggests that melatonin can have a significant effect on autonomic regulation in humans, especially in reducing sympathetic system responses.

An interaction between the RAAS and the pineal gland has been reported. Indeed, an angiotensin-melatonin axis has been identified [167]. This axis is separate from the endocrine RAAS system and is a local RAAS identified in the brain of rats. Angiotensinogen, the precursor of the RAAS, has been identified in pineal glial cells and angiotensin type 1B receptors have been found to be localized in pinealocytes [168]. Baltatu et al., [169] demonstrated that locally produced Ang II in cultured pineal glands from adult Wistar rats acts on pinealocyte AT1 receptors and modulates melatonin synthesis via TPH, which is the rate-limiting enzyme in melatonin synthesis (**Figure 1.14**) [167]. In *in vivo* studies using Wistar rats and *in vitro* studies (pineal gland culture), the use of an Ang-II antagonist inhibited the synthesis of melatonin from the pineal gland as well as caused decreases in the enzymatic pathway involving melatonin (5-hydroxytryptophan, serotonin, 5-hydroxyindole acetic acid and N-acetylserotonin) [169]. Increased melatonin synthesis by Ang II may be a self-defense mechanism against the deleterious effects of Ang II, including hypertension and pathologic remodeling [115]. These studies together indicate that Ang II increases pineal melatonin synthesis.

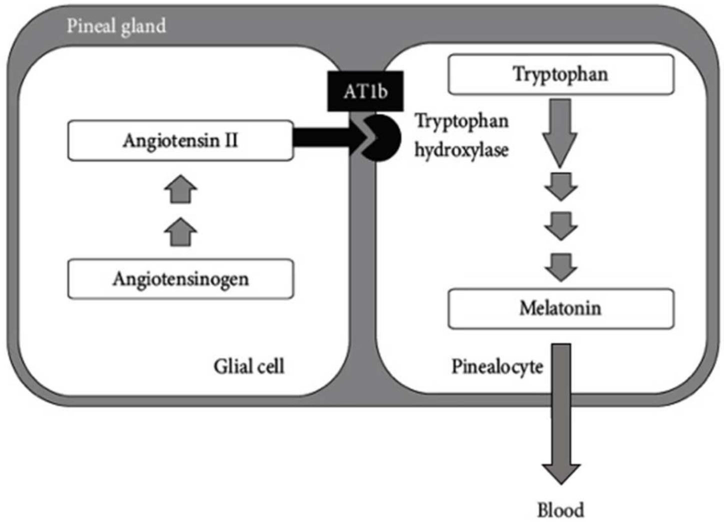

Figure 1.14 Pineal RAAS interacts with melatonin synthesis. Adapted from Source: Campos et al. [167]

1.5.4 Melatonin and Endothelial Function

Melatonin has been shown to improve underlying mechanisms that lead to the optimization of endothelial function. At a cellular level, Pogan et al., [170] observed that melatonin improved internal Ca^{2+} mobilization and agonist-evoked Ca^{2+} entry in aortic SHR cells. Pogan also reported that melatonin reversed the inhibitory effect of free radicals on internal Ca^{2+} release in aortic cells from Sprague Dawley rats (SDR) and bovine aortic endothelial cells. This is important since free radicals are known to disturb the Ca^{2+} signaling process in endothelial cells, which is crucial for enhancement of eNOS activity and thus, increased NO production and subsequent vasodilation [18,124,170]. Melatonin treatment was given to SHR and Wistar rats for 4 weeks and reductions in MAP, systolic and diastolic BP, and HR were observed in the SHR. Melatonin improved maximal relaxation of mesenteric beds to a calcium ionophore and enhanced vasodilation induced by ACh and calcium ionophore of aortic rings from SHR [171]. Shao et al., [172] investigated the effects of melatonin incubation in human umbilical vein endothelial cells that were exposed to pressure to simulate hypertension. They observed decreased levels in endothelin and angiotensin levels, and elevations in eNOS and NO levels in the supernatant. Genetic analysis revealed that the adenylate cyclase-modulating G-protein-coupled receptor signaling pathway was upregulated and adenylate cyclase inhibiting G-protein-coupled receptor signaling pathway was downregulated, demonstrating an increase in cAMP leading to NO synthesis. Aortic rings from male New Zealand rabbits incubated with melatonin potentiated the endothelium-dependent vasodilation induced by ACh and inhibited the vasoconstrictor effects to NEpi and phenylephrine. This corresponded with decreases in lipid peroxides and increases in GSH, SOD, NO and cGMP [173]. In aortic rings from aged Wistar male rats and in diabetic rats, melatonin also restored the vasorelaxation response to ACh and reduced the contractility response to phenylephrine [174,175]. Improvements in the endothelium-dependent vasodilation responses to ACh and restorations in impaired SNP-induced vasorelaxation have also been demonstrated by melatonin in aortic rings preincubated with a high glucose solution from pancreatectomized rats [176,177]. Similarly, rabbits that were fed a high-fat diet and given melatonin for 12 weeks experienced improvements in endothelial vasorelaxation to ACh in the abdominal aorta. Attenuations in morphological arterial lesions characteristic of atherosclerosis and vascular remodeling were also seen in this study [178].

Oxidative stress likely plays a role in the effect of melatonin on endothelial function. In a high fat-induced diabetes rat model, the administration of melatonin for 2 weeks reduced oxidative stress (plasma MDA), increased serum NO levels and improved endothelium-dependent relaxation induced by ACh in the abdominal aorta [179]. Melatonin supplementation protected both rats and humans from the negative effects induced by smoke. Melatonin reduced vascular injury in these smokers via reductions in endothelial adhesion markers, vasoconstrictors and oxidative stress, and increases in eNOS, leading to increased NO bioavailability [180]. In male adult Wistar rats with induced hypertension via L-NAME (NO synthase inhibitor), melatonin supplementation for 4 weeks ameliorated oxidative load, arterial remodeling and endothelium-derived-constricting factor (EDCF)-signaling [181]. Similarly, in SHR that were simultaneously given L-NAME and melatonin for 6 weeks, melatonin prevented the increase in BP, decreased renal ADMA (NOS inhibitor) concentrations and the concentration of oxidative damaged DNA products, and restored NO production compared to those SHR given L-NAME alone [182].

In conclusion, as shown in *in vitro* studies and preclinical models, melatonin can improve NO production and signaling and decrease oxidative load leading to an improvement of endothelial function [125]. Another pathway by which melatonin may influence vasodilation and hence endothelial function is direct Ca^{2+} channel blocking as Satake et al., [183] showed in aortic rings from Wistar rats [183]. Satake et al. demonstrated that melatonin induced vasodilation was in part endothelium-dependent and due to an increased production in cGMP. However, human studies have not evaluated melatonin as a potential treatment strategy to improve endothelial function, even less so in the context of a high sodium diet.

1.5.5 Melatonin's role as an antioxidant

Melatonin attenuates radical formation *in vitro*, which is known as radical avoidance. Melatonin not only scavenges ROS, reactive nitrogen species (RNS) and organic radicals, but also upregulates antioxidant enzymes and downregulates pro-oxidant enzymes (Table 1). Melatonin's scavenging effects are not related to its interaction with melatonin receptors while its effects of antioxidant and prooxidant enzymes are mediated by receptors [106,115,122,123].

Table 1.1 Effects of melatonin on ROS and RNS, and on antioxidant and pro-oxidant enzymes.

ROS/RNS/enzyme	Effect of melatonin
ROS/RNS	
Hydrogen peroxide	↓
Hydroxyl radical	↓
Singlet oxygen	↓
Nitric oxide	↓
Peroxynitrite anion	↓
Antioxidative enzyme	
Superoxide dismutase	↑
Catalase	↑
Glutathione peroxidase	↑
Glutathione reductase	↑
Glucose-6-phosphate dehydrogenase	↑
Gamma-glutamylcysteine synthase	↑
Pro-oxidative enzymes	
Nitric oxide synthase	↓
Lipo-oxygenase	↓

RNS: reactive nitrogen species; ROS: reactive oxygen species. Source: Adapted from Tengattini et al.[122]

Melatonin has several advantages over other antioxidants. It is both lipid and aqueous soluble, and therefore able to cross cell membranes and the blood-brain barrier with ease [106,115,122,123]. Thus, melatonin can protect proteins, lipids and DNA against oxidative stress [184]. Another advantage is the so-called melatonin antioxidant cascade or free radical scavenging cascade in which melatonin's secondary and tertiary metabolites can also neutralize ROS and RNS by donating their electrons and sometimes even more effectively than melatonin itself [184]. This not only amplifies the positive effect of a single melatonin molecule but also allows for a bigger scavenging spectrum. While a classic antioxidant can scavenge up to one ROS, one molecule of melatonin can scavenge up to 10 ROS [106,110,185]. Melatonin is known to be a terminal or suicidal antioxidant, which means that it cannot participate in redox cycling (i.e., a molecule that undergoes the processes of reduction and oxidation). Classical antioxidants (e.g., vitamin C and E) donate electrons and they exhibit both reduced and oxidized forms and this pathway often occurs at the expense of GSH. Oxidized forms of classical antioxidants can also oxidize other molecules and therefore they are prooxidants [186]. In the case of melatonin, once it has been oxidized, it cannot be reduced because it forms stable end-products when it reacts with free radicals; this leads to the termination of the radical reaction chain and avoids the formation of prooxidants [185–188]. Melatonin's total antioxidant capacity has been considered in some instances higher than other well-known classic antioxidants, such as vitamin E and C, β-carotene and garlic oil in *in vivo* and *in vitro* settings [189–195].

In animal studies, melatonin has been shown to ameliorate oxidative stress. Melatonin administration for 6 weeks reduced intracellular levels of O_2^- in tubular epithelial cells and mononuclear and renal MDA in SHRs [144]. In pulmonary hypertensive newborn sheep that were given melatonin for 8 days, an improvement in the vasodilatory capacity of the small pulmonary arteries via endothelium-dependent and endothelium-independent pathways was reported. This was associated with enhanced NO-dependent and NO-independent vasodilator components and increases in NO bioavailability. Melatonin further reduced pulmonary oxidative stress (nitrotyrosine, 4-HNE and 8-isoprostanes) and increased antioxidant capacity (SOD-2 transcript and protein expression, CAT transcript and activity, and GPx1 expression)[196] . In the plasma of these pulmonary hypertensive newborn sheep, melatonin reduced 8-isoprostanes levels, a product of lipid peroxidation, and increased total antioxidant capacity [196]. To date, there is only one study that has studied melatonin under the context of high dietary sodium. In a high-salt induced hypertension model in rats, 8 weeks of melatonin supplementation prevented kidney injury as shown by reductions in glomerular damage and in oxidative stress parameters including O_2^- and mRNA $p67^{phox}$ expression levels. $p67^{phox}$ is a one of the cytosolic subunits and regulator of the NADPH oxidase complex, a major source for ROS [197].

The positive outcomes of melatonin counteracting oxidative stress in humans have also been widely reported. In hypertensive older adults under diuretic monotherapy, the administration of 5 mg of oral melatonin for 30 days 1 h before bedtime increased erythrocytes' SOD-1 and CAT activities and reduced MDA levels

[198]. BP was not reported in that study. In elderly type II diabetic patients, 5 mg of oral melatonin supplementation for 30 days decreased erythrocytic MDA levels and increased SOD-1 activity [199]. The effect of 5 mg/day of melatonin supplementation for one month on the erythrocytes of healthy young and old adults was examined. MDA levels were decreased and SOD1 levels and glutathione reductase were increased in both groups after one month [200]. Two months of 5 mg melatonin supplementation in patients with metabolic syndrome increased erythrocytes' CAT activity and decreased thiobarbituric acid reactive substances, along with a decrease in BP compared to baseline values [201]. Melatonin supplementation for a month lowered levels of oxidative stress expressed as increases in erythrocyte GPx levels and decreases in MDA in obese adults that were on a calorie-restricted diet [202]. Ten mg of oral melatonin daily for 12 weeks resulted in increases in plasma GSH and NO and decreases in MDA, protein carbonyl and serum high sensitivity C-reactive protein levels in type II diabetic patients with coronary heart disease [203]. In healthy adult males, the administration of a total of 15 mg melatonin in the days prior to a bout of strenuous exercise, increased CAT and GPx erythrocyte activities along with total antioxidant status and reduced urinary isoprostanes and 8-hydroxy-2'-deoxyguanosine (8-OHdG) compared to those athletes that did not take melatonin. 8-OHdG is released during DNA repair from oxidative damage and is a known oxidative stress biomarker [204]. These studies altogether showcase the effect of melatonin on oxidative stress by reducing prooxidative and increasing antioxidant molecules. Whether melatonin supplementation can restore oxidative stress levels under a high sodium diet has not been studied.

1.5.6 Melatonin safety studies

Melatonin is considered safe. Galley et al.,[205] evaluated adverse effects in healthy volunteers consuming doses ranging from 20 up to 100 mg of melatonin a day. Only mild transient drowsiness and sleepiness after taking melatonin were reported and these symptoms resolved within 6 hours. Participants were followed up to a week and no participant reported nausea, headache, vomiting, diarrhea or abdominal pain at any time. Another study evaluated the safety of 1 g of melatonin a day for a month in humans with hyperpigmented skin and examination of endocrine, hepatic, hematopoietic and renal markers throughout the study exhibited no abnormalities [206]. 60 mg/day of oral melatonin for up to 13 months and 300 mg/day of rectal melatonin for up to 2 years have been given to amyotrophic lateral sclerosis patients and no side effects were reported [207,208]. Smaller doses of melatonin have been used in both healthy volunteers for 3 days and a month, and in clinical populations, such as metabolic syndrome for 2 months and diabetics for 3 months, with no side effects [202,203,209,210]. The only consideration when taking melatonin is that it should be administered at night before bedtime to simulate the endogenous night-time melatonin release, otherwise it could disrupt the circadian rhythm and cause symptoms such as fatigue.

1.6 Concluding remarks

High dietary sodium is known to cause negative effects beyond increases in BP. These effects include impairments of vascular function, increases in oxidative stress and BP reactivity to different physiological perturbations. Melatonin is a potent antioxidant, and it has improved mechanisms leading to proper vascular function in vitro. Melatonin also modulates the ANS via inhibition in sympathetic responses and activation of the parasympathetic system. Thus, melatonin is a promising strategy to combat the deleterious effects of high dietary sodium. High dietary sodium is a modifiable risk factor for CVD, which remains the leading cause of mortality in the country.

Chapter 2

THE EFFECTS OF MELATONIN ON BLOOD VESSEL FUNCTION DURING HIGH DIETARY SODIUM

2.1 Introduction

Americans average sodium consumption is ~3500 mg/day while the Dietary Reference Intakes recommend ≤ 2300 mg/day [2]. Consuming a high sodium diet (HSD) has been historically related to increases in blood pressure (BP) and hypertension (HTN) development, and multiple organs, such as the vasculature, are negatively affected as well [9,211]. Animal studies have showed that HSD cause vascular dysfunction [72–74,76]. Human studies have corroborated these findings. Specifically, our group has demonstrated that a HSD induces vascular dysfunction at both the macrovascular and microvascular level independently of changes in BP [79,81].

One mechanism responsible for this HSD-induced dysfunction is oxidative stress. In particular, superoxide (O_2^-) is thought to be the central reactive oxygen species (ROS) involved [72–74,76]. Both Greaney et al., [81] and Ramick et al., [82] reported that normotensive adults that underwent a HSD recovered microvascular function with ascorbic acid (a non-specific ROS scavenger), and tempol (O_2^- scavenger), respectively. Furthermore, oxidative stress has been linked to vascular disease directly as it decreases nitric oxide (NO) production or bioavailability [34]. NO has a major vasodilatory role and protects against atherosclerosis in different ways [212]. NO inhibits leukocyte and platelet aggregation along with adhesion to the vessel wall, and protects smooth muscle cells from exposure to platelet-derived growth factors [212]. A HSD reduces NO bioavailability through different pathways. HSD causes decreases in antioxidant enzymes (superoxide dismutase), increases in O_2^- and in endothelial cell stiffness, which reduce endothelial nitric oxide synthase (eNOs), the enzyme responsible for NO production [10].

The investigation of antioxidant supplementation is a reasonable strategy to combat increases in ROS and vascular dysfunction under a HSD, as attempts to decrease sodium consumption in the population have failed [11]. Antioxidants may diminish the risk for atherosclerosis and HTN development, and subsequent cardiovascular disease (CVD), which remains the leading accuse of mortality nationwide [213]. One antioxidant is melatonin, which is a hormone synthesized in the mammalian pineal gland mostly at night [122]. Numerous studies show that long term (>1 month) melatonin supplementation counteracts increased oxidative stress in clinical populations, such as those with hypertension, diabetes or metabolic syndrome [198–201,203]. These antioxidant benefits are accompanied by improvements in endothelial function [170,172,179,197,214]. *In vitro*, melatonin has been shown to increase NO levels and expression levels of eNOS activity in human umbilical vein endothelial cells cultured under pressure to simulate HTN [172]. *In vivo*, melatonin has mitigated adhesion molecules levels, increased eNOS protein expression and prevented endothelial dysfunction in Sprague-Dawley rats under chronic hypoxia [214]. Interestingly, a link exists between CVD and melatonin as evidenced by reductions in nocturnal synthesis, circulating levels of melatonin, and its urinary metabolite (6-sultatoxymelatonin) in various CVD populations, for example heart failure or coronary heart disease [132–134].

Furthermore, the incidence for cardiac events is higher in the early morning when circulating melatonin levels are significantly lower [138–140]. Melatonin supplementation also has positive effects on BP [146–149,215]. Three-four weeks of melatonin supplementation has improved nocturnal BP and BP dipping in hypertensive but also normotensive adults [146–149,215]. To date, there is limited evidence on the effects of melatonin in the human vasculature, and no work in adults consuming a HSD. It is also unknown whether melatonin administration can reduce oxidative stress levels in humans consuming a HSD. The effects of melatonin supplementation under a HSD are unknown.

The primary aim of this study was to determine the effects of melatonin supplementation on macrovascular and microvascular function after 10 days of a HSD. Our second aim was to determine the effects of melatonin on ROS levels after 10 days of a HSD. We hypothesized that melatonin supplementation would increase vascular function as well as decrease ROS on HSD compared to a HSD alone. As an exploratory aim, we investigated the effects of melatonin supplementation on BP and nocturnal dipping after 10 days of a HSD. We hypothesized that melatonin supplementation would decrease BP and increase nocturnal BP dipping on a HSD compared to a HSD alone.

2.2 Methods

2.2.1 Study design

This intervention was a randomized double-blind placebo-controlled crossover design. The study consisted of two 10-day interventions of high sodium combined with either melatonin or placebo. The study protocol and procedures were approved by the Institutional Review Board of the University of Delaware (ID# 1534734) and conform to the provisions of the Declaration of Helsinki. Participants were recruited from the University of Delaware campus and the surrounding community through online advertisements. Informed consent was obtained prior to enrollment in the study. The study was registered on clinicaltrials.gov (NCT04325191).

2.2.2 Participant screening

Healthy normotensive men and women between the ages of 18-45 were recruited to participate in the study. After participants signed the informed consent, they completed a medical history questionnaire, a menstrual cycle form (women only), and a global physical activity questionnaire (GPAQ)[216] . Their height (Holtain Harpenden Stadiometer), weight (Seca 876), waist and hip circumferences, and seated BP were measured. Inclusion criteria included a systolic BP <130mmHg, diastolic BP <80mmHg and body mass index (BMI) between 18.5-29.9 kg/m^2. Exclusion criteria included the following: history of cardiovascular disease and hypertension; liver or kidney disease; malignant cancer; mood disorders (including depression, anxiety, seasonal affective disorder, manias) and/or taking any medications for those conditions; sleep disorder and/or night shift work; pregnancy or breastfeeding; use of tobacco products; ADHD medications use; antibiotics, melatonin or other antioxidant use in the past 3 months; significant changes in weight (≥10 lbs. gain or loss) in the last 6 months; highly trained endurance athletes; abnormal creatinine value (<0.76 and >1.27 mg/dL for men, <0.57 and >1.00 mg/dL for women) and/or a glomerular filtration rate (GFR) (<90 mL/min/1.73); and consumption of less than 2000 mg of sodium per day in their habitual diets. All participants were vaccinated against COVID-19 at the time of enrollment in the study.

2.2.2.1 Blood analysis and habitual diet and sleep measurements

A venous blood sample was collected and sent to LabCorp to examine complete blood count, metabolic panel, and lipid profile. Participants were then allocated to an intervention (A or B) using the randomize function in Excel, which was created by another member of the research team. They were also given a 3-day diet record, which was used to analyze their habitual dietary intake. Participants were asked to record their intake over two weekdays and one weekend day. During the days in which they recorded the diet, they wore a wrist accelerometer (Motionlogger Micro watch, Ambulatory Monitory Inc, Tokyo, Japan) to quantify habitual sleep parameters.

2.2.3 Familiarization visit

Participant brought the wrist accelerometer and the 3-day diet record to the familiarization visit, which served to familiarize the participant with measurements performed during the experimental visit on Day 10. The diet record was reviewed by a trained researcher with the participant and diet records were analyzed using Nutrition Data System for Research software (NDSR, 2020, University of Minnesota, MN).

2.2.3.1 Macrovascular function

Brachial artery flow-mediated dilation (FMD) was performed according to established guidelines [217,218]. This measure was an assessment of macrovascular function. After the participant had rested for 20 minutes in a supine position, longitudinal images of the brachial artery and Doppler blood velocities were recorded proximal to the antecubital fossa using a 10 MHz linear phased array ultrasound transducer (Logic e, GE, Boston, MA). After recording baseline images for 1 min, a BP cuff that had been placed just below the antecubital space was rapidly inflated to 200 mmHg for 5 mins (AG101 rapid cuff inflator, Hokanson, Bellevue, WA). After releasing the cuff, there is a vigorous increase in blood flow (reactive hyperemia) which increases shear stress on the vessel wall. Images and blood velocity were recorded for 2 extra minutes following cuff release. Baseline diameter was determined as the average diameter recorded over the baseline. Peak diameter was determined as the largest 3 second rolling average diameter achieved during reactive hyperemia. Images were analyzed across complete heart cycles using automated edge-detection software (Cardiovascular Suite, Quipu, Italy). Absolute FMD was determined as the difference between peak and baseline diameter. Relative FMD was determined as the absolute FMD expressed as a percentage of the baseline diameter. Shear rate area under the curve (AUC) to peak was calculated from cuff release up to peak reactive hyperemia diameter. The distance from the distal portion of the cuff to the base of thumb and from the proximal side of the cuff to the distal side of the ultrasound probe were measured to ensure the cuff placement was kept the same across data collections. Ultrasound images were recorded at the same depth. Anatomical landmarks were used to place the ultrasound in the same location ensuring inter-visit reproducibility.

2.2.3.2 Microvascular function

Near infrared spectroscopy (NIRS) and vascular occlusion test (VOT) was performed as a microvascular function test. The NIRS device (PortaMon, Artinis, Elst, The Netherlands) is a wireless, non-invasive, dual-wavelength continuous wave system that uses the modified Beer-Lambert law and spatially resolved spectroscopy (SRS) to calculate relative concentrations of oxygenated and deoxygenated hemoglobin and myoglobin and total hemoglobin and myoglobin in the microcirculation and in the muscle cytoplasm [219]. When combined with VOT, which consists of arterial occlusion and reoxygenation, the PortaMon provides information on how well muscle can obtain oxygen from the capillaries and how well the microvasculature can respond to the occlusion stimulus (i.e., microvascular reactivity and function)[220] . The NIRS protocol followed established guidelines [30]. The PortaMon was placed on the proximal/medial portion of the forearm and secured with tape and a black neoprene sleeve to prevent movement and external light interference. After recording baseline values for 3 minutes, a BP cuff placed just above the elbow rapidly inflated to 200 mmHg for 5 mins (AG101 rapid cuff inflator, Hokanson, Bellevue, WA). Images were recorded for 5 minutes following cuff release. The PortaMon quantified the tissue saturation index (TSI), which is the ratio of oxygenated hemoglobin and myoglobin to total hemoglobin and myoglobin expressed as a percentage. TSI is considered an absolute parameter for muscle oxygenation and reflects the balance between O_2 supply and demand [30,219]. During cuff inflation, TSI was reduced, which is directly related to O_2 muscle consumption since oxygen-rich blood was not flowing to the forearm and can be considered an indirect measure of skeletal muscle resting metabolic rate [221]. This is known as slope 1 or the desaturation rate/slope. During the cuff deflation, reactive hyperemia ensued causing TSI to increase and then return to baseline. This is known as slope 2 or resaturation rate/slope. An abnormal (slower and flatter) slope 2 represents impaired microvascular reactivity and endothelial dysfunction at the microcirculatory level, is correlated with FMD % and offers more clinical utility than TSI alone [33,222]. The Portamon was connected to the laboratory computer via Bluetooth for data acquisition (10 Hz) via Oxysoft software (Artinis). Results were analyzed offline. Baseline TSI was calculated as the average TSI 1 minute before the onset of arterial cuff occlusion. Microvascular reactivity was determined as TSI reperfusion slope (slope 2), quantified with linear regression as the average upslope from cuff deflation until the participant reached their respective baseline value. Microvascular function was determined as the TSI AUC from the baseline value achieved during reactive hyperemia until end of the test. Microvascular function is also reported as half time to peak hyperemia ($T_{1/2}$) following occlusion. The lowest TSI (TSImin) obtained during cuff inflation was taken as a measure of the extent of the ischemic insult. Maximal TSI (TSImax) was calculated as the highest TSI value reached following cuff deflation. The TSI amplitude was calculated as the difference between the maximum TSI value reached after cuff deflation and the minimum TSI value reached during cuff occlusion. Hyperemic reserve was calculated as the difference between baseline and peak TSI values presented as a percentage of the baseline. The distances between the Portamon and the base of the thumb and between the Portamon and the distal portion of the BP cuff were measured to guarantee that the Portamon placement was kept consistent across data collections.

2.2.4 Dietary interventions, sodium, and melatonin supplementation

The study consisted of two 10-day interventions separated by a washout period of at least 14 days. During the washout period, the participants were instructed to resume their usual life routines. During both 10-day periods, participants consumed salt pills and they either supplemented with 10 mg of melatonin (HSD+MEL) or lactose placebo (HSD+PL) daily. Both the melatonin and lactose pills were manufactured by the SaveWay compounding pharmacy (Newark, DE) and were matched in weight and appearance. Researchers and participants were blinded to the content of the pills. Participants took the melatonin/placebo 30 minutes before they planned to go to sleep, including the night prior to the experimental visit. The proposed dosage of melatonin has been used with no reported side effects in both clinical and healthy populations [202,203,209]. The total of 6900 mg/day of sodium in both 10-day interventions was achieved by taking 12 enteric coated, slow-release salt pills (Purecaps USA- Empty White Vegetarian and Vegan Delayed Release/ Acid-Resistant, Size 00), each containing ~380 mg, and by consuming 2300 mg of sodium in the diet. 6900 mg/day of sodium is consistent with our prior studies and shown to impair vascular function in normotensive humans [79,81]. Participants were asked to space out the sodium pills and take them with meals to avoid possible gastrointestinal issues. For the first three days of the intervention, participants gradually increased the number of pills they took and for days 4-9, they were instructed to consume 12 pills daily. Research staff provided participants with instructions and strategies to consume a 2300 mg sodium diet during both interventions. Participants were provided with a paper printout of two-dimensional food models adapted from Van Horn et al, [223,224] to help with estimating portion sizes. Participants recorded their dietary intake during the last three days of the intervention (days 7, 8 and 9) and were instructed to replicate their diet as much as possible during both trials. Participants were given a melatonin/placebo pill form, a sodium pill form, and a side effect form for each 10-day intervention to ensure compliance and note potential side effects. They were also given a 3-day diet record, which they completed during days 7, 8 and 9 of the 10-day intervention. The diet record was reviewed by a trained researcher with the participant and diet records were analyzed using Nutrition Data System for Research software (NDSR, 2020, University of Minnesota, Minneapolis). This 3-day diet record was used to assess diet reproducibility and compliance along with 24 h urine collection starting on day 9. A schematic representation of the study design is presented in **Figure 2.1**.

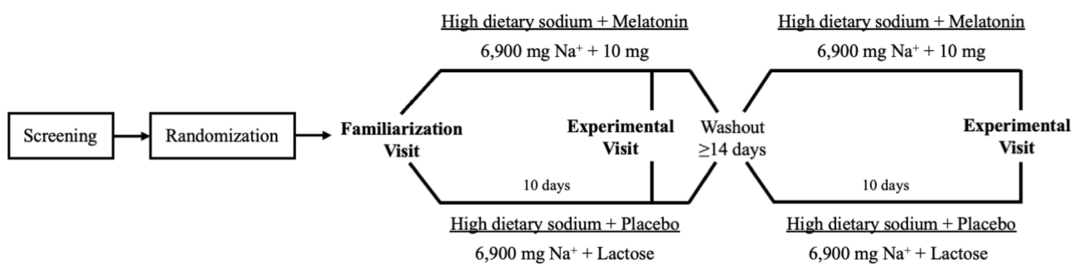

Figure 2.1 Schematic representation of the intervention

2.2.5 Ambulatory BP and urine collection

Participants wore a 24-h ambulatory BP monitor (Oscar 2, SunTech Medical, Morrisville, NC) on their upper arm on day 8 [225]. The BP cuff was programmed to inflate every 20 minutes during the day and every 30 minutes during the night. BP readings were downloaded using AccuWin Pro 4. At least 75% of the readings needed to be successful for the measurement to be valid [226]. The 24-hour urine collection started on day 9. Urine was assessed for urine volume, urine specific gravity (Goldberg Brix Refractometer, Reichert Technologies, Depew, NY), electrolyte concentrations (EasyElectrolyte Analyzer, Medica, Bedford, MA), and osmolality (Advanced 3D3 Osmometer, Advanced Instruments, ON, Canada) from a mixed aliquot from the 24-hour collection container. Urine flow rate was calculated and used to determine 24-hour sodium excretion. For urine collections to be considered valid, they had to be collected within 20-28 h, there could not be ≥2 missed collections, and the urine volume could not be <500 mL [227–229]. The first morning void was collected separate from the 24 h urine collection in the morning of day 10 to assess melatonin compliance by measuring urinary 6-sulfatoxymelatonin (ELISA kit, Alpco, Salem, NH). A 4 Parameter Logistics (4PL) curve fit was used to obtain the calibrator curve with SoftMax Pro Software (Molecular Devices).

2.2.6 Physical activity monitoring and scoring

Participants wore an accelerometer (ActiGraph wGT3X-BT, Pensacola, FL) on the hip for the entire duration of both interventions except to sleep and activities involving water (e.g., swimming, showers). Participants recorded the times when the accelerometer was worn, and not worn, to improve compliance and data quality. Data was accepted if there were at least 4 days with 10 hours of wear time [230]. Participants returned the device at the experimental visit on day 10. Accelerometer data was analyzed using ActiLife software. Wear time was validated using the Troiano algorithm [231] and activity variables were calculated using the Freedson Combination 1998 algorithm [232].

2.2.7 Sleep monitoring and scoring

Participants wore wrist accelerometers (Motionlogger Micro watch, Ambulatory Monitory Inc, Tokyo, Japan) on the non-dominant wrist for 9 days and 9 nights, except for activities involving water (e.g., swimming, showers). Data were collected in the zero-crossing mode (ZCM) and saved in 1-minute epochs. The University of California San Diego (UCSD) algorithm, which is validated to produce accurate and reliable sleep estimates relative to polysomnography [233], along with Action W-2 software (Ambulatory Monitoring, Inc., Ardsley, NY) were used to score the data. To improve data quality, participants completed a sleep diary before bed and upon waking every day during the sleep monitoring period. Data was accepted if the accelerometer was worn for at least 7 days out of the 9 days [234].

Variables of interest were sleep duration, sleep variability and sleep efficiency. Sleep duration was defined as the total time spent asleep from sleep onset to wake onset. We present the mean (quantified for each night that the accelerometer was worn) of the sleep duration. Sleep variability was quantified as the standard deviation (SD) of sleep duration across the 9-day sleep monitoring period. Sleep efficiency was calculated as the total sleep time expressed as a percentage of the total time spent in bed.

2.2.8 Experimental visit

Participants reported to the laboratory on day 10 of each intervention following a 9 h fast, no caffeine or alcohol for at least 12 hours and not exercising for 24 hours. Upon arrival to the laboratory, weight was measured (Seca 876). After 5 min of rest, seated BP was assessed in triplicate and averaged (dominant arm; Dash 2000, GE medical systems, Chicago, IL). A venous blood sample was collected to assess hematocrit (Sure prep™ capillary tubes, Clay Adams spun in a microcentrifuge at 1,950g for 5 min, Legend Micro 17, Thermo Sorvall) hemoglobin (Hb 201+, HemoCue, Angelholm, Sweden), serum electrolytes (EasyElectrolyte Analyzer, Medica, Bedford, MA), plasma osmolality (Advanced 3D3 Osmometer, Advanced Instruments, ON, Canada) and lipid profile (LabCorp, Burlington, NC). Experimental visits were scheduled at the same time for both interventions and early in the morning (ranging from 7AM – 10AM). The temperature of the room was kept between 20°-24° C.

2.2.8.1 Oxidative stress measurement

Electron paramagnetic resonance (EPR) was used to measure O_2^- in whole blood samples using the EMXnano (Bruker) [235]. Blood was processed immediately after it was drawn. Whole blood samples were incubated in a buffer solution for 10 min at 37 °C. This buffer solution contained phosphate buffer saline (PBS) (Corning, NY) with diethylenetriaminepentaacetic acid (DTPA) (100 μM) and 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) (0.2 mM). DTPA is a chelating agent that prevents fenton chemistry. O_2^- is a highly reactive radical with a very short half-life, which makes its detection challenging. CMH is a spin probe that reacts with O_2^- to form nitroxide CM (free radical) that has a half-life of several hours and is easily detected by EPR. The samples were placed in pre-prepared polytetrafluoroethylene (PTFE) tubes, flash-frozen in liquid nitrogen and immediately stored at -80°C. All samples were measured in triplicate and averaged. The EPR acquisition parameters were similar to those reported in the literature [236] and include:

microwave frequency = 9.65 GHz; center field = 3436 G; sweep width = 150 G; sweep time = 20 s; microwave power = 0.316 mW; receiver gain: 40 dB; modulation amplitude = 3.0 G; number of scans = 4; microwave attenuation= 35 dB; and time constant = 10.24 ms. All samples were measured in triplicate and averaged. O_2^- concentration was quantified as nitroxide molarity (mol/L) and number of spins, which represents the number of nitroxides, or number of free radicals.

2.2.8.2 Macrovascular function

Brachial artery flow-mediated dilation (FMD) was performed as previously described. See section 2.2.3.1.

2.2.8.3 Microvascular function

Near infrared spectroscopy (NIRS) and vascular occlusion test (VOT) was performed as previously described. See section 2.2.3.2.

2.2.9 Statistical analysis

Our primary outcome was brachial artery FMD at the end of the melatonin and placebo interventions. Secondary outcomes included TSI reperfusion slope and TSI AUC during the NIRS technique. An a priori power analysis with an alpha of 0.05, 95% power, effect size 0.83 and assuming a difference in FMD of at least 1% at the end of the two arms, calculated an initial sample size of 21 participants using a paired samples t-test (G* Power). This difference in FMD is based off our results using dietary potassium to attenuate the effect of sodium [237]. An improvement of 1% in

FMD is clinically significant since a recent meta-analysis associates this change with a decrease of 8–10% in overall CVD risk over 4 years [21]. JMP Pro 16.0.0 (SAS, Cary, NC) was used to carry out the analysis. Data were assessed for normality, linearity, homoscedasticity, and multicollinearity. Data are expressed as mean ± standard deviation (SD).

2.3 Results

Twenty-four participants took part in this study. Participant screening characteristics are presented in **Table 2.1**. We had equal distribution of men and women. Participants were young, normotensive with a normal BMI. Participants were mostly Caucasian and Asian. Participants average total physical activity was 5.4 ± 3.0 hours per week, which is equivalent to 29.0 ± 17.7 METs hours per week. They spent 8.6 ± 3.5 hours per day sitting or reclining.

Screening blood chemistry is presented in **Table 2.2**. Additional blood work is presented in **Table A.1** (Appendix A). Fasting blood work was within normal limits. Habitual dietary intake is presented in **Table 2.3**. We calculated levels of ROS in 16 participants. Molarity was a mean of $7.4 \times 10^{-5} \pm 4.5 \times 10^{-5}$ mmol/L and spins were $8.2 \times 10^{15} \pm 5.5 \times 10^{15}$. Habitual sleep parameters were collected in 8 participants. Sleep duration was a mean of 458.8 ± 33.6 min, sleep duration SD was 64.2 ± 12.9 min and sleep efficiency was 94.8 ± 2.9 %. **Table 2.4** shows vascular function results assessed by brachial artery FMD and NIRS-VOT during their habitual diet in the familiarization visit.

Table 2.1 Screening characteristics

Demographic data	
N, men/women	13/11
Race, Asian/Black/Caucasian	11/1/12
Anthropometrics	
Age, yr	27.0 ± 2.8
Weight, kg	69.5 ± 10.9
Height, cm	170.4 ± 9.9
BMI, kg/cm^2	23.8 ± 1.9
Hemodynamics	
Systolic BP, mm Hg	111 ± 8
Diastolic BP, mm Hg	67 ± 7
MAP, mm Hg	82 ± 7
Heart rate, bpm	67 ± 11

Data are expressed as means ± SD. BMI: body mass index; BP: blood pressure; MAP: mean arterial pressure.

Table 2.2 Screening blood chemistry

Complete blood count	
WBC, $x10^3$/uL	5.5 ± 1.5
RBC, $x10^6$/uL	4.8 ± 0.5
Hemoglobin, g/dL	14.2 ± 1.3
Hematocrit, %	42.4 ± 4.2
Metabolic panel	
Glucose, mg/dL [b]	73.7 ± 16.6
BUN, mg/dL	12.3 ± 3.8
Creatinine, mg/dL	0.8 ± 0.2
eGFR if Non-African American, mL/min/1.73 [b]	109.7 ± 13.8
eGFR if African American, mL/min/1.73 [c]	128 ± 0
BUN/Creatinine	14.6 ± 3.8
Sodium, mmol/L	140.8 ± 1.8
Potassium, mmol/L [b]	4.3 ± 0.4
Chloride, mmol/L	102.1 ± 2.3
Lipid profile	
Total Cholesterol, mg/dL	186.6 ± 29.7
Triglycerides, mg/dL	96.7 ± 41.2
HDL Cholesterol, mg/dL	59.4 ± 14.8
VLDL Cholesterol, mg/dL	17.7 ±7.0
LDL Cholesterol, mg/dL	109.4 ± 29.0
Non-HDL Cholesterol, mg/dL	127.1 ± 32.2

Data are expressed as means ± SD. BUN: blood urea nitrogen; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; LDL: low-density lipoprotein; RBC: red blood cell; VLDL: very-low-density lipoprotein; WBC: white blood cells. [a]n=22; [b]n=21; [c]n=1.

Table 2.3 3-day average of habitual dietary intake

Energy intake, kcal/day	2019.7 ± 539.5
Total carbohydrate, g/day	248.9 ± 77.3
Total protein, g/day	100.3 ± 33.7
Total fat, g/day	76.9 ± 18.2
Added sugar, g/day	27.8 ± 17.1
Total fiber, g/day	23.5 ± 11.5
Saturated fat, g/day	23.5 ± 5.9
Sodium, mg/day	3802.4 ± 1511.9
Potassium, mg/day	2834.6 ± 1023.8
Calcium, mg/day	927.0 ± 276.4
Magnesium, mg/day	353.3 ± 125.8
Vit A (RAE), mcg/day	648.7 ± 433.8
Vit C, mg/day	78.3 ± 64.8
Iron, mg/day	16.4 ± 6.2
Zinc, mg/day	11.4 ± 3.4
Tryptophan, g/day	1.2 ± 0.4
Alcohol, g/day	7.3 ± 21.5

Data are expressed as means ± SD. n=24.

Table 2.4 Habitual vascular function

Brachial artery FMD [a]	
Baseline brachial artery, mm	3.6 ± 0.7
Peak brachial artery, mm	3.9 ± 0.6
Absolute FMD, mm	0.3 ± 0.1
Relative FMD, %	8.2 ± 5.4
Time to peak diameter, s	55.2 ± 10.5
Shear rate AUC to peak, a.u.	37004 ± 14004
NIRS-VOT [b]	
Baseline TSI, %	66.7 ± 3.4
TSImin, %	34.3 ± 11.1
TSImax, %	77.6 ± 3.4
TSI amplitude, %	43.3 ± 11.7
Hyperaemic reserve, %	16.5 ± 4.3
$T_{1/2}$, s	12.9 ± 3.8
TSI slope 1, %/s	-0.1 ± 0.01
TSI slope 2, %/s	0.2 ± 0.05
AUC TSI, a.u.	209041 ± 24618

Data are expressed as means ± SD. AUC: area under the curve; FMD: flow-mediated dilation; NIRS-VOT: near-infrared spectroscopy with vascular occlusion test; $T_{1/2}$: half time to peak hyperemia; TSI: tissue oxygenation index; TSImax: maximum TSI; TSImin: minimum TSI. [a]n=20; [b]n=18.

Table 2.5 presents hemodynamics, anthropometrics, blood, and urine results from the experimental visit at day 10 of both interventions. There were no differences at the end of each intervention for any of the variables except for free water clearance that was trending to increase in the melatonin group without reaching statistical significance. 6-sulfatoxymelatonin was greater in the melatonin supplementation group.

Table 2.5 Participant characteristics at the end of each intervention

	HSD+PL	HSD+MEL	P value
Laboratory hemodynamics and anthropometrics			
Systolic BP, mm Hg	114 ± 9	115 ± 8	0.73
Diastolic BP, mm Hg	69 ± 7	68 ± 7	0.34
MAP, mm Hg	84 ± 7	84 ± 7	0.60
Heart rate, bpm	73 ± 12	71 ± 13	0.49
Weight, kg	71 ± 11	71 ± 11	0.37
Blood chemistry			
Serum Sodium, mmol/L [a]	139.9 ± 1.9	140.5 ± 3.0	0.41
Serum Potassium, mmol/L [a]	3.9 ± 0.4	3.9 ± 0.3	0.88
Serum Chloride, mmol/L [a]	105.7 ± 1.5	106.1 ± 2.7	0.45
Plasma osmolality, mOsm/kg/H_2O [b]	290.6 ± 7.6	290.6 ± 4.9	0.99
Hemoglobin, g/dL [c]	13.9 ± 1.7	13.7 ± 1.3	0.48
Hematocrit, % [c]	42.2 ± 4.4	41.8 ± 3.6	0.61
Total Cholesterol, mg/dL [d]	174.1 ± 31.9	172.3 ± 33.8	0.53
HDL Cholesterol, mg/dL [d]	55.9 ± 13.8	57.1 ± 15.7	0.36
VLDL Cholesterol, mg/dL [d]	17.5 ± 7.7	16.8 ± 6.9	0.47
LDL Cholesterol, mg/dL [d]	100.6 ± 25.1	98.4 ± 30.6	0.41
Non-HDL Cholesterol, mg/dL [d]	118.1 ± 29.4	115.2 ± 34.9	0.29
Triglycerides, mg/dL [d]	94.1 ± 48.6	90.1 ± 41.4	0.50
Urine chemistry			
Urinary sodium excretion, mmol/24h [a]	326.5 ± 98.3	296.6 ± 93.4	0.22
Urinary potassium excretion, mmol/24h [a]	53.3 ± 20.1	47.5 ± 17.5	0.16

Table 2.5 continued

Urine osmolality, mOsm/kg [e]	573.0 ± 211.9	554.5 ± 236.2	0.62
Urine flow rate, mL/min [a]	1.7 ± 0.8	1.7 ± 1.1	0.97
Urine specific gravity [d]	1.01 ± 0.005	1.01 ± 0.006	0.72
Free water clearance [f]	-1.3 ± 0.8	-1.1 ± 0.8	0.06
6-sulfatoxymelatonin, ng/mL [g]	44.5 ± 27.0	420.6 ± 217.9	0.001

Data are expressed as means ± SD. BP: blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MAP: mean arterial pressure; HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose); VLDL: very-low-density lipoprotein. [a]n=22; [b]n=19; [c]n=23; [d]n=21; [e]n=20; [f]n=17; [g]n=18.

Results from the 3-day diet record, three days prior to the experimental visit on day 10, are shown in **Table 2.6**. Participants consumed similar kilocalories, macronutrients, or micronutrients in both interventions demonstrating that they followed a similar diet.

Table 2.6 Average dietary intake of last 3 days of each intervention

	HSD+PL	**HSD+MEL**	**P value**
Energy intake, kcal/day	2098.6 ± 725.6	1944.1 ± 555.9	0.24
Total carbohydrate, g/day	231.7 ± 127.0	224.7 ± 76.0	0.71
Total protein, g/day	97.9 ± 45.6	94.8 ± 52.3	0.74
Total fat, g/day	82.9 ± 28.0	75.0 ± 24.4	0.20
Added sugar, g/day	30.3 ± 23.9	32.2 ± 26.5	0.72
Total fiber, g/day	21.8 ± 16.2	22.2 ± 11.4	0.88
Saturated fat, g/day	25.3 ± 9.5	23.2 ± 8.6	0.31
Sodium, mg/day	3421.9 ± 1512.7	3239.6 ± 1367.6	0.61
Potassium, mg/day	2702.5 ± 1492.3	2572.6 ± 863.9	0.54
Calcium, mg/day	883.9 ± 359.6	851.7 ± 349.8	0.66
Magnesium, mg/day	330.9 ± 156.9	324.8 ± 126.7	0.78
Vit A (RAE), mcg/day	636.1 ± 280.3	669.8 ± 638.1	0.80
Vit C, mg/day	93.2 ± 102.1	95.9 ± 93.2	0.86
Iron, mg/day	15.2 ± 6.5	14.3 ± 4.4	0.45
Zinc, mg/day	11.2 ± 4.9	10.9 ± 3.2	0.62
Tryptophan, g/day	1.2 ± 0.5	1.1 ± 0.6	0.75
Alcohol, g/day	8.3 ± 22.1	2.2 ± 7.3	0.16

Data are expressed as means ± SD. HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose). N=24.

We did not observe differences in sleep duration, sleep duration variability and sleep efficiency during each intervention assessed objectively by sleep accelerometers (**Table 2.7**). Additionally, we did not observe any differences when sleep measures were subjectively assessed with the PSQI (**Table 2.7**). We quantified physical activity via actigraphy. There were no differences in sedentary, light, moderate, vigorous, or very vigorous physical activity assessed by accelerometer during each intervention. Steps were similar during each intervention and participants also wore the devices for the same duration (**Table 2.7**).

Table 2.7 Sleep and physical activity parameters during each intervention

	HSD+PL	**HSD+MEL**	**P value**
Sleep metrics [a]			
Duration, min	473.0 ± 43.1	461.5 ± 46.6	0.64
Duration SD, min	79.7 ± 33.2	66.8 ± 28.8	0.29
Sleep efficiency, %	95.1 ± 2.6	94.6 ± 2.6	0.42
PSQI [b]			
Component 1: Sleep quality	0.6 ± 0.5	0.8 ± 0.6	0.19
Component 2: Sleep latency	0.6 ± 0.6	0.8 ± 0.7	0.27
Component 3: Sleep duration	0.4 ± 0.5	0.6 ± 0.6	0.21
Component 4: Sleep efficiency	0.2 ± 0.5	0.5 ± 0.9	0.23
Component 5: Sleep disturbance	1 ± 0.4	0.9 ± 0.4	0.33
Component 6: Use of sleep drugs	0 ± 0	0 ± 0	-
Component 7: Daytime dysfunction	0.5 ± 0.6	0.6 ± 0.6	0.27
Global Score	3.4 ± 1.7	4.0 ± 1.9	0.07
Physical activity [c]			
Sedentary, min/day	653.8 ± 79.9	645 ± 67.8	0.48
Light PA, min/day	124.3 ± 24.2	124.4 ± 29.8	0.99
Moderate PA, min/day	36.8 ± 16.3	34.6 ± 18.6	0.37
Vigorous PA, min/day	2.7 ± 2.6	2.7 ± 3.9	0.99
Very vigorous PA, min/day	1.6 ± 6.4	2.8 ± 11.5	0.32
Steps /day	6553 ± 1769	6121 ± 3010	0.48
Time accelerometer worn, min/day	819.3 ± 87.8	802.7 ± 84.5	0.28

Data are expressed as means ± SD. HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose); PA: physical activity; PSQI: Pittsburg Sleep Quality Index. [a]n=12; [b]n=21; [c]n=19.

24-h ambulatory BP monitor results are shown in **Figure 2.2** and **Table 2.8**. There were no differences in 24-hour MAP nor during just awake hours or sleep hours at the end of each intervention (**Figure 2.2**). We observed a decrease in nocturnal systolic pressure in the melatonin group but there were no other differences in other peripheral BP variables. There were no differences in overall, awake, or asleep central blood pressure variables. Regarding nocturnal BP dipping, we observed a greater systolic dipping in the melatonin group compared to the placebo group (HSD+PL: 13.3 ± 7.4 %, HSD+MEL: 16.2 ± 6.8 %; **p=0.04).** Diastolic nocturnal dipping (HSD+PL: 20.9 ± 9.3 %, HSD+MEL: 22.9 ± 7.9 %; p=0.37), central systolic nocturnal dipping (HSD+PL: 11.9 ± 7.3 %, HSD+MEL: 13.9 ± 6.1 %; p=0.23) and central diastolic nocturnal dipping (HSD+PL: 20.2 ± 8.3 %, HSD+MEL: 21.8 ± 6.7 %; p=0.43) remained unchanged at the end of each intervention.

Table 2.8 Ambulatory blood pressure measurements at the end of each intervention

	HSD+PL	HSD+MEL	P value
Peripheral hemodynamics			
Overall SBP, mm Hg	117 ± 9	114 ± 8	0.21
Overall DBP, mm Hg	65 ± 6	65 ± 5	0.49
Awake SBP, mm Hg	121 ± 9	120 ± 9	0.67
Awake DBP, mm Hg	69 ± 5	69 ± 5	0.97
Asleep SBP, mm Hg	106 ± 12	101 ± 9	**0.02**
Asleep DBP, mm Hg	56 ± 9	54 ± 7	0.26
Central hemodynamics			
Overall cSBP, mm Hg	106 ± 8	104 ± 6	0.17
Overall cDBP, mm Hg	67 ± 6	66 ± 5	0.34
Awake cSBP, mm Hg	110 ± 8	109 ± 7	0.54
Awake cDBP, mm Hg	71 ± 6	71 ± 5	0.76
Asleep cSBP, mm Hg	97 ± 11	94 ± 8	0.09
Asleep cDBP, mm Hg	58 ± 9	56 ± 7	0.23

Data are expressed as means ± SD. cDBP: central diastolic blood pressure; cSBP: central systolic blood pressure; DBP: diastolic blood pressure; SBP: systolic blood pressure. HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose). N=19.

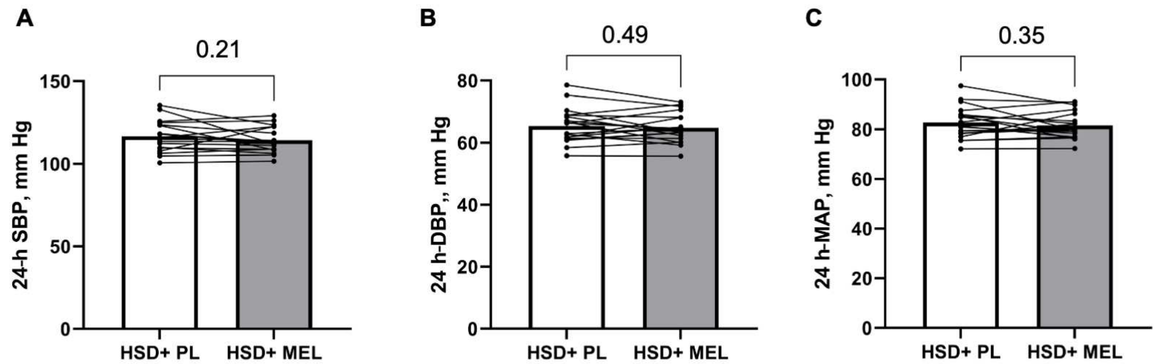

Figure 2.2 24-h ambulatory blood pressure monitor results at the end of the high sodium diet plus placebo intervention (HSD+PL) and at the end of the high sodium diet plus melatonin intervention (HSD+MEL). A) 24-h mean arterial pressure (MAP); B) 24-h systolic blood pressure (SBP); C) 24-h diastolic blood pressure (DBP). N=19. Data are presented as individual values.

Results on macrovascular function as assessed by brachial artery FMD and from microvascular function as assessed by NIRS-VOT at the end of each intervention are presented in **Table 2.9**, **Figure 2.3** and **2.4**. We did not observe any differences in baseline brachial artery diameter or baseline TSI at the end of each intervention. We did not observe significant differences in macrovascular or microvascular function at the end of each intervention.

Table 2.9 Vascular function at the end of each intervention

	HSD+PL	**HSD+MEL**	**P value**
Brachial artery FMD [a]			
Baseline brachial artery diameter, mm	3.5 ± 0.7	3.5 ± 0.7	0.48
Peak brachial artery diameter, mm	3.7 ± 0.7	3.8 ± 0.7	0.78
Absolute FMD, mm	0.2 ± 0.1	0.2 ± 0.1	0.59
Time to peak diameter, s	46.7 ± 11.4	49.3 ± 13.1	0.64
NIRS-VOT [b]			
Baseline TSI, %	66.8 ± 3.6	66.3 ± 4.1	0.52
TSImin, %	32.2 ± 10.1	32.9 ± 13.1	0.62
TSImax, %	77.1 ± 5.1	77.8 ± 6.0	0.31
TSI amplitude, %	44.9 ± 14.1	44.9 ± 17.4	0.99
$T_{1/2}$, s	12.2 ± 3.1	11.3 ± 2.5	0.23

Data are expressed as means ± SD. AUC: area under the curve; FMD: flow-mediated dilation; HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose); NIRS-VOT: near-infrared spectroscopy with vascular occlusion test; TSI: tissue oxygenation index; $T_{1/2}$: half time to peak hyperemia; TSImax: maximum TSI; TSImin: minimum TSI. [a]n=23; [b]n=20.

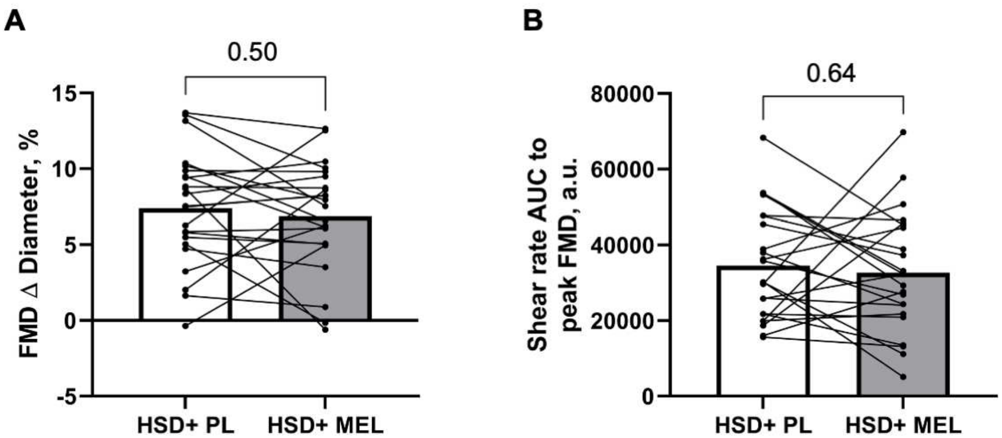

Figure 2.3 Macrovascular function assessed via brachial artery flow-mediated dilation (FMD) at the end of the high sodium diet plus placebo intervention (HSD+PL) and at the end of the high sodium diet plus melatonin intervention (HSD+MEL). A) Relative FMD; B) Shear rate area under the curve (AUC) to peak reached during reactive hyperemia. N=23. Data are presented as individual values.

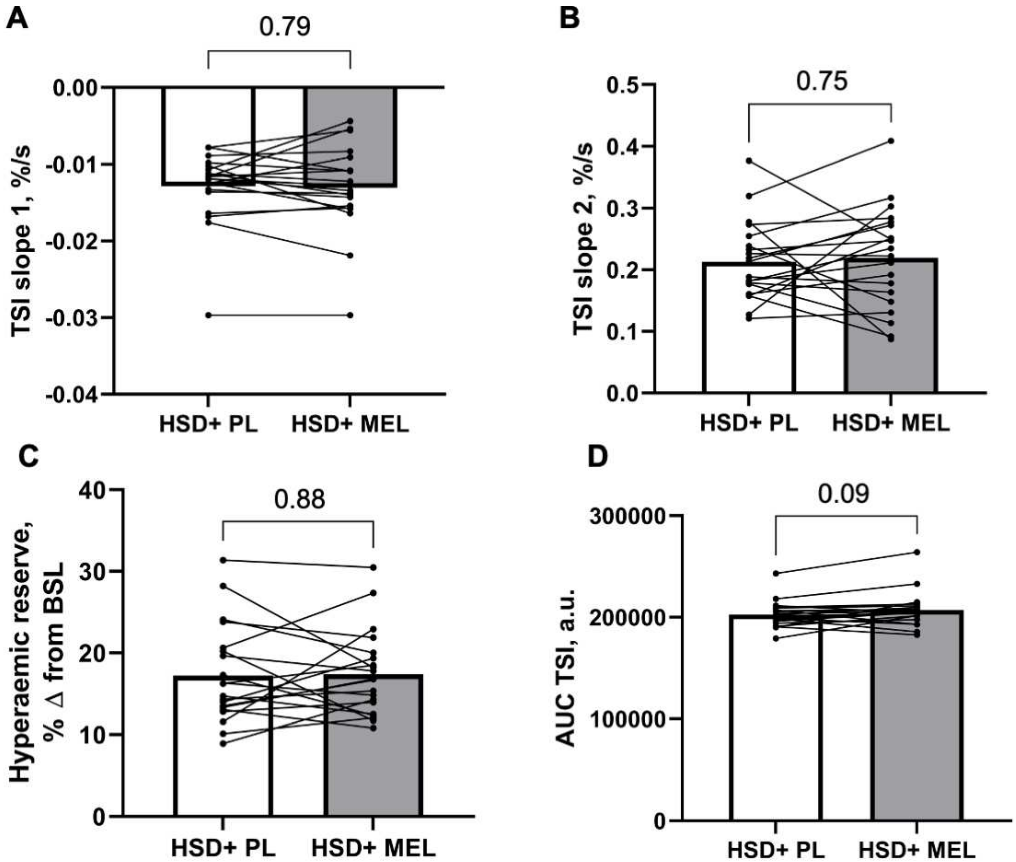

Figure 2.4 Microvascular function assessed via near infrared spectroscopy during vascular occlusion test (NIRS-VOT) at the end of the high sodium diet plus placebo intervention (HSD+PL) and at the end of the high sodium diet plus melatonin intervention (HSD+MEL). A) Slope 1 indicating desaturation rate; B) Slope 2 indicating resaturation rate; C) Hyperaemic reserve; D) Hyperaemic area under the curve (AUC). N=20. Data are presented as individual values.

We evaluated ROS as a potential mechanism by which melatonin may exert its effects. The concentration and number of free radicals at the end of each intervention are depicted in **Figure 2.5**. We did not observe differences in any of the variables at the end of each intervention.

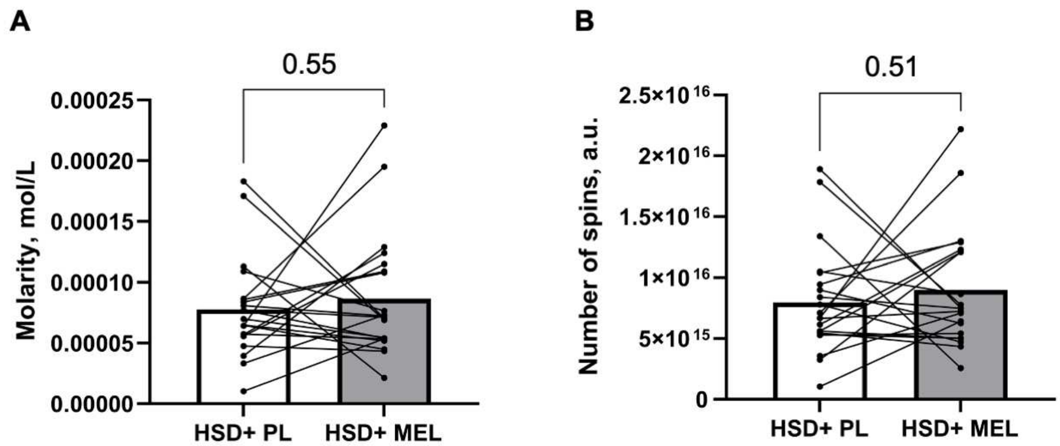

Figure 2.5 Free radicals at the end of the high sodium diet plus placebo intervention (HSD+PL) and at the end of the high sodium diet plus melatonin intervention (HSD+MEL). A) Molarity indicating the concentration of free radicals; B) Number of spins indicating absolute number of free radicals. N=21. Data are presented as individual values.

2.4 Discussion

This study was the first randomized controlled trial carried out in young, healthy, normotensive men and women to investigate the effects of melatonin supplementation on 1) vascular function and 2) ROS during a HSD. We hypothesized that melatonin supplementation while consuming a HSD would 1) increase vascular function and 2) decrease ROS compared to a HSD alone. The primary novel findings are that melatonin supplementation does not improve vascular function nor change the generation of ROS during an HSD in this cohort of young healthy normotensive humans. Thus, both hypotheses were rejected.

We evaluated melatonin as an antioxidant to combat vascular dysfunction imposed by a HSD. Extensive animal work has shown that this hormone improves vascular function *in vivo* and *in vitro* [170,172,179,197,214]. Melatonin has restored endothelial dysfunction in diabetic rats [179] and rats with chronic intermittent hypoxia [214]. It has also prevented renal injury in hypertensive rats that were fed a HSD [197]. Human studies are scarce, however. We assessed vascular function, both at the macrovascular and the microvascular level, at the end of two 10-day periods in which participants followed either a HSD with melatonin or a HSD with placebo. In contrast to the animal literature and to our hypothesis, we did not observe differences at the end of the melatonin supplementation group and the placebo group for any of our vascular function variables. This is the first study evaluating the effects of melatonin in vascular function in humans and a direct comparison with animal studies to explore the lack of an effect is difficult. One reason may be the *in vitro* nature of animal studies, which don't account for the whole organism, eliminating other important factors that may indeed play a role in the final response. Another reason is the discrepancy between the dosages. We administrated 10 mg a day while animal studies administer a dose that would correspond to 300 mg for a 60-kg human [197,238].

Some human studies have evaluated the use of antioxidants to overcome vascular dysfunction in the context of a HSD. We have demonstrated that ascorbic acid, apocynin (inhibitor of NADPH oxidase or NOX) and Tempol can mitigate microvascular dysfunction imposed by a HSD [81,82]. Baric et al. [239], demonstrated that oral supplementation of an antioxidant cocktail (vitamins C and E) prevented microvascular dysfunction induced by a 7-day HSD and restored oxidative status in young healthy individuals. We could not replicate these results using melatonin. A potential reason for this discrepancy could be the low absorption of oral melatonin, reported to be only 3% with great interindividual variation in a study carried out in young healthy males [240]. Furthermore, the average elimination half-life in these male subjects was 54 min reaching baseline plasma melatonin levels approximately 5 h after consumption [240]. It is possible that our null findings could be attributed to the timing of our experimental visits which occurred in the morning, when melatonin is no longer in the system. It is noteworthy to highlight that we did observe an increase in melatonin's urinary metabolite demonstrating that our participants were compliant, and which corresponds to a greater nocturnal plasma peak, in the melatonin supplementation group.

Despite a lack of effect in our study, the powerful antioxidant properties of melatonin are well-known [106,115,122,123]. Melatonin increases antioxidant enzymes and decreases ROS as seen in improvements in superoxide dismutase (SOD) and catalase (CAT) activity levels and decreases in malondialdehyde (MDA) levels in erythrocytes from treated hypertensive older adults and elderly type II diabetics [198,199]. We did not observe differences in the concentration or quantity of free radicals at the end of each of our interventions, however. Several reasons could have contributed to our results. First, despite using a lower dosage (5 compared to 10 mg), their interventions lasted longer (one or two months compared to 10 days) [198,199,201,203]. Secondly, most human studies are performed in clinical populations. There is limited evidence about the effect of melatonin on free radicals in healthy individuals. One study in healthy males that underwent a strenuous race showed a decrease in oxidative damage in those that had taken melatonin prior to the race [204]. Third, it is worth pointing out differences in the methodology to quantify free radicals. We utilized EPR, which allows for the precise measurement of ROS by using spin probes that react directly with specific free radicals [235]. Other studies have used lipid peroxidation techniques or activity of antioxidant enzymes which provide more variable results [198,199].

Melatonin is known to exert antihypertensive effects even in normotensive young individuals [146–149,215]. In agreement with the literature and in line with our hypothesis, we report greater decreases in peripheral nighttime systolic blood pressure and increases in nighttime systolic dipping in the melatonin group compared to the placebo under a HSD. While both groups have a normal dipping value (between 10-20%), these are clinically important findings as prospective studies have observed a relation between declines in nocturnal BP and a increases in CVD mortality even in non-hypertensive individuals [241] .Overall and daytime blood pressure values remained unchanged at the end of each intervention. We did not observe any central blood pressure changes either. Although we did not evaluate the pathways behind these BP decreases, several mechanisms may be responsible. These include indirect regulation of blood pressure via the central nervous system and catecholamine secretion modulation, antioxidant and anti-inflammatory effects, relaxation of smooth muscle in blood vessels, NO production and Ca^{2+} signaling [116,152,153]. Melatonin has also been reported to have antilipidemic effects. Melatonin supplementation has improved total cholesterol and triglyceride levels in different populations [201,242]. Because of the relation between these lipids and increases in BP and declines in macrovascular function, we evaluated lipid levels at the end of both 10-day interventions [201] but we did not observe any changes. There was also no difference in fat consumption nor other macronutrients and micronutrients suggesting that diet did not influence these results. Finally, melatonin is traditionally known for being an important physiological sleep regulator and for improving different sleep parameters in those suffering from sleep disturbances and disorders, and it also may have beneficial effects on a healthy population [243,244]. Sleep improvement in turn has been related to an enhancement in vascular function, while sleep restriction has been related to endothelial dysfunction development [245–247]. Hence, we quantified sleep along with physical activity objectively with actigraphy since both are two important determinants of vascular function, but no differences were found [245–248].

In this study, we used salt pills to increase sodium intake over two 10-day periods. Participant compliance to the salt pills was assessed via a 24-h urine collection and a 3-day diet record, one day and three days prior to the experimental visit, respectively. 24-h urinary sodium excretion, urine osmolality and sodium consumption were similar at the end of each intervention showing compliance to the salt pills. Indeed, in general, the macronutrient and micronutrient intakes were similar at the end of each intervention, indicating that participants consumed similar foods. In particular, we did not observe differences in tryptophan consumption, a precursor of melatonin [249]. We used melatonin pills and lactose pills as a placebo in each of the 10-day interventions. Compliance to the melatonin pills was assessed using 6-sulfatoxymelatonin collected from the urine of the morning of the experimental visit. Urinary 6-sulfatoxymelatonin was increased in the melatonin supplementation group as expected.

This study comes with some limitations. We did not control for menstrual cycle phase, however there is literature that shows no difference in FMD between distinct phases of the menstrual cycle in premenopausal women [250]. Melatonin has been shown to increase the activity and concentration of endogenous antioxidant enzymes while sodium has been shown to decrease these. However, in this study we did not evaluate the effect of melatonin on antioxidants during the HSD. We also did not evaluate the effect of melatonin on inflammation which melatonin has been shown to decrease [214]. Furthermore, we do not know whether the HSD alone caused vascular dysfunction and increased ROS since we did not take baseline measurements. However, our purpose was to compare melatonin to placebo under a HSD. While we could not quantify the change in plasma melatonin levels at night, we did assess melatonin's urinary metabolite, which is highly correlated with the former [113,251]. Lastly, our sample consisted of young healthy normotensive adults and the results are not generalizable to other populations. Further studies could address whether these results are applicable to disease populations.

2.5 Conclusions

In this randomized cross-over, double-blinded controlled trial, we sought to determine whether melatonin supplementation could improve vascular function and reduce free radicals under a HSD compared to a placebo. Our findings showed that melatonin supplementation did not improve macrovascular or microvascular function and did not decrease free radicals under a HSD in young healthy normotensive adults. Interestingly, we observed decreases in nighttime systolic BP and increases in nighttime systolic dipping. This is important as melatonin could have significant clinical implications for populations with elevated BP. Future studies should replicate this experiment with a different melatonin dose, explore mechanisms beyond free radicals' formation, such as inflammation and antioxidant defense, and extrapolate to other populations.

Chapter 3

THE EFFECTS OF MELATONIN ON BLOOD PRESSURE REACTIVITY DURING HIGH DIETARY SODIUM

3.1 Introduction

High sodium diets (HSD) have historically been related to increases in resting blood pressure (BP) [211]. Not only resting BP, but BP responses to different physiological perturbations, a term named BP reactivity, are also exaggerated during a HSD [83,88]. Some of these commonly used perturbations include exercise, for example the isometric handgrip exercise (IHG), and exposure to cold, using the cold pressor test (CPT). A study in adult normotensive rats fed a HSD for 2-3 weeks experienced a greater increase in mean arterial pressure (MAP) during exercise [83]. These results have been replicated in humans as well. Healthy Chinese adults experienced increased BP reactivity to the CPT after 1 week of a HSD [86]. Consumption of salt pills for one week also produced an increase in MAP during IHG in healthy young normotensive participants [89]. This exacerbated increase in BP reactivity induced by a HSD is known to be linked to the development of hypertension (HTN) [66,252].

Average sodium consumption in the U.S. clearly surpasses the recommendations, and education programs and awareness campaigns to lower sodium consumption to the recommended level have shown inconsistent results[2,11]. Hence, exploring strategies that can ameliorate these greater increases in BP is imperative in order to decrease HTN risk and combat cardiovascular disease (CVD), which is the leading cause of mortality in the U.S. [213]. In fact, CVD have been linked to melatonin, a hormone released by the pineal gland predominantly at night. Melatonin nocturnal synthesis, circulating levels of melatonin, and its urinary metabolite (6-sultatoxymelatonin) are reduced in various CVD populations [132–134]. Furthermore, the incidence for cardiac events is higher in the early morning when circulating melatonin levels are significantly lower [138–140].

Melatonin has been shown to regulate the autonomic nervous system (ANS) via inhibition of the sympathetic system and activation of the parasympathetic system. Carotid-femoral pulse wave velocity (PWV), which assesses arterial stiffness, and BP decreased in healthy young men one hour after consuming melatonin [160]. Pulsatility index, an indicator of vasomotor tone and resistance to flow, along with circulating norepinephrine values were also decreased in healthy adults after melatonin consumption [148,161]. Muscle sympathetic nerve activity (MSNA), a direct index of sympathetic activity in humans, during orthostatic stress was decreased after oral melatonin consumption in healthy young participants while BP reactivity was not decreased during IHG and CPT. However, this was not a chronic supplementation study and IHG was performed at 30% of their maximal voluntary contraction (MVC) [163]. It is not known if a higher percentage of MVC might elicit a different response. Finally, no study has evaluated post exercise ischemia (PEI) as a physiological stressor in the context of melatonin under a HSD.

Melatonin has been shown to modulate the ANS but whether it can ameliorate an increase of BP reactivity, more specifically during IHG and PEI, and CPT under a

HSD, is not known. The purpose of this study was to determine the effects of melatonin consumption on BP reactivity after 10 days of HSD. We hypothesized that melatonin supplementation would decrease BP reactivity during IHG and PEI, and CPT on HSD compared to HSD alone.

3.2 Methods

3.2.1 Study design

This intervention was a randomized double-blind placebo-controlled crossover design. The study consisted of two 10-day interventions of high sodium combined with either melatonin or placebo. The study protocol and procedures were approved by the Institutional Review Board of the University of Delaware (ID# 1534734) and conform to the provisions of the Declaration of Helsinki. Participants were recruited from the University of Delaware campus and the surrounding community through online advertisements. Informed consent was obtained prior to enrollment in the study. The study was registered on clinicaltrials.gov (NCT04325191).

3.2.2 Participant screening

Healthy normotensive men and women between the ages of 18-45 were recruited to participate in the study. After participants signed the informed consent, they completed a medical history questionnaire, a menstrual cycle form (women only), and a global physical activity questionnaire (GPAQ)[216] . Their height (Holtain Harpenden Stadiometer), weight (Seca 876), waist and hip circumferences, and seated BP were measured. Inclusion criteria included a systolic BP <130mmHg, diastolic BP

<80mmHg and body mass index (BMI) between 18.5-29.9 kg/m^2. Exclusion criteria included the following: history of cardiovascular disease and hypertension; liver or kidney disease; malignant cancer; mood disorders (including depression, anxiety, seasonal affective disorder, manias) and/or taking any medications for those conditions; sleep disorder and/or night shift work; pregnancy or breastfeeding; use of tobacco products; ADHD medications use; antibiotics, melatonin or other antioxidant use in the past 3 months; significant changes in weight (≥10 lbs. gain or loss) in the last 6 months; highly trained endurance athletes; abnormal creatinine value (<0.76 and >1.27 mg/dL for men, <0.57 and >1.00 mg/dL for women) and/or a glomerular filtration rate (GFR) (<90 mL/min/1.73); and consumption of less than 2000 mg of sodium per day in their habitual diets. All participants were vaccinated against COVID-19 at the time of enrollment in the study.

3.2.2.1 Blood analysis and habitual diet and sleep measurements

A venous blood sample was collected and sent to LabCorp to examine complete blood count, metabolic panel, and lipid profile. Participants were then allocated to an intervention (A or B) using the randomize function in Excel, which was created by another member of the research team. They were also given a 3-day diet record, which was used to analyze their habitual dietary intake. Participants were asked to record their intake over two weekdays and one weekend day. During the days in which they recorded the diet, they wore a wrist accelerometer (Motionlogger Micro watch, Ambulatory Monitory Inc, Tokyo, Japan) to quantify habitual sleep parameters.

3.2.3 Dietary interventions, sodium, and melatonin supplementation

The study consisted of two 10-day interventions separated by a washout period of at least 14 days. During the washout period, the participants were instructed to resume their usual life routines. During both 10-day periods, participants consumed salt pills and they either supplemented with 10 mg of melatonin (HSD+MEL) or placebo (lactose)(HSD+PL) daily. Both melatonin and lactose pills were manufactured by the SaveWay compounding pharmacy (Newark, DE) and were matched in weight and appearance. Researchers and participants were blinded to the content of the pills. Participants took the melatonin/placebo 30 minutes before they planned to go to sleep, including the night prior to the experimental visit. The proposed dosage of melatonin has been used with no reported side effects in both clinical and healthy populations [202,203,209]. The total of 6900 mg/day of sodium in both 10-day interventions was achieved by taking 12 enteric coated, slow-release salt pills (Purecaps USA- Empty White Vegetarian and Vegan Delayed Release/ Acid-Resistant, Size 00), each containing ~380 mg, and by consuming 2300 mg of sodium in the diet. 6900 mg/day of sodium is consistent with our prior studies and shown to impair vascular function in normotensive humans [79,81]. Participants were asked to space out the sodium pills and take them with meals to avoid possible gastrointestinal issues. For the first three days of the intervention, participants gradually increased the number of pills they took and for days 4-9, they were instructed to consume 12 pills daily. Research staff provided participants with instructions and strategies to consume a 2300 mg sodium diet during both interventions. Participants were provided with a paper printout of two-dimensional food models adapted from Van Horn et al, [223,224] to help with estimating portion sizes. Participants recorded their dietary intake during the last three days of the intervention (days 7, 8 and 9) and were instructed to replicate their diet as much as possible during both trials. Participants were given a melatonin/placebo pill form, a sodium pill form, and a side effect form for each 10-day intervention to ensure compliance and note potential side effects. They were also given a 3-day diet record, which they completed during days 7, 8 and 9 of the 10-day intervention. The diet record was reviewed by a trained researcher with the participant and diet records were analyzed using Nutrition Data System for Research software (NDSR, 2020, University of Minnesota, Minneapolis). This 3-day diet record was used to assess diet reproducibility and compliance along with 24 h urine collection starting on day 9. A schematic representation of the study design is presented in **Figure 3.1.**

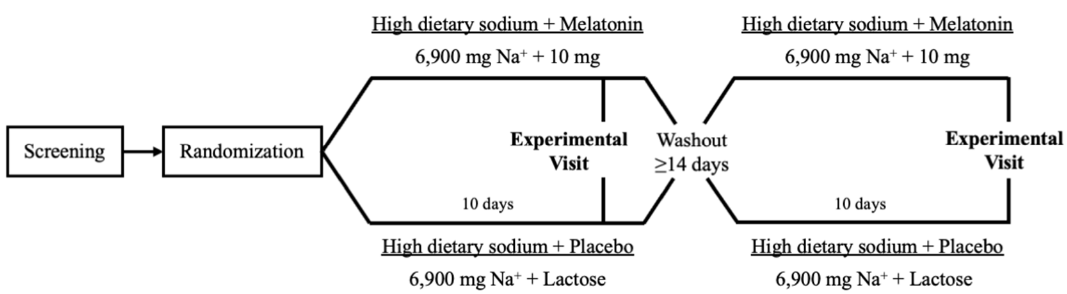

Figure 3.1 Schematic representation of the intervention

3.2.4 Urine collection

The 24-hour urine collection started on day 9. Urine was assessed for urine volume, urine specific gravity (Goldberg Brix Refractometer, Reichert Technologies, Depew, NY), electrolyte concentrations (EasyElectrolyte Analyzer, Medica, Bedford, MA), and osmolality (Advanced 3D3 Osmometer, Advanced Instruments, ON, Canada) from a mixed aliquot from the 24-hour collection container. Urine flow rate was calculated and used to determine 24-hour sodium excretion. For urine collections to be considered valid, they had to be collected within 20-28 h, there could not be ≥2 missed collections, and the urine volume could not be <500 mL [227–229]. The first morning void was collected separate from the 24 h urine collection in the morning of day 10 to assess melatonin compliance by measuring urinary 6-sulfatoxymelatonin (ELISA kit, Alpco, Salem, NH). A 4 Parameter Logistics (4PL) curve fit was used to obtain the calibrator curve with SoftMax Pro Software (Molecular Devices).

3.2.5 Physical activity monitoring and scoring

They wore an accelerometer (ActiGraph wGT3X-BT, Pensacola, FL) on the hip for the entire duration of both interventions except to sleep and activities involving water (e.g., swimming, showers). Participants recorded the times when the accelerometer was worn, and not worn, to improve compliance and data quality. Data was accepted if there were at least 4 days with 10 hours of wear time [230]. Participants returned the device at the experimental visit on day 10. Accelerometer was analyzed using ActiLife software. Wear time was validated using the Troiano algorithm [231] and activity variables were calculated using the Freedson Combination 1998 algorithm [232].

3.2.6 Sleep monitoring and scoring

Participants wore wrist accelerometers (Motionlogger Micro watch, Ambulatory Monitory Inc, Tokyo, Japan) on the non-dominant wrist for 9 days and 9 nights, except for activities involving water (e.g., swimming, showers). Data were collected in the zero-crossing mode (ZCM) and saved in 1-minute epochs. The University of California San Diego (UCSD) algorithm, which is validated to produce accurate and reliable sleep estimates relative to polysomnography [233], along with Action W-2 software (Ambulatory Monitoring, Inc., Ardsley, NY) were used to score the data. To improve data quality, participants completed a sleep diary before bed and upon waking every day during the sleep monitoring period. Data was accepted if the accelerometer was worn for at least 7 days out of the 9 days [234].

Variables of interest were sleep duration, sleep variability and sleep efficiency. Sleep duration was defined as the total time spent asleep from sleep onset to wake onset. We present the mean (quantified for each night that the accelerometer was worn) of the sleep duration. Sleep variability was quantified as the standard deviation (SD) of sleep duration across the 9-day sleep monitoring period. Sleep efficiency was calculated as the total sleep time expressed as a percentage of the total time spent in bed.

3.2.7 Experimental visit

Participants reported to the laboratory on day 10 of each intervention following a 9 h fast, no caffeine or alcohol for at least 12 hours and not exercising for 24 hours. Upon arrival to the laboratory, weight was measured (Seca 876). After 5 min of rest, seated BP was assessed in triplicate (dominant arm; Dash 2000, GE medical systems, Chicago, IL). The average is reported in this paper. A venous blood sample was collected to assess hematocrit (Sure prep™ capillary tubes, Clay Adams spun in a microcentrifuge at 1,950g for 5 min, Legend Micro 17, Thermo Sorvall) and hemoglobin (Hb 201+, HemoCue, Angelholm, Sweden), serum electrolytes (EasyElectrolyte Analyzer, Medica, Bedford, MA), and plasma osmolality (Advanced 3D3 Osmometer, Advanced Instruments, ON, Canada) and lipid profile (LabCorp, Burlington, NC). Due to unknown effects of COVID-19 in our measurements, our experimental visits were scheduled 2 weeks post vaccination or infection. Experimental visits were scheduled at the same time for both interventions and occurred early in the morning (ranging from 7AM – 10AM). The temperature of the room was kept between 20°-24° C.

BP was measured at the middle finger of the participant's non-dominant hand non-invasively on a beat-by-beat basis via finger photoplethysmography at the heart level (NOVA, Finapres Medical Systems, Enschede, The Netherlands). Beat-by-beat BP correlates with intra-radial artery BP measures [253]. A single-lead ECG was used to measure heart rate and a respiratory belt transducer (Pneumotrace, Stoelting, Wood Dale, IL) was placed around their thorax to avoid Valsalva. Participants underwent two different physiological perturbations.

3.2.7.1 Isometric Handgrip Exercise and Post-Exercise Ischemia

Participants performed three maximal voluntary contractions (MVC) with their dominant hand with one-minute rest in between trials by squeezing a grip force transducer device (ADInstruments, Colorado Springs, CO) at maximal effort. The three attempts were averaged and used to calculate the 40% relative work rate. After the calibration period, a 10 min baseline period occurred followed by isometric handgrip exercise (IHG), which consisted of a static voluntary contraction for 2 min at 40% MVC. Participants were provided visual feedback during the duration of the isometric exercise and were asked to provide their effort level using the Rating of Perceived Exertion (RPE) scale at baseline and at the end of each minute [254]. Brachial BP was assessed as well using an automated oscillometric upper arm BP cuff placed on the right arm (Dash 2500, GE Healthcare, Milwaukee, WI) to compare with finger cuff values. During the last 5 s of exercise, a cuff on the right upper arm was inflated to 240 mmHg and remain inflated for 3 min and 15 s. The inflation of the upper arm cuff began PEI. The cuff was then released, and measures were recorded during recovery for an additional 2 minutes. The additional 15 s of PEI were incorporated to account for the potential effects of the initial decrease in BP when exercise is immediately stopped.

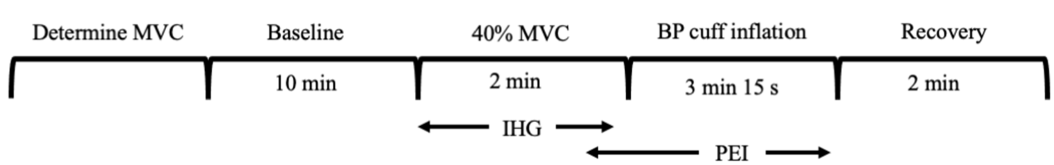

Figure 3.2 Isometric Handgrip exercise and Post-Exercise Ischemia protocol. IHG: isometric handgrip exercise; MVC: maximum voluntary contraction; PEI: post-exercise ischemia.

3.2.7.2 Cold pressor test

After the participant recovered to baseline values following the IHG trial, a 2 min baseline for CPT was started, then their dominant hand was submerged in a slurry ice/water mixture (1-5°C) ensured with a digital thermometer for 2 min [61,255]. Additional measures were taken for 2 extra min during recovery. The CPT evaluates BP reactivity due to a marked vasoconstriction caused by the cold mixture [57].

3.2.8 Statistical analysis

Beat-to-beat BP and ECG signals were recorded continuously with an acquisition software (LabChart Pro 8, ADInstruments, Colorado Springs, CO) at 1000 Hz and stored for offline analysis. The data was adjusted to each cardiac cycle. Baseline BP was calculated using the average MAP of 1 min overall baseline prior to the start of the exercise. Maximum BP was determined as the average MAP of the last 30 s of IHG and the last 3 min of PEI. BP recorded in the first 15 s of PEI was not included in the analysis as a subject experiences a robust and transient decrease in BP [256]. **Figure 3.2** depicts the IHG and PEI protocol in this study. Baseline BP for the CPT was calculated using the average of the 1 min baseline. Maximum BP was determined as the average MAP of the last 30 s of the CPT. For the IHG/PEI and the CPT protocols, BP reactivity was reported as delta change and as the percent change (delta change expressed as a percent of baseline BP) [256].

Our primary outcome was the difference in the change in MAP at the of the melatonin and the placebo interventions. Muller et al. [166], observed that a one-time administration of 3 mg of melatonin decreased MAP by 3 mm Hg in response to a mental stress challenge compared to placebo. Considering that our intervention was not acute, and our dosage was higher (10 mg), we ran a more conservative a priori power analysis. With an alpha of 0.05, 95% power, effect size 1 and assuming a percent difference in MAP of 3% (approximately 2 mm Hg standard deviation) at the end of the two arms, the necessary sample size was 16 participants using a paired samples t-test (G* Power). JMP Pro 16.0.0 (SAS, Cary, NC). Data were assessed for normality, linearity, homoscedasticity, and multicollinearity. Data are expressed as mean ± standard deviation (SD).

3.3 Results

Twenty participants took part in this study. Participant screening characteristics are presented in **Table 3.1**. We had an equal distribution of men and women. Participants were young and normotensive with a normal BMI. Participants were mostly Caucasian and Asian. Participants average total physical activity was 5.5 ± 3.2 hours per week, which is equivalent to 30.0 ± 18.2 METs hours per week. They spent 8.2 ± 3.4 hours per day sitting or reclining.

Screening blood chemistry is presented in **Table 3.2**. Fasting blood work was within normal limits. Habitual dietary intake is presented in Table **3.3**. Habitual sleep parameters were collected in 8 participants. Sleep duration was a mean of 471.8 ± 34.1 min, sleep duration SD was 75.3 ± 32.7 min and sleep efficiency was 94.9 ± 3.0 %.

Table 3.1 Screening characteristics

Demographic data	
N, men/women	11/9
Race, Asian/Black/Caucasian	8/1/11
Anthropometrics	
Age, yr	27.0 ± 2.9
Weight, kg	70.3 ± 11.3
Height, cm	170.2 ± 10.1
BMI, kg/cm^2	24.1 ± 1.8
Hemodynamics	
Systolic BP, mm Hg	112 ± 8
Diastolic BP, mm Hg	67 ± 7
MAP, mm Hg	82 ± 7
Heart rate, bpm	63 ± 8

Data are expressed as means ± SD. BMI: body mass index; BP: blood pressure; MAP: mean arterial pressure. n=20.

Table 3.2 Screening blood chemistry

Complete blood count	
WBC count, x10^3/uL	5.4 ± 1.5
RBC count, x10^6/uL	4.8 ± 0.5
Hemoglobin, g/dL	14.3 ± 1.3
Hematocrit, %	42.8 ± 3.9
Metabolic panel	
Glucose, mg/dL [b]	72.1 ± 16.7
BUN, mg/dL	12.6 ± 3.8
Creatinine, mg/dL	0.9 ± 0.2
eGFR if Non-African American, mL/min/1.73 [b]	107.9 ± 13.5
eGFR if African American, mL/min/1.73 [c]	128 ± 0
BUN/Creatinine	14.6 ± 4.1
Serum Sodium, mmol/L	140.8 ± 1.8
Serum Potassium, mmol/L [b]	4.3 ± 0.4
Serum Chloride, mmol/L	101.4 ± 1.9
Lipid profile	
Total Cholesterol, mg/dL	191.6 ± 29.9
Triglycerides, mg/dL	96.7 ± 43.5
HDL Cholesterol, mg/dL	59.8 ± 15.9
VLDL Cholesterol, mg/dL	17.7 ± 7.5
LDL Cholesterol, mg/dL	114.0 ± 30.2
Non-HDL Cholesterol, mg/dL	131.7 ± 33.8

Data are expressed as means ± SD. BUN: blood urea nitrogen; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; LDL: low-density lipoprotein; RBC: red blood cell; VLDL: very-low-density lipoprotein; WBC: white blood cells. [a]n=18; [b]n=17; [c]n=1.

Table 3.3 3-day average of habitual dietary intake

Energy intake, kcal/day	2098.8 ± 520.8
Total carbohydrate, g/day	241.9 ± 69.6
Total protein, g/day	100.1 ± 31.2
Total fat, g/day	78.0 ± 19.3
Added sugar, g/day	26.8 ± 18.1
Total fiber, g/day	22.7 ± 11.5
Saturated fat, g/day	23.8 ± 6.4
Sodium, mg/day	3836.7 ± 1491.4
Potassium, mg/day	2820.5 ± 1062.7
Calcium, mg/day	909.9 ± 267.9
Magnesium, mg/day	350.2 ± 128.5
Vit A (RAE), mcg/day	639.1 ± 471.2
Vit C, IU/day	83.3 ± 68.4
Iron, mg/day	15.6 ± 4.8
Zinc, mg/day	11.5 ± 3.3
Tryptophan, g/day	1.2 ± 0.4
Alcohol, g/day	8.7 ± 23.4

Data are expressed as means ± SD. n=20.

Table 3.4 shows hemodynamics, anthropometrics, blood, and urine results from the experimental visit at day 10 of both interventions. There were no differences at the end of each intervention for any of the variables. 6-sulfatoxymelatonin was greater in the melatonin supplementation group.

Table 3.4 Participant characteristics at the end of each intervention

	HSD+PL	HSD+MEL	P value
Laboratory hemodynamics and anthropometrics			
Systolic BP, mm Hg	114 ± 10	115 ± 8	0.85
Diastolic BP, mm Hg	69 ± 7	68 ± 6	0.39
MAP, mm Hg	84 ± 7	83 ± 6	0.71
Heart rate, bpm	70 ± 11	69 ± 12	0.68
Weight, kg	71 ± 11	71 ± 11	0.23
Blood chemistry			
Serum Sodium, mmol/L [a]	140.1 ± 1.8	140.9 ± 3.1	0.24
Serum Potassium, mmol/L [a]	3.9 ± 0.4	3.9 ± 0.2	0.74
Serum Chloride, mmol/L [a]	105.6 ± 1.5	105.9 ± 2.7	0.56
Plasma osmolality, mOsm/kg/H_2O [b]	290.4 ± 8.1	290.0 ± 4.8	0.79
Hemoglobin, g/dL [c]	14.1 ± 1.7	13.8 ± 1.4	0.71
Hematocrit, % [c]	42.4 ± 4.4	41.9 ± 3.7	0.75
Total Cholesterol, mg/dL [c]	175.4 ± 32.6	174.7 ± 34.1	0.86
HDL Cholesterol, mg/dL [c]	55.8 ± 14.3	56.7 ± 16.1	0.63
VLDL Cholesterol, mg/dL [c]	16.8 ± 6.9	16.8 ± 7.2	0.94
LDL Cholesterol, mg/dL [c]	102.7 ± 25.5	101.2 ± 30.8	0.97
Non-HDL Cholesterol, mg/dL [c]	119.6 ± 30.4	115.6 ± 34.8	0.95
Triglycerides, mg/dL [c]	89.6 ± 41.3	90.4 ± 41.9	0.89
Urine chemistry			
Urinary sodium excretion, mmol/24h [a]	320.9 ± 94.6	282.1 ± 85.8	0.19
Urinary potassium excretion, mmol/24h [a]	53.8 ± 19.5	47.2 ± 17.1	0.26

Table 3.4 Continued

Urine osmolality, mOsm/kg [d]	551.6 ± 222.4	534.7 ± 251.4	0.99
Urine flow rate, mL/min [d]	1.7 ± 0.8	1.7 ± 1.1	0.71
Urine specific gravity [d]	1.01 ± 0.005	1.01 ± 0.006	0.67
Free water clearance [e]	-1.2 ± 0.8	-1.0 ± 0.8	0.13
6-sulfatoxymelatonin, ng/mL [d]	42.8 ± 26.9	439.2 ± 209.6	0.001

Data are expressed as means ± SD. BP: blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MAP: mean arterial pressure; HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose); VLDL: very-low-density lipoprotein. [a]n=18; [b]n=15; [c]n=19; [d]n=17; [e]n=14.

Results from the 3-day diet record, three days prior to the experimental visit on day 10, are shown in **Table 3.5**. Participants consumed similar kilocalories, macronutrients, or micronutrients in both interventions demonstrating that they followed a similar diet.

Table 3.5 Average dietary intake of last 3 days of each intervention

	HSD+PL	HSD+MEL	P value
Energy intake, kcal/day	2089.3 ± 780.1	2053.9 ± 528.3	0.65
Total carbohydrate, g/day	225.6 ± 133.6	235.2 ± 78.9	0.56
Total protein, g/day	99.5 ± 45.7	98.9 ± 54.2	0.76
Total fat, g/day	84.1 ± 28.4	80.6 ± 22.3	0.58
Added sugar, g/day	31.8 ± 25.9	35.7 ± 27.7	0.36
Total fiber, g/day	21.3 ± 17.5	22.1 ± 12.3	0.84
Saturated fat, g/day	26.1 ± 9.8	25.1 ± 7.8	0.83
Sodium, mg/day	3293.8 ± 1361.9	3353.3 ± 1207.4	0.92
Potassium, mg/day	2657.6 ± 1556.7	2621.3 ± 901.6	0.73
Calcium, mg/day	853.3 ± 348.3	850.0 ± 316.3	0.51
Magnesium, mg/day	326.4 ± 167.5	331.9 ± 134.3	0.91
Vit A (RAE), mcg/day	614.2 ± 229.4	724.3 ± 675.0	0.43
Vit C, mg/day	93.1 ± 105.8	104.8 ± 99.6	0.57
Iron, mg/day	14.8 ± 6.8	14.7 ± 4.4	0.71
Zinc, mg/day	11.5 ± 5.2	11.3 ± 2.9	0.59
Tryptophan, g/day	1.2 ± 0.5	1.1 ± 0.6	0.63
Alcohol, g/day	7.8 ± 22.9	2.7 ± 7.9	0.23

Data are expressed as means ± SD. HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose). n=20.

We did not observe differences in sleep duration, sleep variability and sleep efficiency during each intervention assessed by sleep accelerometers (**Table 3.6**). We did not observe any differences either with the PSQI (**Table 3.6**). We quantified physical activity via actigraphy. There were no differences in sedentary, light, moderate, vigorous, or very vigorous physical activity assessed by accelerometer during each intervention. Steps were similar during interventions and participants also worn the devices for the same amount of time (**Table 3.6**).

Table 3.6 Sleep and physical activity parameters during each intervention

	HSD+PL	HSD+MEL	P value
Sleep metrics [a]			
Duration, min	471.9 ± 39.2	463.3 ± 50.6	0.65
Duration SD, min	75.9 ± 32.9	53.3 ± 13.8	0.13
Sleep efficiency, %	94.6 ± 2.6	94.3 ± 2.8	0.51
PSQI [b]			
Component 1: Sleep quality	0.6 ± 0.5	0.7 ± 0.7	0.19
Component 2: Sleep latency	0.7 ± 0.6	0.7 ± 0.7	0.43
Component 3: Sleep duration	0.4 ± 0.5	0.4 ± 0.7	0.43
Component 4: Sleep efficiency	0.2 ± 0.4	0.4 ± 0.7	0.42
Component 5: Sleep disturbance	1 ± 0.4	0.9 ± 0.4	0.33
Component 6: Use of sleep drugs	0 ± 0	0 ± 0	-
Component 7: Daytime dysfunction	0.4 ± 0.6	0.6 ± 0.6	0.27
Global Score	3.3 ± 1.9	4 ± 1.9	0.17
Physical activity [c]			
Sedentary, min/day	642.6 ± 80.4	635.9 ± 63.2	0.66
Light PA, min/day	125.8 ± 26.3	124.9 ± 33.3	0.89
Moderate PA, min/day	35.5 ± 15.7	33.9 ± 18.7	0.57
Vigorous PA, min/day	2.5 ± 2.6	2.1 ± 3.7	0.59
Very vigorous PA, min/day	2.0 ± 7.2	3.6 ± 12.9	0.32
Steps /day	5900 ± 3216	6415 ± 1544	0.51
Time accelerometer worn, min/day	808.5 ± 92.4	791.7 ± 87.2	0.37

Data are expressed as means ± SD. HSD+MEL: high sodium diet plus melatonin; HSD+PL: high sodium diet plus placebo (lactose); PA: physical activity. [a]n=9; [b]n=18; [c]n=15.

During IHG, average calculated MVC was not different at the end of each intervention (HSD+PL: 279 ± 121 N, HSD+MEL: 283 ± 120 N; p=0.72). To ensure that participants did exert 40% of their MVC during the 2 min of IHG, we compared the force they exerted with the calculated 40% of MVC within the same intervention. Participants did not reach the calculated 40% of their MVC in either of the interventions. During the placebo intervention, participants exerted an average force of 102.7 N (standard deviation was 42.9 N), while the calculated 40% was an average of 111.7 N (standard deviation was 49.2 N) (p=0.0002). During the melatonin intervention, participants exerted an average force of 102.3 N (standard deviation was 46.4 N), while the calculated 40% was an average of 113.2 N (standard deviation was 47.9 N) (p=0.0001). Achieved force during the 2 min of IHG was not different at the end of each intervention (HSD+PL: 102.7 ± 42.9 N, HSD+MEL: 102.3 ± 46.4 N; p=0.91). We measured effort at baseline, and at the end of each minute of the IHG with the RPE scale. There were no differences in RPE at baseline (HSD+PL: 6 ± 0 N, HSD+MEL: 6 ± 0 N; p=1), end of minute 1 (HSD+PL: 12 ± 2 N, HSD+MEL: 13 ± 1 N; p=0.24) and end of minute 2 (HSD+PL: 16 ± 2 N, HSD+MEL: 15 ± 2 N; p=0.81).

Regarding IHG, there were no differences in baseline MAP (HSD+PL: 84 ± 6 mm Hg, HSD+MEL: 85 ± 9 mm Hg; p=0.50), SBP (HSD+PL: 118 ± 8 mm Hg, HSD+MEL: 118 ± 12 mm Hg; p=0.91) or DBP (HSD+PL: 67 ± 7 mm Hg, HSD+MEL: 69 ± 8 mm Hg; p=0.28), at the end of each intervention. MAP responses calculated as delta change and percent change to IHG are depicted in **Figure 3.3**. There were no differences in the responses in MAP. SBP and DBP responses were not different either. SBP delta change (HSD+PL: 30 ± 18 mm Hg, HSD+MEL: 30 ± 11 mm Hg; p=0.96), SBP percent change (HSD+PL: 25 ± 15 %, HSD+MEL: 26 ± 11 %; p=0.93), DBP delta change (HSD+PL: 25 ± 12 mm Hg, HSD+MEL: 24 ± 8 mm Hg; p=0.66), and DBP percent change (HSD+PL: 38 ± 18 %, HSD+MEL: 36 ± 12 %; p=0.55) were similar at the end of each intervention.

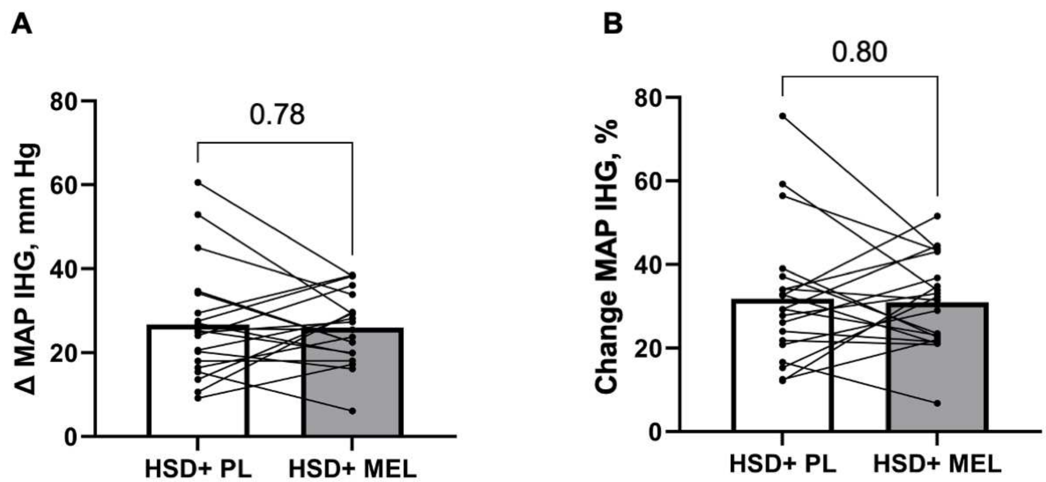

Figure 3.3 Blood pressure responses to handgrip exercise (IHG) at the end of the high sodium diet plus placebo intervention (HSD+PL) and at the end of the high sodium diet plus melatonin intervention (HSD+MEL). A) Mean arterial pressure (MAP) delta change; B) Mean arterial pressure (MAP) percent change. n=20. Data are presented as individual values.

MAP responses calculated as delta change and percent change to PEI are depicted in **Figure 3.4**. There were no differences in the responses in MAP. SBP and DBP responses were not different either. SBP delta change (HSD+PL: 27 ± 14 mm

Hg, HSD+MEL: 28 ± 14 mm Hg; p=0.82), SBP percent change (HSD+PL: 23 ± 12 %, HSD+MEL: 24 ± 13 %; p=0.72), DBP delta change (HSD+ PL: 20 ± 9 mm Hg, HSD+MEL: 19 ± 7 mm Hg; p=0.91), and DBP percent change (HSD+PL: 30 ± 14 %, HSD+MEL: 29 ± 11 %; p=0.77) were similar at the end of each intervention.

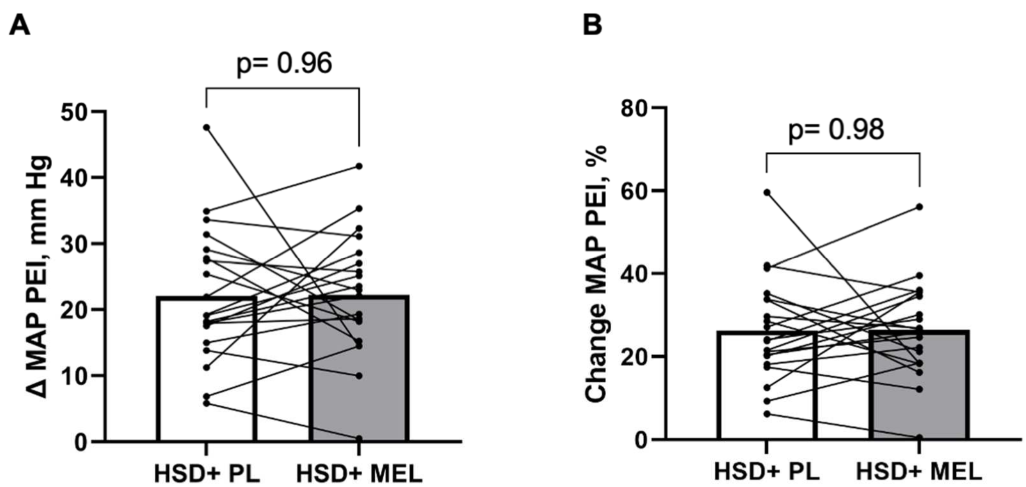

Figure 3.4 Blood pressure responses to post-exercise ischemia (PEI) at the end of the high sodium diet plus placebo intervention (HSD+PL) and at the end of the high sodium diet plus melatonin intervention (HSD+MEL). A) Mean arterial pressure (MAP) delta change; B) Mean arterial pressure (MAP) percent change. n=20. Data are presented as individual values.

Regarding the CPT, there were no differences in baseline MAP (HSD+PL: 89 ± 7 mm Hg, HSD+MEL: 89 ± 6 mm Hg; p=0.91), SBP (HSD+PL: 122 ± 9 mm Hg, HSD+MEL: 122 ± 8 mm Hg; p=0.98) or DBP (HSD+PL: 72 ± 8 mm Hg, HSD+MEL: 73 ± 6 mm Hg; p=0.87), at the end of each intervention. There were no differences in the MAP responses calculated as delta change and percent change to CPT (**Figure**

3.5). SBP and DBP responses were not different either. SBP delta change (HSD+PL: 29 ± 20 mm Hg, HSD+MEL: 27 ± 22 mm Hg; p=0.53), SBP percent change (HSD+PL: 24 ± 17 %, HSD+MEL: 22 ± 18 %; p=0.37), DBP delta change (HSD+PL: 21 ± 14 mm Hg, HSD+MEL: 20 ± 12 mm Hg; p=0.53), and DBP percent change (HSD+PL: 29 ± 20 %, HSD+MEL: 27 ± 17 %; p=0.46) were similar at the end of the interventions.

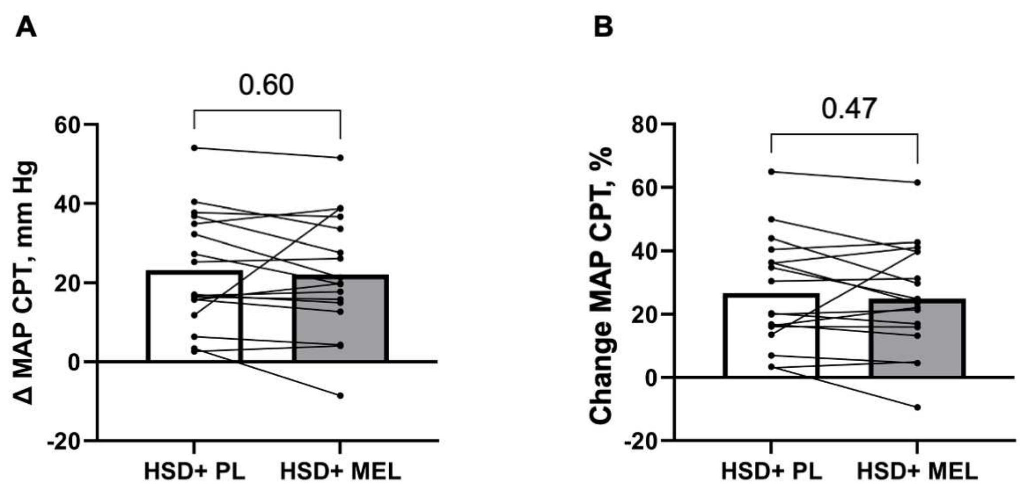

Figure 3.5 Blood pressure responses to the cold pressor test (CPT) at the end of the high sodium diet plus placebo intervention (HSD+PL) and at the end of the high sodium diet plus melatonin intervention (HSD+MEL). A) Mean arterial pressure (MAP) delta change; B) Mean arterial pressure (MAP) percent change. n=17. Data are presented as individual values.

3.4 Discussion

This study was the first randomized crossover, double-blind, controlled trial carried out in humans to investigate the effects of melatonin supplementation on BP reactivity. We hypothesized that melatonin supplementation with a HSD would decrease BP reactivity to three different physiological perturbations compared to a HSD alone. The primary novel finding is that melatonin supplementation does not decrease BP responses to IHG, PEI and CPT during a HSD compared to HSD alone in this cohort of young healthy normotensive humans. Thus, our hypothesis was rejected.

Studies evaluating the effects of melatonin on BP reactivity in humans are scarce and the different perturbations used in these studies make comparisons challenging. In healthy young participants, a one-time 3 mg dose of melatonin did not change beat-to-beat BP responses to IHG (2 min at 30% of their MVC) and CPT (2 min) compared to the placebo intervention [163]. We hypothesized that a higher force production would elicit different results. Therefore, we used 40% of MVC, however we found that our participants, although very close to 40%, could not maintain this level of force, which could have potentially affected our results. We were also interested in evaluating PEI as a physiological perturbation since this measure isolates the metaboreflex from central command and mechanoreflex, which are other components of the exercise pressor reflex (EPR) [257]. Despite not observing any differences in BP reactivity at the end of the placebo and melatonin interventions under PEI, it allowed us a deeper evaluation of the ANS. Cook, et al., [164] found that 3 mg of melatonin did not change beat-to-beat BP responses to head-down rotation, another perturbation to increase sympathetic responses, in the same cohort. Interestingly, both studies reported decreases in MSNA, but not in BP responses [163,164]. The authors hypothesized that melatonin could affect baroreceptor unloading only in specific perturbations, but not to IHG and CPT [163]. Our results are in line with these previous studies, which observed no changes at the end of the melatonin and placebo arms. Future studies should examine BP responses to different perturbations, such as submaximal exercise. Finally, and opposite to our results, 3 mg of oral melatonin attenuated beat-to-beat MAP responses induced by mental stress (mental arithmetic) in young healthy participants [166]. Hence, BP responses may be stimulus specific.

The literature highlights that melatonin may impact sympathetic output through diverse pathways. Some of these pathways overlap with the proposed mechanisms by which a HSD increases BP. First, a HSD is known to increase the excitability of neurons in the rostral ventrolateral medulla (RVLM), while melatonin epigenetically modifies the area postrema neurons, which inhibit the activity of the RVLM [93–95,103,124]. The RVLM is responsible for integration and regulation of sympathetic vasomotor tone; its stimulation causes an increase in sympathetic nervous activity outflow [93–95,100–103]. Second, a HSD may cause increases in extracellular sodium and plasma osmolality, whose levels are sensed by neurons in the circumventricular organs in the brain [99]. These organs are connected to the paraventricular nucleus (PVN) of the hypothalamus, which projects to the RVLM. Melatonin is known to stimulate (GABA)-ergic signaling inhibiting the PVN by the SCN, and thereby, inhibiting the RVLM [124]. Although we did not show any influence of melatonin on BP reactivity under a HSD, future research should evaluate the role that the abovementioned brain structures play in this framework.

Other supplements have been evaluated in the context of BP reactivity. In agreement with our results, beetroot juice supplementation, containing nitrates known to have lowering BP properties, did not lower beat-to-beat BP responses to submaximal exercise in healthy young recreationally trained males [258]. Similarly, anthocyanins, a subclass of flavonoids rich in antioxidants, did not lower beat-to-beat BP responses to CPT and mental stress test in untreated hypertensive young males [259]. In contrary to our results, vitamin E supplementation, a known antioxidant, attenuated increases in BP responses to CPT in healthy young men [260]. However, this intervention was longer than ours (4 weeks) and only recruited males aged 19-24 years old.

We used salt pills to increase sodium intake over two 10-day periods. We evaluated participant compliance to the salt pills via a 24-h urine collection and a 3-day diet record, one day and three days prior to the experimental visit, respectively. We did not observe differences in 24-h urinary sodium excretion, urine osmolality and sodium consumption at the end of the interventions, highlighting that our participants were compliant with taking the salt pills in both interventions. Furthermore, participants consumed similar foods in both interventions as shown by no differences in macronutrient and micronutrient intake during interventions. In particular, we did not observe differences in tryptophan consumption, a precursor of melatonin [249]. We confirmed compliance to the melatonin pills by quantifying 6-sulfatoxymelatonin, increased in the melatonin supplementation group, collected from the urine of the morning of the experimental visit.

Melatonin has also been reported to have antilipidemic effects as it has improved total cholesterol and triglycerides levels in different populations [201,242]. Because of the relation between these lipids and increases in BP, we evaluated lipid levels at the end of both 10-day interventions [201] but we did not observe any changes. We further evaluated sleep and physical activity objectively with actigraphy since they are two important factors that may modulate BP responses [243,244,261–264]. We did not find any differences for any of these two outcomes.

There are several limitations in the current study. We did not control for menstrual cycle phase, so the influence of the different levels of hormones in the studied variables remain unknown. Sodium regulation changes during the menstrual cycle [265]. Estrogen could have also changed BP responses depending on the menstrual cycle phase in which data was collected since estrogen may modulate BP in premenopausal females [266–268]. Another factor that may reduce BP is reduced inflammation, [269,270] which is shown to be decreased by melatonin [214]. We did not evaluate the effect of melatonin on inflammation during a HSD and therefore, do not know if inflammation was reduced. Although prior work has demonstrated a relation between a HSD and an exaggerated BP increase [83,86,88],we could not corroborate this in our study as we did not take baseline measurements. However, our purpose was to compare BP responses to these stressors at the end of melatonin and placebo interventions under a HSD. We did not evaluate the other two mechanisms behind the increases in BP and heart rate elicited by the EPR, which are central command and arterial baroreflex [42]. We did not quantify the change in plasma melatonin levels at night but we assessed melatonin's urinary metabolite, which is highly correlated with the former [113,251]. Lastly, our sample consisted of young healthy normotensive adults and the results are not generalizable to other populations. Further studies could address whether these results are applicable to disease populations.

3.5 Conclusions

In this randomized controlled trial, we sought to determine whether melatonin supplementation could decrease BP responses to three different physiological perturbations under a HSD. Our findings showed that melatonin supplementation did not improve BP responses to IGH, PEI or CPT in young healthy normotensive adults. Future studies should explore a different melatonin dose, evaluate the mechanisms behind these responses, and extrapolate the results to other populations.

Chapter 4

CONCLUSION

4.1 Summary

A HSD is known to cause a multitude of negative physiological consequences even in healthy young normotensive individuals. Animal and human studies have reported impairments in vascular function, partially mediated by increases in free radicals and decreases in antioxidant enzymes [8,9]. Evidence shows that a HSD may increase sympathetic reactivity to various physiological stimuli [8,9]. These consequences can lead to development of HTN and atherosclerosis, contributing to the prevalence of CVD, which remains the number one killer in the U.S [5]. Indeed, a HSD is considered one of the leading dietary CVD risk factors [5]. Despite these health concerns, American dietary sodium consumption continues to remain well above the recommendations [4]. Furthermore, behavioral strategies implemented to reduce sodium intake have been unsuccessful [11].

The use of antioxidants to overcome these consequences is a plausible alternative. Melatonin, an antioxidant hormone secreted mostly from the pineal gland at night, has decreased levels of free radicals in clinical and healthy populations [198–201,203]. Evidence shows that melatonin improved vascular function *in vivo* and *in vitro* [170,172,179,197,214] and modulated the ANS through inhibition of the sympathetic system and of the parasympathetic system [148,161,163]. In this innovative study, we evaluated whether melatonin, could increase vascular function, decrease levels of free radicals and BP reactivity under a HSD. In this young healthy normotensive cohort, we did not observe differences in vascular function, free radicals or BP reactivity at the end of the melatonin supplementation trial and the placebo trial. We did observe, however, decreases in nocturnal SBP accompanied by increases in systolic dipping, as reported in other studies [146–149], in the melatonin trial compared to the placebo. The presence of null findings may be attributed to different reasons. First, the dosage and length of the melatonin intervention might not have been long enough. Second, there is currently no standardization in the assessment of free radicals although the method we used is considered the gold standard. Hence, it is difficult to compare our EPR results with data yielded by other techniques. Third, the study design did not allow for control of the menstrual cycle, which could have an impact on the BP reactivity results. Lastly, the study was not designed to evaluate whether sodium caused the aforementioned negative effects. Hence, melatonin supplementation may not be necessary in this group of young, healthy, normotensive participants.

4.2 Perspectives

Although we did not observe changes in vascular function, oxidative stress and BP reactivity, we observed decreases in nocturnal systolic pressures along with increases in systolic dipping in the melatonin trial. The latest are considered predictors of CVD development, hence melatonin may be protective against the risk of future CVD. Studies should explore other mechanisms that may play in role in vascular function, oxidative stress and BP reactivity, such as inflammation or antioxidant capacity, and a different melatonin dose.

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Appendix A

SUPPLEMENTARY TABLE- THE EFFECTS OF MELATONIN ON VASCULAR FUNCTION, OXIDATIVE STRESS AND BLOOD PRESSURE REACTIVITY DURING A HIGH SODIUM

Table A.1 Screening blood chemistry

Complete blood count	
Mean Corpuscular Volume, fL	88.9 ± 3.4
Mean Corpuscular Hemoglobin, pg	29.7 ± 1.6
Mean Corpuscular Hemoglobin Concentration, g/dL	33.4 ± 0.9
Red Cell Distribution Width, %	12.5 ± 0.7
Platelets, x10^3/uL	253.8 ± 54.6
Neutrophils, %	56.1 ± 8.4
Lymphocytes, %	32.5 ± 8.1
Monocytes, %	7.7 ± 1.8
Eosinophils, %	2.5 ± 1.7
Basophils, %	0.9 ± 0.3
Neutrophils (Absolute), x10^3/uL	3.2 ± 1.2
Lymphocytes (Absolute), x10^3/uL	1.8 ± 0.5
Monocytes (Absolute), x10^3/uL	0.4 ± 0.1
Eosinophils (Absolute), x10^3/uL	0.1 ± 0.1
Basophils (Absolute), x10^3/uL	0.03 ± 0.04
Immature Granulocytes, %	0.1 ± 0.4

Table A.1 continued   Screening blood chemistry

Immature Grans (Absolute), x10^3/uL	0.004 ± 0.02
Metabolic panel	
Total Carbon Dioxide, mmol/L	24.0 ± 1.6
Calcium, mg/dL	9.6 ± 0.4
Total protein, Total, g/dL	7.3 ± 0.4
Albumin, g/dL	4.6 ± 0.3
Total globulin, g/dL	2.7 ± 0.4
Albumin/Globulin Ratio	1.8 ± 0.3
Total bilirubin, mg/dL	0.6 ± 0.2
Alkaline Phosphatase, IU/L	61.7 ± 17.5
AST (SGOT), IU/L	20.4 ± 6.7
ALT (SGPT), IU/L	17.8 ± 9.9

Data are expressed as means ± SD. AST: aspartate aminotransferase; ALT: alanine aminotransferase; SGOT: (serum) glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase. n=22.

Appendix B

IRB APPROVAL LETTER

Institutional Review Board
210H Hullihen Hall
Newark, DE 19716
Phone: 302-831-2137
Fax: 302-831-2828

DATE:	December 3, 2021
TO:	Macarena Ramos Gonzalez, MSc
FROM:	University of Delaware IRB
STUDY TITLE:	[1534734-13] Melatonin and Salt on Blood Vessels Function
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	December 3, 2021
EXPIRATION DATE:	December 17, 2022
REVIEW TYPE:	Expedited Review
REVIEW CATEGORY:	Expedited review category # *(9)*

Thank you for your Continuing Review/Progress Report submission to the University of Delaware Institutional Review Board (UD IRB). The UD IRB has reviewed and APPROVED the proposed research and submitted documents via Expedited Review in compliance with the pertinent federal regulations.

As the Principal Investigator for this study, you are responsible for and agree that:

- All research must be conducted in accordance with the protocol and all other study forms as approved in this submission. Any revisions to the approved study procedures or documents must be reviewed and approved by the IRB prior to their implementation. Please use the UD amendment form to request the review of any changes to approved study procedures or documents.
- Informed consent is a process that must allow prospective participants sufficient opportunity to discuss and consider whether to participate. IRB-approved and stamped consent documents must be used when enrolling participants and a written copy shall be given to the person signing the informed consent form.
- Unanticipated problems, serious adverse events involving risk to participants, and all non-compliance issues must be reported to this office in a timely fashion according with the UD requirements for reportable events. All sponsor reporting requirements must also be followed.

Oversight of this study by the UD IRB REQUIRES the submission of a CONTINUING REVIEW seeking the renewal of this IRB approval, which will expire on December 17, 2022. A continuing review/progress report form and up-to-date copies of the protocol form and all other approved study materials must be submitted to the UD IRB at least 45 days prior to the expiration date to allow for the required IRB review of that report.

If you have any questions, please contact the UD IRB Office at (302) 831-2137 or via email at hsrb-research@udel.edu. Please include the study title and reference number in all correspondence with this office.

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