# CHARACTERIZATION OF THE ANTIHYPERTENSIVE AND ANTIOXIDANT PROPERTIES OF HEMP SEEDS *(CANNABIS SATIVA L.)* AND PROTEIN-DERIVED PEPTIDES IN INDIVIDUALS WITH HIGH BLOOD PRESSURE

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## ABSTRACT

The purpose of this study was to produce bioactive peptides in the form of a hemp seed protein hydrolysate (HPH) and assess the antihypertensive and antioxidant properties in comparison to whole hemp seed protein and casein using a clinical trial design. Bioactive peptides were generated using a previously established method utilizing gastrointestinal enzymes (pepsin and pancreatin) and optimized time, temperature, and pH conditions. Thirty-one individuals with hypertension participated in the study, following a randomized crossover design. They consumed the three treatments (50 g casein, 50 g hemp seed protein, or 45 g hemp seed protein + 5 g HPH) for six weeks each with 2 weeks washout period in between treatments. During the study, 24-hr blood pressure (BP) was measured at various timepoints and blood samples were collected to determine the level of enzymes and biomarkers involved in the BP regulation. The treatment containing HPH showed greater BP lowering effect compared to whole hemp protein and casein. This hypotensive effect was observed for both 24-hr systolic and diastolic BP. Although, we could not differentiate the effects of hemp protein and HPH on angiotensin converting enzyme, renin and nitric oxide, the peptides demonstrated an ability to enhance the levels of superoxide dismutase and catalase while reducing plasma total peroxides and reactive oxygen species to a greater extent than hemp protein and casein. Furthermore, the attenuation of 24-hrBP was correlated with the increased level of epoxy oxylipins, which are involved in the relaxation of smooth muscle cells and subsequently vasorelaxation. These findings propose, for the first time from a clinical trial that hemp seed bioactive peptides may have a potential role as hypotensive and antioxidant agents as an alternative therapy for individuals with hypertension.

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| | |
|---|---|
| 24-hrDBP | 24-hour diastolic blood pressure |
| 24-hrSBP | 24-hour systolic blood pressure |
| AA | Arachidonic acid |
| ABPM | Ambulatory blood pressure monitoring |
| ABTS•$^{+}$ | 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid |
| ACE | Angiotensin converting enzyme |
| ADA | Ardenic acid |
| ADMA | Asymmetric dimethylarginine |
| AL | Alcalase |
| ALA | Alfa linolenic acid |
| ARBs | Angiotensin II receptor blockers |
| Arg | arginine |
| AT-I | Angiotensin I |
| AT-II | Angiotensin II |
| BMI | Body mass index |
| BP | Blood pressure |
| CAT | Catalase |
| Cooh-AA | carboxy-arachidonic acid |
| COX | Cyclooxygenase |
| CVD | Cardiovascular disease |
| CYP | Cytochrome P450 |

| | |
|---|---|
| DBP | Diastolic blood pressure |
| DHA | Docosa hexaenoic acid |
| DGLA | dihomo-gamma-linolenic acid |
| DiHDoPE/DiHDPE; | dihydroxydocosapentaenoic acid |
| DiHETE | dihydroxy-eicosatetraenoic acid |
| DiHETrE | dihydroxy-eicosatrienoic acid |
| DiHODE | dihydroxyoctadecadienoic acid |
| DiHOME | dihydroxy-octadecenoic acid |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| eNOS | Endothelial nitric oxide synthase |
| EPA | Eicosapentaenoic acid |
| EpDoPE | epoxydocosapentaenoic acid |
| EpETE | epoxy-eicosatetraenoic acid |
| EpETrE | epoxy-eicosatrienoic acid |
| EpODE | epoxy-octadecadienoic acid |
| EpOME | epoxy-octadecenoic acid |
| ET-1 | Endothelin-1 |
| FA | Fatty acid |
| FAPGG | *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine |
| FI | Fluorescence Intensity |
| FL | Flavourzyme |
| FRAP | Ferric reducing antioxidant power |

| | |
|---|---|
| GCP | Good clinical practice |
| GIT | Gastrointestinal tract |
| GLA | Gamma-linolenic acid |
| GPX | Glutathione peroxidase |
| GSH | Glutathione |
| GSSG | Glutathione disulfide |
| HDoHE | Hydroxy-docosahexaenoic acid |
| HEPE | hydroxy-eicosapentaenoic acid |
| HHTrE | hydroxy-heptadecatrienoic acid |
| HETE | hydroxy-eicosatetraenoic acid |
| HETrE | hydroxy-eicosatrienoic acid |
| HODE | hydroxy-octadecadienoic acid |
| HOTrE | hydroxy-octadecatrienoic acid |
| HPLC | high-performance liquid chromatography |
| HR | Heart rate |
| HRSA | Hydroxyl radical scavenging activity |
| HSH | hemp seed hydrolysate |
| HSP | Hemp seed protein |
| HSP+ | HSP plus HSP hydrolysate |
| HXB3 | hepoxilin B3 |
| k DPA | keto-docosapentaenoic acid |
| LA | Linoleic acid |

| | |
|---|---|
| LOX | Lipoxygenase |
| LTs | Leukotrienes |
| LPS | Lipopolysaccharides |
| LXA4 | lipoxin A4 |
| MA, | Mead acid |
| MAP | Mean arterial pressure |
| MCA | Metal chelating activity |
| MDA | Malondialdehyde |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NASH | Nonalcoholic steatohepatitis |
| NCT | National Clinical Trial |
| NO | Nitric oxide |
| NON-ENZ | non-enzymatic |
| $O2.^{-}$ | superoxide anion radical |
| $^{\cdot}OH$ | hydroxyl radical |
| $ONOO^{-}$ | peroxynitrite |
| ORAC | oxygen radical absorbance capacity; |
| oxoETE | oxo-eicosatetraenoic acid |
| oxoODE | oxo-octadecadienoic acid |
| oxoOTrE | oxo-octadecatrienoic acid |
| PG | Prostaglandin |
| PGI2 | Prostacyclin |

| | |
|---|---|
| PTPs | Plasma total peroxides |
| PUFAs | Polyunsaturated fatty acids |
| RAS | Renin-angiotensin system |
| RAAS | Renin-angiotensin-aldosterone system |
| RDA | Recommended dietary allowance |
| ROO• | Peroxyl radical |
| ROS | Reactive oxygen species |
| RSA | Radical scavenging activity |
| SBP | Systolic blood pressure |
| SHRs | Spontaneously hypertensive rats |
| SOD | Superoxide dismutase |
| SPE-LC-MS/MS | Solid phase extraction-liquid chromatography MS/MS |
| sEH | Soluble epoxide hydrolase |
| SEM | Standard error of the mean |
| TH | Thermolysin |
| THC | Tetrahydrocannabinol |
| TNF-α | Tumor necrosis factor |
| TriHOME | trihydroxy-octadecenoic acid |
| Tx | Thromboxanes |

# CHAPTER I

# GENERAL INTRODUCTION

## INTRODUCTION

High blood pressure (BP) or hypertension has affected a considerable proportion of the global population and is described as when both the systolic and diastolic BP (SBP and DBP) is consistently above 130/80 mm Hg (1). Epidemiological studies have reported that more than 30% of the world population, which accounts for over one billion people have hypertension (2). According to the American Heart Association report in 2017, nearly 50% of the United States population have hypertension but half of those are not medically diagnosed. High BP is the major risk factor for the occurrence of several serious medical conditions such as cardiovascular disease (CVD), chronic kidney disease, vision impairment, and, cognitive disorder, and is the most common cause of global disability and mortality (3). Primary hypertension accounts for at least 90% of diagnosed cases and several factors including genetic predisposition and environmental risk factors have been identified in developing hypertension, however, the exact cause remains unknown (1,4).

The renin–angiotensin–aldosterone system (RAAS) is the major hormonal cascade that plays a critical role in regulating systemic vascular resistance and BP (5). The first step of the RAAS begins with release of renin from granular cells of the renal juxtaglomerular tissue that is triggered by low BP. Renin stimulates the formation of the hormone angiotensin I by cleaving angiotensinogen produced by the liver. Afterward, angiotensin I generates angiotensin II (Ang II) by the action of the enzyme angiotensin-converting enzyme (ACE) produced in the lungs. Ang II affects BP through several actions that lead to promoting the development of arterial vasoconstriction, increased aldosterone secretion, sodium reabsorption, and sympathetic activity from the central nervous system. Therefore, activation of the RAAS results in BP elevation (6). Nevertheless, endothelial function is controlled by a complex integrated system to maintain normal BP. An increase in BP will result in the activation of the enzyme endothelial nitric oxide synthase (eNOS) by bradykinin (a peptide produced by endothelial cells) to produce nitric oxide (NO) (7).

NO is a well-known neurotransmitter and the most potent vasodilator involved in BP regulation. NO promotes the relaxation of smooth muscle cells in the wall of blood vessels that leads to increased blood vessel dilation. Bradykinin and Ang II modulate NO synthesis in opposite ways where the former stimulates, and the latter inhibits NO production (8). The vasorelaxation mediated by NO counters the BP rise initially induced by RAAS activation (9). However, overactivation of the RAAS or insufficient generation of NO can lead to excessive vasoconstriction and subsequently increased BP. Thus, NO plays a crucial role in the regulation of blood flow and BP and when it is not adequately synthesized, it can result in the development of various pathological conditions that include hypertension (10,11). A number of medical interventions have aimed to address the occurrence of this imbalance in order to prevent or manage pathologic conditions like hypertension.

Pharmacological treatment of high BP is categorized into four main classes according to their site or mechanism of action. One of the most common classes of these medications includes ACE inhibitors, angiotensin II receptor blockers (ARBs), and direct renin antagonists that targets different steps of the RAAS (12). Regardless of the therapeutic advances in hypertension treatment, health-related complications associated with drug interventions are still on the rise (4). Also, consumption of these medications has some side effects that vary from common adverse effects such as cough, excessive low BP (hypotension), fatigue, and azotemia to rare side effects like angioedema (13). Adverse effects and disruption of the patient's daily activities due to treatment or associated negative side effects may result in poor adherence to hypertension medications. Studies have shown that the rate of poor adherence to antihypertensive medications can be as high as 50% and it is estimated that about half of patients discontinue the medical therapy within the first year (14). Medication non-adherence contributes not only to poor BP control but also, to increased risk of cardiovascular morbidity and mortality, and heart failure. As a consequence, the combination of pharmacological intervention and lifestyle modifications are both recommended in the treatment of high BP to lower the health-related risks associated with hypertension and alleviate its impact on a patient's quality of life (15,16). Lifestyle adjustments may be the only necessary intervention in the prevention and treatment of high BP in those with above-optimal BP or stage 1 hypertension without other risk factors (17).

An unhealthy diet, a sedentary lifestyle, obesity, smoking, and excessive alcohol intake are the environmental factors that contribute to hypertension. Hence, adopting a healthy lifestyle that includes a balanced diet, routine exercise, minimum alcohol consumption, and not smoking is the most important approach to the primary prevention of hypertension or addressing the health-related complications associated with high BP (18). Among dietary components, increasing protein consumption and its effect on hypertension prevention and treatment have been investigated in many observational and clinical trials. A meta-analysis of 40 clinical trials involving 3,277 participants in total showed that increased dietary protein intake regardless of animal or plant source was correlated with significant reductions in the average of SBP and DBP when compared with carbohydrates (19).

Numerous research investigations have extensively assessed the effect of the different sources of protein on BP. However, the majority of these studies have only compared protein with a carbohydrate control, and clinical trials directly comparing animal and vegetable sources on protein with respect to BP regulation are scarce. Additionally, the findings from observational studies are inconsistent and cannot distinguish between the superiority of plant or animal protein sources in hypertension prevention or treatment (20). Proteins with plant origin have shown a positive impact on BP in several studies (21–23). INTERMAP, a cross-sectional epidemiological trial on 4680 individuals aged 40 to 59 years in four countries, revealed an inverse association between plant protein consumption and BP (24). In a clinical trial, 40 g of soybean protein consumption compared to complex carbohydrate control, reduced both SBP and DBP (-4.31 and - 2.76 mmHg respectively) in people with mild hypertension (25).

The findings have demonstrated that particular amino acids within the protein structure are mainly responsible for the beneficial effects of proteins in lowering BP. Some examples of these amino acids are cysteine, glutathione (tripeptide), glutamate, and arginine. These amino acids have exerted the potential in lowering or inhibiting various factors that contribute to hypertension, like insulin resistance, reduced synthesis of nitric oxide, abnormal RAAS function, increased production of free radicals such as reactive oxygen species (ROS), and oxidative stress, and increased formation of advanced glycation end products (26). Also, leucine improves skeletal muscle protein synthesis and increases insulin sensitivity by suppressing hepatic gluconeogenesis (27,28). Moreover, taurine and tryptophan have shown antioxidant and anti-

inflammatory properties, which may help to alleviate sympathetic nervous system activity (29,30). As specific amino acids or peptides are primarily responsible for the health advantages of proteins, enzymatic hydrolysis of food protein structure in order to generate bioactive peptides has become increasingly popular in various research studies (31).

Enzymatically derived peptides from food proteins, also known as bioactive peptides, have shown therapeutic potential for numerous degenerative diseases such as CVD, inflammation, and cancer. These peptides have exhibited health-enhancing characteristics that rely on the extent of absorption and bioavailability of their intact forms in selective tissues, which is determined by the peptide composition (31). Several bioactive peptides derived from various animal and plant sources of food proteins have been investigated for their possible health-promoting properties such as antioxidant, anti-inflammatory, antihypertensive, antimicrobial, and immunomodulatory impacts. The most common sources of animal protein utilized for the production of bioactive peptides include milk proteins, casein, and whey as well as egg, fish, and meat proteins whereas the plant protein sources used are soybean, pea, beans, hemp seed, canola, chia seed, and flaxseed (32–42). However, in comparison to other plant sources of protein, hemp seed protein (HSP) derived bioactive peptides are less known in terms of their possible positive effects on health (43).

Hemp seed (*Cannabis sativa L.*) is a plant from the Cannabaceae family that is believed to have Central Asian origin. Hemp seeds contain 20-25% protein which consists of albumin and edestin with all essential amino acids and in particular high levels of arginine and glutamine (44). Unlike most plant sources of protein for instance soybean, HSP is highly digestible (45). Some studies have suggested that HSP-derived bioactive peptides may possess functional properties with therapeutic potential in chronic diseases such as hypertension (46). In spite of that, the bioactive ingredients derived from HSP have not been commercially utilized as health promoting agents in Canada. On the other hand, the existing literature on HSP bioactive peptides is very narrow and limited to *in vitro* and animal studies and there is no clinical trial conducted on the effects of these peptides in humans. The lack of extensive research on HSP and subsequently its bioactive peptides is mainly because of the historical restrictions on the cultivation of hemp products due to the presence of a psychoactive compound known as tetrahydrocannabinol (THC). After almost

60 years, in 1990, Canada lifted the limitations of hemp crops for varieties with THC levels of less than 0.3% (44,47).

Despite the increasing interest in exploring the impacts of HSP since then, it still requires further clinical investigations first to validate the observed preclinical health effects and second to unravel the underlying mechanisms by which HSP and its bioactive peptides exert their beneficial impacts. Also, HSP-derived bioactive peptides may have additional or unique physiological functions that are independent of HSP and need to be clarified. So, in our study, hypertensive men and women incorporated 50 g/d protein from HSP, HSP with added bioactive peptides or casein control for 6 weeks each, in a double-blind, randomized, crossover, controlled trial with the focus on 24-hr SBP and DBP and circulating biomarkers involved in BP regulation. This study was conducted at the Richardson Centre for Food Technology and Research (RCFTR) in Winnipeg, Manitoba.

**OBJECTIVES**

The main goal of this research is to broaden the overall understanding of the effects of HSP on BP and generate knowledge on HSP hydrolysate-derived bioactive peptides consumption in individuals with high BP and compare their effects with casein. This study will focus on the blood-circulating biomarkers associated with BP regulation.

Specific objectives were to:

1. Evaluate the impact of two interventions: consuming 50 g/day of HSP alone or in combination with bioactive peptides over a 6-week period, in comparison to a casein control, on 24-hour SBP and DBP.
2. Assess the effects of HSP alone versus HSP combined with bioactive peptides on 24-hr SBP and DBP.
3. Determine the impacts of HSP treatments, relative to casein, on plasma enzymes associated with BP regulation.
4. Investigate the effects of HSP treatments, on circulating antioxidant or oxidative biomarkers connected to hypertension, compared to casein.
5. Examine the link between blood oxylipins and BP after consumption of HSP treatments, relative to casein.

## HYPOTHESES

The proposed hypotheses to be investigated are:

1. Consumption of HSP alone or together with HSP-derived bioactive peptides will reduce the 24-hr SBP and DBP compared to casein in hypertensive individuals.
2. Consumption of HSP plus HSP-derived bioactive peptides will have a greater lowering effect on the 24-hr SBP and DBP compared with HSP intake.
3. Consumption of HSP treatments, relative to casein, will downregulate plasma enzymes involved in hypertension.
4. HSP treatment intake will modify antioxidant or oxidative biomarkers associated with BP regulation compared to casein.
5. Consumption of HSP treatments, relative to casein, will alter circulating oxylipin-derived metabolites known to be involved in BP regulation.

## SIGNIFICANCE

Our research is anticipated to assist with the advancement of knowledge on plant sources of proteins and in particular hemp seed. The research on hemp seed is considered an innovative area especially in Canada as one of the largest hemp producers in the world. Furthermore, the use of hemp seed as a sustainable source of protein has gained a growing interest in exploring its various health-related benefits such as its potential effects in hypertension management. On top of that, hemp seed has shown the ability as a promising product for the generation of bioactive peptides with enhanced beneficial properties. Knowledge expansion in this area not only will aid in the prevention and treatment of health issues but also will provide a tremendous opportunity for economic growth and help Canada's position as a leader in the global hemp industry.

## OUTLINE OF THIS THESIS

This thesis adopts a manuscript style, comprising four manuscripts following the General Introduction (Chapter I) and Literature Review (Chapter II). Chapter II presents recent literature on the effects of plant proteins and derived bioactive peptides, focusing on their antioxidant and antihypertensive properties. The initial manuscript (Chapter III) delineates our study protocol, while the subsequent research manuscripts focus on distinct objectives. Chapter IV delves into the antihypertensive effects of hemp seed protein and its hydrolysate, covering objectives 1, 2,

and 3. Chapter V addresses the antioxidant effects as per objective 4, and Chapter VI examines oxylipin profile analyses related to objective 5. Manuscript 1 has been published, Manuscript 2 is undergoing final peer review, and Manuscripts 3 and 4 are in submission. Transitions between chapters ensure a cohesive flow throughout the thesis. Chapter VII (General Discussion and Conclusion) summarizes the thesis, highlighting key findings and the novelty of the research. It concludes by addressing the limitations and suggesting future directions.

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## TRANSITION STATEMENT 1

In chapter I, we delved into the complexities of hypertension, unraveling the role of RAAS and the crucial significance of NO in blood pressure regulation. Addressing this global health concern involved exploring the impact of lifestyle changes, dietary choices, and the challenges associated with poor adherence to hypertension medications. In the following chapter, we shift our focus to plant proteins and bioactive peptides, delving into their antioxidant and antihypertensive properties. Our lens narrows from the systemic effects of hypertension to the molecular level interactions. Chapter II introduces us to the diverse world of dietary plant proteins, uncovering their functional attributes, and highlighting the emergence of bioactive peptides as potential therapeutic agents. We aim to reveal the potential of these plant-derived bioactive peptides in managing oxidative stress and hypertension. Our exploration extends beyond theoretical considerations to the practical realm, emphasizing the need for clinical trials and technological advancements. While chapter I highlighted the global impact and challenges of hypertension, chapter II provides a closer examination of the promising avenues within plant-based nutrition. We are set to move intentionally into practical insights and potential actions, holding the promise of gaining a thorough understanding of hypertension and discovering inventive approaches for its effective management.

# CHAPTER II

## MANUSCRIPT 1

## PLANT PROTEINS AND BIOACTIVE PEPTIDES: ANTIOXIDANT AND ANTIHYPERTENSIVE PROPERTIES

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**ABSTRACT**

Plant proteins have been widely recognized as healthier alternatives to animal sources of protein. The beneficial effects of plant proteins on human health have always been the center of attention from different perspectives such as their protective effects against high blood pressure and oxidative stress. Consumption of proteins of plant origin provides ample health-related benefits while they are typically more sustainable, safe, and cost-effective compared to animal sources. The advantages of plant proteins have made them great candidates for consumption in diets and from which to generate bioactive peptides with enhanced properties when compared to the parent protein. Since plant protein-derived bioactive peptides possess various significant functions including antioxidative and antihypertensive effects in the human body, they are considered promising ingredients with great potential to act as health-promoting agents. The most common methods to produce bioactive peptides are enzymatic proteolysis, chemical hydrolysis, fermentation, genetic engineering techniques, and traditional chemical synthesis. The technique, enzyme type, protein substrate, enzyme: protein ratio, and hydrolysis conditions used are all factors that affect the peptide composition of the resulting hydrolysate. Antioxidant properties of bioactive peptides are associated with their radical scavenging, inhibition of lipid peroxidation, and metal ion chelation activities while their blood pressure reducing activities occur mainly through inhibition of angiotensin-converting enzyme and renin activities. The objective of this review is to discuss the antioxidant and hypotensive activities of plant-derived protein bioactive peptides and the mechanism by which they exert their health-promoting and disease-preventing properties. In addition, some knowledge gaps and suggestions for further research will be discussed.

## DIETARY PLANT PROTEINS AND HEALTH BENEFITS

The two major protein sources in the diet are animal and plant materials. The demand for protein is of special attention due to the impressive health benefits associated with consumption including curbing hunger, maintaining a healthy body weight, preventing muscle breakdown, improving cardiovascular health, and diabetes management (1). The push for the supply of high protein content products in the marketplace on the one hand and the adverse environmental impacts associated with animal protein production on the other hand, have made considering the consumption of more plant-based proteins crucial. Based on the current dietary guidelines, the recommended dietary allowance (RDA) of protein for an adult is set at 0.8 g per kg of body weight; however, it is recommended that 10-35% of daily calories come from protein to meet the requirements of individuals with different needs and choices.

There are RDAs established for the total protein and each essential amino acid intake, which can be fulfilled by consuming different protein sources. However, nutrition recommendations do not offer guidance on the proportionate inclusion of animal and plant proteins in the human diet (2,3). Other than less negative ecosystem consequences correlated with the consumption of proteins from plant sources, the production of plant proteins is much more cost-effective when compared to animal proteins from the consumer's perspective (4). In addition, cultural and religious affiliations and ethical beliefs have resulted in consumers' preference for plant proteins over animal sources (5).

It is also important to keep in mind that plant protein sources are linked with many health benefits further due to the existence of non-protein constituents within the food matrix such as micronutrients (vitamins and minerals) and bioactive compounds like the polyphenols (6). Plant-based proteins can come from one of these four groups: nuts (almond, walnut, and hazelnut), legumes (chickpea, lentil, and soy), seeds (hemp, sesame, and pumpkin), and cereals (rice, oat, and barley) (7). Dietary guidelines provide clear advice on protein consumption of foods mostly of plant origin. For instance, the Canadian Diabetes Association's clinical practice has recommended the consumption of dietary pulses to help maintain healthy glycemic levels in patients with type 2 diabetes (8). As a result, environmental sustainability, economic efficiency, and health-related benefits linked to plant proteins have made plant-based sources of protein excellent alternates to animal proteins.

The long-term health-promoting and chronic disease-preventing properties of plant proteins have been extensively evaluated in the last few years. Numerous studies have focused on the potential impact of plant proteins on lowering cardio-metabolic risk factors and they are suggested as an effective alternative to animal proteins in the diet to help decrease cardiovascular disease risk factors in the adult population (9). A meta-analysis of randomized control trials revealed that the replacement of 35% of dietary protein with plant-based equivalents instead of animal proteins resulted in significant improvements in diabetes markers including HbA1c, fasting glucose, and insulin levels (8). The association between the risk of colorectal cancer with the type of protein in the diet has been evaluated in a prospective Danish cohort where the authors reported that substituting animal proteins with plant proteins in the diet may be a preventive approach for colorectal cancer in individuals with same genetic polymorphism (10).

A large number of studies with a focus on the functional properties of plant proteins have shown the beneficial effects of plant protein ingredients on postprandial blood glucose, insulin, and/or hunger and satiety-regulating hormones (11,12). In addition, the research evaluating the potential link between protein consumption and mortality has demonstrated a significant inverse correlation between plant protein intake and all-cause mortality by 10% and cardiovascular and stroke-related mortality in both genders (13,14).

Proteins from vegetable sources like beans, nuts, seeds, and cereals have also exhibited antioxidant and antihypertensive activities in several studies (15,16). The legumes that are widely studied for their antioxidant activities are lentils, peas, chickpeas, and beans (17). Various tools have been used to identify the free radical scavenging potential of different plant-based proteins including the hydrogen-donating compounds that are neutralizers of aqueous phase radicals and chain-breaking compounds that act as antioxidants toward lipid peroxyl radicals (18,19). The amino acid constituent of the protein is responsible for the antioxidant activity of the protein. Aromatic amino acids such as tyrosine, phenylalanine, and tryptophan, and the sulfur-containing amino acid such as cysteine, are recognized as antioxidant agents due to their potential to donate protons to neutralize toxic free radicals (20).

Multiple studies have evaluated and compared the blood pressure (BP)-reducing effects of plant versus animal proteins. For instance, the cohort Chicago Western Electric Study followed more than 1700 men for 8 years and found out that plant-based protein consumption was inversely

associated with both systolic and diastolic blood pressure (SBP and DBP, respectively) whereas intake of the animal proteins was positively correlated with BP (21). Similar to this research, the results from the PREMIER lifestyle modification study on mildly hypertensive US adults demonstrated a negative association between plant protein intake and BP. However, there was no correlation with animal protein consumption (22). Similarly, the INTERMAP (International Population Study on Macronutrients and Blood Pressure) study showed a negative association between plant protein intake and BP. This multicenter research was carried out in four different countries (United Kingdom, United States, China, and Japan) and evaluated more than 4500 men and women (16).

The functionality of a protein depends on its intrinsic and/or extrinsic physicochemical properties that are influenced by molecular characteristics such as surface charge and hydrophobicity, molecular weight, and secondary, and tertiary structures in their intact states (23–25). On a global scale, the high demand for plant proteins is positively associated with the demand for high quality and functionality of food proteins. Thus, novel protein modification approaches that have the ability to modify the chemical, biophysical, and surface-active characteristics of proteins and subsequently enhance their functionality are recommended. Such protein-manipulating approaches that can improve protein ingredients include protein extraction, isolation, fractionation, and modification (1,26).

## BIOACTIVE PEPTIDES

Bioactive peptides can be produced as a result of protein manipulation techniques. The smaller size of these peptides distinguishes them from the parent protein, however, antioxidant and antihypertensive properties of bioactive peptides are other distinguishing factors that are altered during the hydrolysis process (27,28). Bioactive peptides are naturally encrypted inside the parent protein structure and need to be released through distinct techniques and commercialized as nutraceuticals to be able to exhibit their functional properties.

## TECHNOLOGIES TO PRODUCE BIOACTIVE PEPTIDES

Bioactive peptides are conventionally isolated during enzymatic proteolysis through gastrointestinal digestion or by using *in vitro* proteolytic enzymes or chemical hydrolysis. Microbial fermentation, genetic engineering techniques, and traditional chemical synthesis are

other approaches used in the production of bioactive peptides (29). These methods belong to one of the two categories: top-down and bottom-up methods. The top-down method refers to the generation of bioactive peptides through the separation and purification of raw protein such as the enzymatic hydrolysis method whereas the bottom-up process involves using amino acids in synthesizing specific polypeptide sequences including chemical synthesis and genetic techniques. The enzyme-catalyzed hydrolysis of protein is the most commonly utilized method in the production of bioactive peptides.

Commercially, pancreatin, trypsin, papain, pepsin, alcalase, chymotrypsin, bromelain, and pronase are widely used enzymes to convert proteins into bioactive peptide sequences (30). The optimum pH, temperature, and duration of enzyme activity are crucial factors to consider when preparing protein hydrolysates. For instance, Girgih et al. (2014) utilized pepsin and pancreatin to generate bioactive peptides from hemp seed proteins. In their study, hemp seed protein was treated with pepsin at pH 2.0, 37°C, and 2 h. Subsequently, it was further hydrolyzed with pancreatin at pH 7.5, 37°C, and 4 h to simulate the digestive process in the gastrointestinal tract (GIT). Following hydrolysis, the resulting digest was centrifuged, and the solid residue discarded. The supernatant was freeze-dried to obtain a protein hydrolysate (31). The enzyme specificity determines the enzyme-protein interactions and thus the hydrolysate profile and the number and size of the peptides that are produced. In addition to the enzyme used for hydrolysis, the protein substrate and hydrolysis conditions such as temperature, pH, reaction time, enzyme:protein ratio, and ion concentration are all factors that affect the hydrolysate peptide composition (30).

The functional properties of bioactive peptides are associated with their structure, and therefore, the condition under which the hydrolysate is produced is the key determinant of their functional attributes (32). To be more specific, health-promoting properties and biological activities of bioactive peptides are influenced by their distinct characteristics such as amino acid composition, sequence, size, charge, structure, and their interaction with each other and other molecules such as carbohydrates and lipids (Lacou et al., 2016). For example, the effectiveness of antioxidant peptides is mainly due to the presence of specific types of amino acids, such as hydrophobic (Leu, Val, Ala, Pro, Phe), aromatic (Tyr, Trp, His), sulfur-containing (Cys, Met), acidic (Glu), and basic (Lys) amino acids. Hydrophobic amino acids enhance the solubility of peptides in lipid

environments and increase interactions between the peptides and free radicals, accordingly promoting their ability to suppress lipid peroxidation. In addition, hydrophobic amino acids improve the contact between peptides and polyunsaturated fatty acids. Other amino acids that possess phenolic hydroxyl like Tyr and indolyl groups like Trp directly act as hydrogen donors, seize free radicals and effectively scavenge them. The formation of dense aromatic rings in Phe, Tyr, and Trp residues of peptides enhances the chelation of metal ions by the peptides. The presence of imidazole ring in histidine with hydrogen and electron donating ability is responsible for strong metal ion chelating, hydroxyl radicals scavenging and lipid peroxidation inhibiting capacities of peptides containing His residues. Also, adding Pro and Leu to the N-terminal of dipeptide (His-His) considerably improved the antioxidant capacity of peptides by synergistically incorporating the antioxidant properties of hydrophobic and aromatic amino acids (33,34).

Moreover, it has been shown that many bioactive peptides have the capacity to affect different targets and elicit various physiological impacts and are called multifunctional peptides. Multifunctional peptides exert at least two distinct properties that could include antioxidant, anti-inflammatory, hypotensive, anti-diabetic, hypocholesterolemic, immunomodulatory, and antibacterial activities (35). As an example, bioactive peptides derived from soybeans have exhibited both hypocholesterolemic and hypoglycemic, while peptides obtained from hemp seeds and lentil proteins have displayed antioxidant properties along with the ability to lower blood pressure (36–39). Compared to monofunctional peptides, multifunctional peptides are superior due to their cost-effectiveness and lower adverse impacts (35).

Bioactive peptides which consist of protein fragments of 2-20 amino acids with low molecular weight, exert their health-promoting effects on bodily functions including cardiovascular, immune, nervous, and digestive systems (40,41). When ingested, the intestinal barrier allows the smaller peptides to cross the membrane intact into the blood flow utilizing a peptide carrier system called peptide transporter 1 (PepT1). PepT1 is the main transporter involved in dipeptide and tripeptide absorption and facilitates their uptake from the intestinal lumen into the intestinal cells against their concentration gradient (42). In comparison to smaller peptides, absorption of larger peptides consisting of more than three amino acids can be mediated by a receptor or non-receptor-mediated endocytosis. Also, these peptides can cross the epithelial barriers intact through paracellular or transcellular pathways (43). The stability and bioavailability of the

bioactive peptides depend on their structural characteristics including amino acid profile, chain size, and hydrophobicity. Hence, the food matrix into which bioactive peptides are incorporated is another determining factor that can affect the bioaccessibility and bioavailability of bioactive peptides through chemical modifications (44,45).

## BIOACTIVE PEPTIDES OF PLANT ORIGIN, FUNCTIONAL PROPERTIES, AND BIOLOGICAL ACTIVITIES

Enzymatic hydrolysis can enhance protein functionality by producing a broad variety of bioactive peptides (46). Plant sources have priority over animal-based proteins in bioactive peptides production owing to the fewer risks associated with their consumption, nutritional benefits, economically reasonable costs, and large industrial-scale production. Health-enhancing and disease-risk reduction potential of plant proteins has gained increasing interest in the last few years particularly due to their antioxidant, antihypertensive, antidiabetic, hypocholesterolemic, immunomodulatory, antimicrobial, opiate, and hepatoprotective properties (47). However, the beneficial effects of plant-based bioactive peptides are not limited to physiological activities and lowering the risk of chronic diseases. They also possess several important functional characteristics including emulsifying and foaming capacities, and water and oil retention properties due to the hydrophilic/lipophilic properties, structural adaptability, and size and sequence of amino acid composition. Hence, plant-derived bioactive peptides have great potential to be utilized in designing novel functional foods and nutraceuticals (48–50).

## ANTIHYPERTENSIVE EFFECTS OF PLANT-BASED BIOACTIVE PEPTIDES, MECHANISM OF ACTION

Primary (essential) hypertension is one of the most important public health issues that affect 1.13 billion of the world's adult population. The etiology of hypertension is multifactorial, but gene-environment interactions are the major contributing factors (51,52). Studies have demonstrated a strong association between uncontrolled high BP and subsequent serious health complications such as cardiovascular diseases (CVDs) including heart attack, stroke, and heart failure as well as kidney disease, peripheral vascular disease, and vision loss (US Institute of Medicine Committee on Public Health Priorities to Reduce and Control Hypertension, 2010). Hypertension has also been recognized as one of the major preventable risk factors for death, and therefore, it is of

utmost importance to control high BP to avoid hypertension-induced cardiovascular and renal morbidity and mortality (53).

Despite the global burden and the considerable research on hypertension, progress in hypertension management has been slow. This has been shown by the worldwide trends in hypertension prevalence owing to the fact that current medications are reasonably effective so, there is a lack of scientific discoveries and new inventions related to hypertension control (54). Commonly prescribed medications used to lower BP have some side effects including cough, diarrhea or constipation, dizziness, tiredness, headache, depression, and erectile dysfunction (55). The adverse effects contributed by these drugs are the most important causes of poor medication adherence (56).

In long-term surveillance of hypertensive patients in terms of their adherence to antihypertensive medications, 9.3% of the patients reported severe adverse effects of the medications as the causes of non-compliance. Hence, an additional 23.4% of the patients' non-adherence to the medication occurred as a result of possible side effects. Although no death was reported in correlation with the medications' side effects, almost 1% of the patients were hospitalized due to the adverse effects of hypotensive drugs (57). Thus, the side effects of current BP-reducing medications on one side and the possible risks associated with the non-adherence to hypertension therapy on the other side justify and necessitate the application of alternative approaches that are safer with less or no side effects in patients with hypertension. Therefore, the identification and promotion of functional foods as therapeutic substitutions with protective effects against high BP and its metabolic consequences are of substantial interest (58). Such compounds with a low risk of harmful side effects and the potential to express targeted responses with low toxicity even when delivered at high doses play a significant role in hypertension research and therapy (43).

It is globally accepted that exercise and weight control are the first-line treatment strategies for hypertension, however, the importance of plant protein consumption has not yet reached a consensus. The results from one cross-sectional study comparing sedentary vegans with endurance athletes on a Western diet have revealed that a plant-based diet had a more BP-reducing effect when compared to physical activity (59). Antihypertensive effects of plant protein-derived bioactive peptides have been explored by focusing on their molecular targets and the underlying mechanism by which bioactive peptides show their beneficial effects against high

blood pressure. To understand the molecular targets of antihypertensive peptides, first we need to know the pathophysiology of hypertension (60).

The renin-angiotensin system (RAS) is a hormonal system that gets activated when blood pressure drops. Renin is the first component of the RAS that is released from the kidneys in response to hypotension. Renin then acts to cleave angiotensinogen - a plasma protein produced by the liver into angiotensin I (AT-I). The conversion of AT-I to angiotensin II (AT-II) is catalyzed by angiotensin-converting enzyme (ACE), which is expressed primarily in the lungs. AT-II is a hormone and it binds to the AT-II receptors in blood vessels and tissues to exert various effects. AT-II is a potent vasoconstrictor and acts directly on vascular muscle cells to increase peripheral resistance and ultimately raise BP. ACE is also involved in the cleavage of bradykinin (a vasodilator), resulting in vascular contraction without adequate relaxation (61,62).

In terms of antihypertensive properties of plant-based bioactive peptides, arginine is a non-essential amino acid that has been particularly studied for its indirect vasodilatory effect through the production of nitric oxide (NO). As a vasodilator, NO stimulates the blood vessels to relax, causing increased blood flow, leading to BP reduction. The concentration of arginine in various plant-based proteins and protein hydrolysates is different, which could be one of the contributing factors to their different BP-reducing potentials (3). In the INTERMAP study, the intake of certain amino acids including glutamate, cysteine, proline, phenylalanine, and serine was considerably higher in the participants who consumed high plant but low animal proteins. The results showed that both the SBP and DBP were significantly lower in this group of participants when compared with the volunteers that consumed low plant but high animal protein intakes (16).

Table 2.1. represents various studies about the antihypertensive effects of bioactive peptides from plant protein sources. The literature on the antihypertensive effects of plant proteins and their bioactive peptides are mainly based on *in vitro* and animal studies (31,62–72) while the number of clinical trials investigating these effects in human, particularly hypertensive patients is limited (73–75). The hypotensive potential of plant-based bioactive peptides particularly on hypertensive rats or human volunteers compared with normotensive animals or human subjects suggests that these peptides could be mainly used as the initial approach to relieve hypertension, especially at the stage 1 or prehypertension condition. A review of the studies presented in Table 2.1. reveals

that plant-based bioactive peptides mainly target the components involved in RAS to exhibit their BP-reducing effects. The number of studies showing ACE-inhibitory effects is more than those that proved the effects of bioactive peptides on renin concentration. Therefore, it seems that ACE is the major target for plant-based peptides to exert their inhibitory effects. Moreover, vasorelaxation molecules involved in the regulation of blood pressure such as NO are considered as the potential targets for BP regulating effects of plant-derived bioactive peptides.

## ANTIOXIDANT EFFECTS OF PLANT-BASED BIOACTIVE PEPTIDES AND MECHANISM OF ACTION

Oxidative stress denotes the imbalance between the production of oxidative compounds and antioxidant defense where the production of free radicals is excessively more than the body's antioxidant-protective mechanisms, which subsequently causes organ and molecular damage (76). A large body of research has demonstrated that an augmented level of free radicals and insufficient antioxidant defense systems are correlated with the development of many chronic conditions such as cardiovascular diseases, cancer, diabetes, and neurodegenerative and inflammatory diseases (77). Fig. 2.1 demonstrates the initiation of reactive oxygen/nitrogen species and free radicals generation, their cell-destructive and tissue-damaging activities, and consequently the development of chronic diseases. It also illustrates the points of bioactive peptides' actions and their inhibitory effects (78,79).

Various endogenous sources including mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells, etc., and exposure to environmental toxins such as cigarette smoke, UV and ionizing radiations, pollutants, heavy metals, and drugs are responsible for the production of free radicals (80–82). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the two categories of free radicals produced in mammals. Although the excessive production of ROS and RNS leads to the reaction of these molecules with lipids, protein, and nucleic acids to cause pathologic conditions and cellular and tissue damage, maintaining a balanced and non-toxic level of ROS plays a vital role in redox signaling in the body. In fact, ROS play a dual role in the regulation of physiological functions, cell signaling, and homeostasis (83,84). Redox signaling includes a wide range of mechanisms that are involved in the regulation of gene expression, metabolic pathways, and signal transductions. The structure and function of proteins depend on the oxidized or reduced state of cysteine residues of proteins mediated by redox reactions

(Fomenko et al., 2008). Formation of toxic and highly reactive oxygen species including superoxide anions (O2•-), hydrogen peroxide (H2O2), hydroxyl radicals (OH•), hydroperoxides (ROOH), alkoxy-peroxy radicals (ROO•, RO•) and peroxynitrite (ONOO-) occurs as a result of an imbalance in the cellular redox system (78,84).

The endogenous antioxidant system is involved in the prevention or reduction of the detrimental effects of oxidative stress on human health. This endogenous system consists of non-enzymatic defense that includes vitamins such as vitamins A, E (α-tocopherol), and C (ascorbate), glutathione, and uric acid as well as antioxidant enzymes including catalase, glutathione peroxidase (GPX) and superoxide dismutase (SOD) (85,86). Under the condition of overproduction of oxidants, neutralizing effects of the endogenous defense system are not entirely sufficient to cope with oxidative stress thus, exogenous antioxidants play a significant role in scavenging the body from free radicals damage (87). Various plant protein sources have exhibited antioxidant activities though hydrolysate-derived bioactive peptides of various fractions may demonstrate higher antioxidant properties (88).

The presence of certain amino acids, such as aromatic (Try, Tyr, and Phe), imidazole (His), and sulfur-containing (Cys and Met) groups, enhances the antioxidative properties of peptides. Since the functionality of the protein source is determined by the sequence of peptides releases in the body, generating tailored bioactive peptides with specific sequences involved in antioxidative mechanisms may strengthen the antioxidative capacity (88). Bioactive peptides particularly from plant sources are of much interest for their ability to exert radical scavenging and metal chelating properties (47). The isolation and further fractionation techniques by which bioactive peptides are produced as well as the protein source from which the peptides are extracted define the antioxidant properties and the extent of their ability to exert these functions (89). For instance, proteolysis of native or heated soy protein with different enzymes including pepsin, papain, chymotrypsin, Alcalase (AL), Protamex, Thermolysin (TH) and Flavourzyme (FL), yielded peptides with different degrees of hydrolysis varying from 1.7 to 20.6%, which subsequently affected their antioxidant activities ranging from 28 to 65% (90).

The protective effects of bioactive peptides against oxidative damage have been widely studied by scientists around the world. Various studies have been carried out to investigate the antioxidant activities of bioactive peptides from plant sources such as peas (91,92), hemp seed

(71,93), date seed (94), potato (95,96), mung bean (97), okra seed (98), chia (72), hemp bran (69), sesame seed (64), and black soy (75). Table 2.1 presents several studies about the antioxidant effects of bioactive peptides from plant protein sources. Pownall et al. (2010) investigated the antioxidant properties of HPLC-fractionated bioactive peptides from pea protein hydrolysate. The fractions with higher contents of hydrophobic and aromatic amino acids eluted later from the HPLC column. In addition, these fractions showed the strongest radical scavenging (DPPH•, OH•, and O2•- and H2O2) and metal chelating properties. Moreover, linoleic acid oxidation was inhibited by these peptide fractions at a significantly higher level than glutathione. This study concluded that the key determinant of the antioxidant activity of peptides derived from pea protein hydrolysates depends on the amounts of their constituent hydrophobic and aromatic amino acids.

Ambigaipalan and Shahidi (2015) in their research on antioxidant properties of date seed protein prepared hydrolysates by using AL, FL, and TH through individual or sequential treatment. They found that the incorporation of hydrolysates in salmon inhibited oxidation by 30% compared to butylated hydroxytoluene with a 9% inhibition rate. Moreover, hydrolysates extracted by using a combination of FL and TH exhibited the highest inhibitory effects on β-carotene oxidation (73.7%). In addition, hydroxyl and peroxyl radical-induced DNA strand scission was inhibited by both hydrolysates and carnosine.

According to the studies that have been carried out, plant proteins act as antioxidant agents through various mechanisms. These mechanisms include 1) hydrogen or electron-donating activities to neutralize ROS/RNS and free radicals, which is related to their peptide bonds and hydroxyl groups; 2) suppressing pro-oxidant enzymes and metal ion chelating activities, which inhibits the formation of ROS/RNS and free radicals; 3) altering enzymatic and non-enzymatic antioxidant gene expressions, which leads to upregulating the functions of endogenous stress and hypertension intricately contribute to the occurrence and advancement of each antioxidant systems including GPX, SOD, catalase, and ascorbate (99).

## INTRICATE RELATIONSHIP BETWEEN OXIDATIVE STRESS AND HYPERTENSION

Oxidative stress and hypertension are intricately associated with each other, and one condition can lead to another and vice versa. Oxidative stress arises when the generation of ROS exceeds

the capacity of the body to offset or neutralize them. Where the body's defense mechanisms are not able to inhibit the chronic and uncontrolled production of ROS, these molecules can result in cell and organ damage and consequently, various pathological conditions such as hypertension and other cardiovascular diseases occur (100).

## OXIDATIVE STRESS AND HYPERTENSION

Multiple mechanisms are involved in the development of hypertension through oxidative stress. Firstly, ROS have the potential to impair the endothelial cells in the blood vessel walls and inhibit their ability to produce NO through endothelial dysfunction. Secondly, oxidative stress initiates the release of pro-inflammatory factors by triggering inflammatory responses that can lead to blood vessel constriction and hypertension. Lastly, oxidative stress contributes to activation of the sympathetic nervous system resulting in elevated heart rate and increased BP (100).

## HYPERTENSION AND OXIDATIVE STRESS

Hypertension itself can contribute to the progression of oxidative stress. On one hand, high BP amplifies ROS generation in the involved tissues like the vascular system and kidneys while on the other hand decreases the antioxidant enzymes level such as SOD and GSH, which consequently reduces the body's ability against the overproduction of ROS. Additionally, the organ injury that occurs in hypertension leads to oxidative stress and inflammation (101).

## MULTIFUNCTIONAL PEPTIDES IN TREATMENT OF OXIDATIVE STRESS AND HYPERTENSION

The complex relationship between oxidative stress and hypertension and the occurrence of both simultaneously provides a compelling rationale for the use of versatile treatment strategies such as multifunctional peptides to address both conditions concurrently. Multifunctional peptides can be designed to demonstrate antioxidant, anti-inflammatory, and vasodilatory properties. When specific multifunctional peptides target oxidative stress and the mechanisms linked to it, they can enhance antioxidant capacity, alleviate inflammation, and re-establish endothelial functions, which ultimately result in BP reduction. Moreover, they can directly lower BP through their inhibitory impact on the sympathetic nervous system and vascular tone. Targeting both oxidative stress and hypertension with a single bioactive peptide has the advantage of receiving synergistic

benefits and amplifies the therapeutic effectiveness compared to addressing each condition distinctly (102,103).

## CONCLUSION AND FUTURE RESEARCH

The popularity of plant protein consumption is growing rapidly thus, the demand is increasing. This trend is expected to grow even more just as the research exploring innovative strategies to generate plant proteins with high quality and enhanced functionality. Bioactive peptides from plant-based foods are valuable sources of functionality beyond their nutritional value.

Despite a vast body of research supporting the beneficial effects of plant-based protein and bioactive peptides against high BP and oxidative stress, the number of clinical trials investigating these effects in humans is limited. The lack of compatibility between some *in vitro* and *in vivo* studies evaluating the physiological and functional properties of plant-based bioactive peptides could be related to the effects of proteases in the gastrointestinal system that breaks down the peptides' structure and lower their bioactivity. Designing clinical trials that investigate the bioavailability of peptides in the body and also developing technologies to enhance the oral bioavailability of bioactive peptides need fundamental research.

Moreover, the most studied food-derived peptides are the bioactive peptides produced from dairy sources and the research on plant protein-derived bioactive peptides is just emerging. In addition, the mechanisms by which bioactive peptides exert their antihypertensive and antioxidant properties are not fully recognized. Identifying the exact components of bioactive peptides and the conditions under which they are produced may help to determine the exact underlying mechanisms associated with their health-promoting activities. Hence, identifying the peptides with multifunctional properties will be of importance in studying the mechanisms by which they exert their beneficial effects.

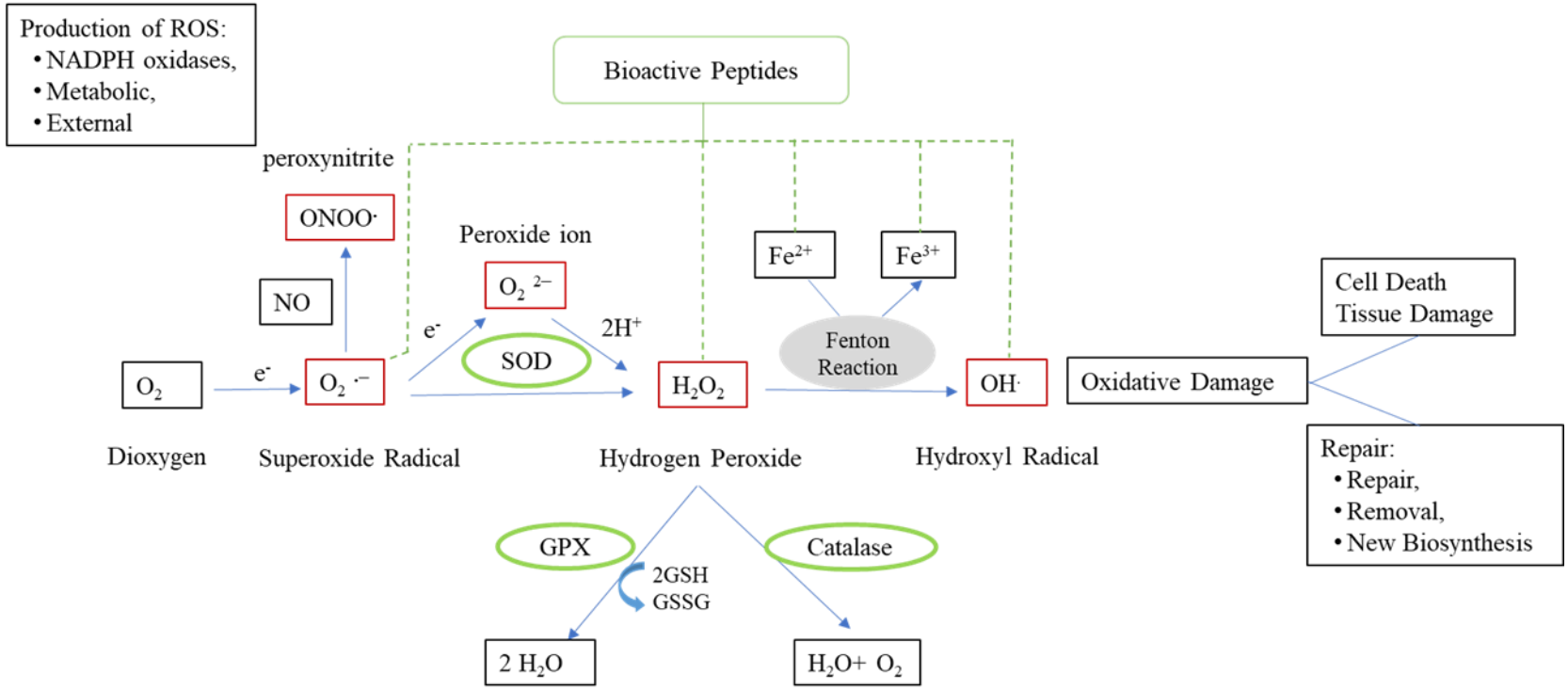
Currently, the number of bioactive peptides approved for a health claim and available in the marketplace is very limited and the products are mainly of animal origin such as Calpis and Evolus that contain dairy milk-derived ACE-inhibitory tripeptides. Utilization of more bioactive peptides with designed health benefits in food products, particularly from plant sources could be one of the trends in the prospects of the global food industry, which aligns with increasing sustainability. Production of pure food-derived bioactive peptides is estimated to grow on a large

scale and bioactive peptides with well-characterized properties are expected to be available on the market as nutraceuticals.

## AUTHORS' CONTRIBUTIONS

The authors' contributions were as follows—REA: conceptualized the research; MS: wrote the first draft of the manuscript; REA and MS: critically reviewed and approved the final version of manuscript.

**Figure 2.1.** Formation of different ROS and RNS from Oxygen and Possible Antioxidant Effects of Bioactive Peptides




In this figure, ROS are denoted in red, while antioxidant enzymes are represented in green. Fe2+, ferrous ion; Fe3+, ferric ion; GPX, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; H2O2, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; O2•-, superoxide radical; OH•, hydroxyl radical; ONOO, peroxynitrite; ROS, reactive oxygen species; SOD, superoxide dismutase.

**Table 2.1.** Studies on Antihypertensive and Antioxidant Effects of Bioactive Peptides from Plant Protein Sources

| Reference | Source | Effects | Model | Mechanism of Actions |
|---|---|---|---|---|
| **(91)** | Pea protein | Antioxidant properties | In vitro | Free radical ($DPPH^{\bullet}$, $OH^{\bullet}$, and $O_2^{\bullet-}$) and $H_2O_2$ scavenging activity, MCA, and inhibition of linoleic acid oxidation |
| **(92)** | Pea protein | Antioxidant properties | In vitro | Inhibition of free radical nitric oxide production |
| **(71)** | Hemp seed protein | Antioxidant and antihypertensive properties | In vitro | Increasing ORAC activity upon hydrolysis, $DPPH^{\bullet}$ radical scavenging, and ACE inhibitory activities |
| **(94)** | Date seed protein | Antioxidant properties | In vitro | Radical scavenging activity, inhibitory effects against $ROO^{\bullet}$ and $OH^{\bullet}$ radical-induced DNA scission, chelating activity toward $Fe^{2+}$ |
| **(62)** | Chia seed protein | Antihypertensive properties | In vitro | ACE inhibitory activity |
| **(95)** | Potato protein | Antioxidant properties | In vitro | $ABTS^{\bullet+}$ radicals scavenging activity and inhibiting lipid oxidation |
| **(96)** | Potato protein | Antioxidant properties | In vitro | Strong $ABTS^{\bullet+}$ radical and $H_2O_2$ scavenging activity |
| **(97)** | Mung bean albumin | Antioxidant properties | In vitro | Iron chelating activity, increased ORAC activity |
| **(98)** | Okra seed meal protein | Antioxidant properties | In vitro | $ABTS^{\bullet+}$ radical and $OH^{\bullet}$ scavenging activity |
| **(40)** | Chia protein | Antihypertensive and antioxidant properties | In vitro | ACE inhibitory activity, $Fe^{3+}$ reducing antioxidant, and $DPPH^{\bullet}$ radical scavenging activity |

| | | | | |
|---|---|---|---|---|
| **(69)** | Hemp bran protein | Antihypertensive and antioxidant properties | In vitro | ACE inhibitory activity, $ABTS^{\bullet+}$, and $DPPH^{\bullet}$ radical scavenging activity, $Fe^{2+}$ chelating activity, $Fe^{3+}$ reducing antioxidant power |
| **(38)** | Hemp seed protein | Antioxidant properties | • In vitro<br>• Animal study | • $DPPH^{\bullet}$ and $OH^{\bullet}$ free radicals scavenging ability and MCA,<br>• Increased plasma SOD and CAT activity, decreased PTPs, preventing metal-catalyzed lipid peroxidation |
| **(39)** | Hemp seed protein | Antihypertensive properties | Animal study | Attenuation of the normal increases in SBP in young growing SHRs, reduction in SBP in adult SHRs, suppressed plasma ACE activity, and renin level in SHRs. |
| **(64)** | Sesame seed protein | Antioxidant and antihypertensive properties | In vitro | Enhanced radical scavenging and MCA, inhibition of linoleic acid oxidation, ACE, and renin inhibitory effects |
| **(73)** | Pea protein | Antihypertensive properties | • Animal study<br>• Clinical trial | • Reductions in SBP, DBP, plasma levels of AT-II, and renal expression of renin mRNA levels<br>• Reductions in SBP |
| **(36)** | Mung bean protein | Antihypertensive properties | Animal study | Reductions in SBP, DBP, MAP, and HR |
| **(68)** | Pigeon pea protein | Antihypertensive properties | Animal study | SBP lowering effect |
| **(35)** | Pea protein | Antihypertensive properties | Animal study | SBP lowering effect |
| **(34)** | Flaxseed protein | Antihypertensive properties | • In vitro<br>• Animal study | • ACE and renin-inhibitory activities<br>• SBP lowering effect |

| | | | | |
|---|---|---|---|---|
| **(33)** | Australian canola protein | Antihypertensive properties | • In vitro<br>• Animal study | • Inhibition of ACE activity and renin<br>• SBP lowering effect |
| **(66)** | Rapeseed protein | Antihypertensive properties | • In vitro<br>• Animal study | • Inhibition of ACE activity and renin<br>• SBP lowering effect |
| **(74)** | Rice bran protein | Antihypertensive properties | Clinical trial | SBP lowering effect |
| **(32)** | Black soy protein | Antihypertensive and antioxidant properties | Clinical trial | SBP lowering effect, increased plasma SOD activity and NO, and decreased plasma MDA |

2,2-diphenyl-1-picrylhydrazyl (DPPH•); 2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS•+); ACE, angiotensin-converting enzyme; AT-II, angiotensin-II; CAT, catalase; DBP, diastolic blood pressure; Fe2+, ferrous ion; Fe3+, ferric ion; H2O2, hydrogen peroxide; HR, Heart Rate; MAP, mean arterial pressure; MDA, malondialdehyde; MCA, metal chelating activity; mRNA, messenger RNA; NO, nitric oxide; OH•, hydroxyl radical; ORAC, Oxygen Radical Absorbance Capacity; PTPs, plasma total peroxides; ROO•, peroxyl radical; SBP, systolic blood pressure; SHRs, spontaneously hypertensive rats; SOD, superoxide dismutase; O2•-, superoxide radical..

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## TRANSITION STATEMENT 2

As we shift our focus from the exploration of plant proteins and bioactive peptides, we will have a closer examination of the practical applications and implications of these findings in the context of hypertension. In Chapter II, we reviewed existing literature to explore the antioxidant and antihypertensive attributes associated with plant proteins and the bioactive peptides derived from them. Building upon this foundational understanding, Chapter III now pivots towards a more practical application of this knowledge. Specifically, this chapter focuses on elucidating the comprehensive protocol of our clinical trial, a significant endeavor aimed at bridging the gap between theoretical insights and tangible interventions. The detailed protocol of our study has been published in the Trials journal, providing a comprehensive resource for those interested in the intricacies of our research. This publication encompasses the objectives and methodology of our clinical trial, offering a roadmap for the systematic exploration of the hypotensive potential associated with the consumption of HSP and HSP+ in comparison to casein protein. The objective of this chapter is to provide with a clear and transparent guide to the planned procedures, ensuring a thorough understanding of our research approach and the anticipated outcomes. As we navigate through the various aspects of our trial protocol, we aim to demystify the scientific process and shed light on the practical steps taken to advance our understanding of the potential benefits of plant-derived proteins in managing hypertension.

In the forthcoming manuscript, it's important to note that certain biomarkers and outcomes were not evaluated in this project. However, to uphold consistency in the published article, we opted not to eliminate these biomarkers from the thesis. Instead, we introduced additional biomarkers related to blood pressure regulation in place of the ones not measured. Additionally, due to constraints on participants' time and the associated burden, we did not conduct assessments on diet and physical activity.

# CHAPTER III

## MANUSCRIPT 2

# A DOUBLE BLIND, RANDOMIZED, CROSS OVER TRIAL PROTOCOL OF WHOLE HEMP SEED PROTEIN AND HEMP SEED PROTEIN HYDROLYSATE CONSUMPTION FOR HYPERTENSION

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**ABSTRACT**

Background: Primary hypertension accounts for almost 95% of all cases of high blood pressure and is a major modifiable risk factor for cardio-vascular diseases. Lifestyle interventions have been shown to prevent hypertension. One of the prominent potential therapeutic lifestyle strategies to prevent or manage hypertension is increasing dietary protein as a macronutrient or as bioactive peptides. An emerging plant-based protein source that may have anti-hypertensive properties is hemp seed.

Methods: A randomized, double-blind, crossover clinical trial will be conducted on 35 hypertensive participants aged 18-75 years, with a BMI between 18.5 and 40 kg/m2, systolic blood pressure (SBP) between 130 and 160 mmHg and diastolic blood pressure ≤ 110 mmHg. The trial will be conducted for 22 weeks and will consist of 3 treatment periods of 6 weeks, separated by 2-week washout periods. The treatments will be consumed twice a day and consist of 50-gram casein, hemp seed protein (HSP), or HSP plus HSP hydrolysate (HSP+). The primary outcome of this trial is 24 hr SBP, measured on the first day of the first phase and the last day of each phase. Office blood pressure and anthropometrics will be determined on the first and last day of each period. Blood samples will be collected on the first and last two days of each treatment phase to be analyzed for specific biomarkers.

Discussion: This trial protocol is designed to evaluate the hypotensive potential of consuming whole HSP, and HSP+, in comparison to casein protein. This study will be the first trial investigating the potential antihypertensive benefit of dietary hemp protein plus bioactive peptides consumption in humans.

Trial registration: National Clinical Trial (NCT), NCT03508895. Registered 28 June 2018 - Retrospectively registered, on the publicly accessible Registry Databank at Clinicaltrials.gov (http://ClinicalTrials.gov)

Keywords: Casein, Hemp seed protein, Bioactive peptides, Hypertension

## BACKGROUND

Hypertension afflicts over 7.3 million Canadians (1) and is the number one modifiable risk factor for death and disability globally (2), accounting for 13.5% of all global premature deaths (3). Over the last 25 years, control of hypertension has increased markedly in Canada (4); however, one-third of the diagnosed hypertensive population remains uncontrolled and many people remain undiagnosed. Uncontrolled hypertension can lead to a spectrum of diseases including stroke, coronary artery disease, as well as heart and kidney failure (5).

The exact etiology of the development and progression of primary hypertension remains uncertain, however, both genetic and lifestyle factors, including diet in particular, have been implicated. Many interconnected contributing factors have been proposed to explain the increase in blood pressure (BP) leading to hypertension. Factors include over-activation of the renin-angiotensin-aldosterone system (RAAS), endothelial dysfunction, elevated sympathetic activation, and other genetic predispositions (6-8). Current therapeutic agents for BP control have proven to be reasonably effective; however, the incidence of mortality due to high BP is still on the rise (9). Consequently, an important need exists for alternative therapeutic strategies to prevent or manage hypertension.

Lifestyle interventions that have been shown to prevent hypertension include maintaining healthy body weight, moderate physical activity, low-sodium diets, reduced alcohol consumption, and adherence to diets rich in fruit and vegetables (10). Another emerging potential therapeutic lifestyle strategy to prevent or manage hypertension is increasing dietary protein (11, 12). Both increases in dietary protein as a macronutrient (12-16) and as smaller doses as bioactive peptides (17-19), have shown the potential to reduce blood pressure. Indeed, evidence exists that plant-based protein sources may be more effective than animal sources, however, these data stem mainly from observational trials and are inconsistent (11, 20). Randomized controlled trials comparing plant-based protein sources to animal-based protein sources are limited (12, 21-23). An essential plant-based protein source that may have anti-hypertensive properties is hemp seed.

Hemp seed (*Cannabis sativa* L.) utilization in foods has been limited due to legislation restricting the cultivation of all Cannabis species on account of the presence of varieties high in the psychoactive compound tetrahydrocannabinol (THC). However, as of 1998 in Canada,

cultivation of hemp seed containing < 0.3% THC has been allowed. Despite heightened consumer demand for hemp food products, due to historical restrictions on the growing of hemp, the nutritional science literature on hemp seed protein (HSP) remains limited (24). HSP has a unique amino acid profile, with high levels of arginine, which makes HSP an ideal protein to consume for BP-reducing effect, or from which to make BP-reducing bioactive peptides (25).

Both observational and intervention trials have identified associations between dietary protein intake and BP (11, 12, 22). A meta-analysis of 40 randomized controlled trials demonstrated that replacing a median of 40 g/day of dietary carbohydrate with dietary protein is associated with small reductions in both systolic −1.76 mm Hg (95% confidence interval, CI: −2.33, −1.20) and diastolic −1.15 mm Hg (95% CI: −1.59, −0.71) BP (SBP and DBP, respectively) (22). The meta-analysis did not find a difference between animal and plant-based protein sources in terms of the BP-lowering effect. However, trials specifically comparing animal proteins to vegetable proteins are limited and with inconsistent results.

The antihypertensive effects of protein may not only be related to the amount of protein in the diet but may also be related to the type of protein. Whether a protein is animal or plant-based is likely not as important as the type of amino acids contained in the protein source in terms of BP-lowering ability. Animal sources of protein are considered complete proteins, in that they contain an adequate proportion of all nine essential amino acids necessary for human dietary needs. Most vegetable sources individually are not considered complete because they are limited in one or more of the essential amino acids. This is important because the specific amino acid content of a protein is likely critical to its antihypertensive potential. Additionally, different protein sources may affect BP through alternate mechanisms depending on their amino acid content. Specific amino acids which have been identified as having potential BP-reducing properties include arginine, cysteine tryptophan, and glutamic acid (26).

Casein is the main protein found in cow's milk (27), with whey protein making up most of the remaining protein. Pal and Ellis (28) investigated the effects of consuming casein or whey protein, compared to glucose, for 12 weeks in 70 men and women using a randomized parallel-designed trial. Whey and casein protein consumption lowered SBP and DBP compared to baseline after 12 weeks and lowered DBP compared to the glucose control. No difference in BP was seen between the two milk protein fractions. Soybean protein, which has been well studied

for its potential BP-reducing effects (15, 16, 29), is a representative vegetable protein with a high arginine content. This high arginine content may be responsible for some of soybean protein's anti-hypertensive properties (26). Unlike soybean, HSP consumption has not been well characterized in terms of blood pressure. HSP has even higher concentrations of arginine than soy, casein, or whey protein, which may make it an ideal candidate to evaluate for BP control (30). Consumer demand for hemp food products is growing and health food retailers, are looking to add hemp foods to their product offerings. However, hemp foods, unlike casein, whey, and soy, have a very limited database of peer-reviewed publications (31-34) outlining the health benefits of consumption due to restrictions on hemp cultivation, which have only recently been lifted in many countries.

Arginine, conditionally an essential amino acid depending on age and health (35), has been shown to reduce BP when supplemented in the diet (35, 36). The main source of endogenous nitric oxide (NO) is L-arginine, which is oxidized to NO and citrulline by the action of endothelial NO synthase (eNOS) (37). However, some protein-incorporated arginine can undergo methylation, and with subsequent proteolysis can yield methyl-arginine compounds such as asymmetric dimethylarginine (ADMA). ADMA is an endogenous inhibitor of NO synthase and has been shown to reduce the sensitivity of NO synthase to L-arginine. However, ADMA inhibition of NO synthase may be overcome with increased dietary arginine consumption. Additionally, arginine rapidly undergoes catabolism by arginase, a metalloenzyme, which hydrolyzes L-arginine to L-ornithine and urea (38). Arginase is, therefore, a key regulator of NO availability, and arginase inhibitors are being investigated for therapeutic potential in the treatment of endothelial function and hypertension (39, 40). As with ADMA, additional dietary arginine consumption may be able to overcome arginase activity, which limits arginine availability for eNOS and NO production.

Typical sources of dietary arginine are meat, fish, and poultry, however, soy protein isolates and HSP also possess higher levels of this amino acid (30, 41), while milk protein is relatively low in arginine (42). Food-based arginine is well tolerated with few side effects, making it a better source of arginine than free L-arginine which has been associated with nausea, gastrointestinal discomfort, and diarrhea (37). HSP, due to its high arginine content, is, therefore, a protein source, which should be investigated for its antihypertensive effects.

Girgih et al. (25) compared the effects of HSP isolate on the prevention and treatment of hypertension in spontaneously hypertensive rats (SHRs). The replacement of 1 % casein on a dry weight basis in the rat diet with HSP isolate was able to prevent the increase in SBP seen in young growing SHRs over 8 weeks and decrease SBP in adult SHRs with established hypertension. Decreased plasma renin concentrations and angiotensin-converting enzyme (ACE) activity were observed with the HSP feeding. Additionally, Girgih et al. (25) produced an HSP hydrolysate from the enzymatic hydrolysis of HSP isolate. Protein hydrolysates contain bioactive peptides, which are typically produced by enzymatic hydrolysis of food proteins to release short peptide sequences (43). However, bioactive peptides can also be produced endogenously through the digestion of dietary proteins (19). Numerous bioactive peptides have been shown to modulate RAAS via inhibition of ACE and renin activity (44). The HSP-derived bioactive peptides made by Grigih et al. (25) were able to lower SBP in adult SHRs with established hypertension and decreased SBP in young growing SHRs to a greater extent than the HSP isolate when substituted for casein at 1 %. The HSP-derived bioactive peptides also reduced renin concentrations and ACE activity more than did the HSP isolate in the SHRs, and even reduced plasma ACE activity in the normotensive control rats.

Since there was no difference in the degree of reduction in SBP in the adult SHRs between HSP isolate and bioactive peptide, but the reduction in ACE activity was much greater with the bioactive peptide (45), it was hypothesized that the intake of HSP isolate was likely lowering BP via a different mechanism than the peptide (25). Indeed, the marked difference in the arginine content between the HSP isolate and the hemp seed protein hydrolysate (HPH) could be responsible for the differing hypotensive mechanisms. The HSP isolate contained more than six times the arginine in the HSP hydrolysate-derived bioactive peptides (25). However, none of the rats were fed a combination of the HSP isolate with additional HSP bioactive peptides to investigate whether the BP-reducing effects would be additive or synergistic.

Bioactive peptides are specific short amino acid sequences that remain inactive when bonded to other amino acids within the primary structure of a food protein, but after release via hydrolysis, the free forms of these peptides can affect biological processes. Bioactive peptides have been shown to act as anti-hypertensive agents by inhibiting in vitro and in vivo renin, ACE, and angiotensin II receptor activities in addition to enhancing blood NO levels (44).

Therefore, there is an interest in characterizing these natural anti-hypertensive compounds so that they can serve as alternatives to anti-hypertensive drugs. Bioactive peptides have the advantage of specificity, with limited side effects, potency, and low toxicity such that they can be consumed at high doses (44, 46, 47). Different types of bioactive peptides, derived from numerous sources including dairy, meat, fish, poultry, canola, buckwheat, algae, and hemp, have shown hypotensive effects in animal models (43, 44).

However, only a limited number of the bioactive peptides that have been characterized in animals and in-vivo have subsequently been tested in human trials (19). Most human trials investigating dietary bioactive peptides have been derived from dairy, with the results from casein and whey-derived bioactive peptide feeding trials being mixed (19, 48). A meta-analysis of human trials investigating dairy-derived bioactive peptides found consumption of 2-5 grams of bioactive peptides a day could yield approx. 5 mmHg and 2 mmHg reductions in SBP and DBP, respectively. It has been suggested that the amino acid composition of the bioactive peptides may be critical to the ability of the peptide to inhibit ACE activity. Bioactive peptides with a positive charge due to arginine or lysine residues are thought to be beneficial for ACE inhibition (49). A 3-week crossover human intervention trial conducted by our team fed 3 grams per day of pea protein hydrolysate led to a 5-6 mmHg reduction in SBP in the second and third weeks compared to placebo (18). To our knowledge, the addition of bioactive peptides on top of increased dietary protein has not been previously investigated in humans.

While there is good evidence that the replacement of dietary carbohydrates with protein has anti-hypertensive effects (11-13, 22), numerous gaps in the research remain. First, comparisons of plant versus animal sources of dietary protein, or high arginine versus low arginine sources of dietary protein are limited (11, 22). Second, the effects of additional dietary protein, where it does not explicitly replace carbohydrates, are not known (22). Third, human trials investigating HSP consumption are limited (32). Fourth, human trials involving non-dairy-derived bioactive peptides are limited (19). Lastly, no trials investigating the potential benefit of increased dietary protein plus bioactive peptides have been completed to our knowledge. To address these knowledge gaps, we propose a clinical trial that would evaluate the anti-hypertensive potential of consuming whole HSP, and HSP+, compared to casein protein using a randomized, double-blinded, and crossover design. This design will be able to address the knowledge gaps identified

above. Although little work has been done in humans in this specific area, for the following reasons, we were not able to perform a pilot trial before the main study; the long-term nature of this study was the main factor that we couldn't consider a pilot study as the first phase. Recruitment of 35 participants and obtaining their consent to enter a long-term trial was a challenge so, recruitment of more people as a pilot group would increase the duration of the study. So, we had to recruit more people to start the main study or have the pilot group on an extra washout period to be able to start the trial over. These participants might lose their interest to continue with the study and the rate of drop out would increase. In terms of testing the procedure, we mainly followed similar clinical trials with other treatments to evaluate the acceptability of the procedure in the following criteria; treatment consumption frequency, treatment consumption under supervision, free-living trial with basic recommendations for physical activity and alcohol consumption, method and frequency of the outcome measurements, method of randomization and follow-up with participants. Acceptability of the treatments was assessed using taste and texture testing with random participants. The sample size was calculated based on the expected change in the main outcome using similar studies that considered 24-hour SBP as their main outcome.

## METHODS/DESIGN

### OBJECTIVES AND HYPOTHESIS

This study aims to assess the hypotensive potential of consuming whole HSP, and HSP+, compared to casein protein in patients with hypertension. Hypotheses to be tested in this study are as follows; Hypothesis one: Consumption of 50 g/day of dietary protein, of any type (casein, HSP, or HSP+) for 6 weeks will lead to reduced 24 hr SBP compared to baseline. Hypothesis two: Consumption of 50 g/day of hemp-based dietary protein (HSP and HSP+) for 6 weeks will reduce 24 hr SBP compared to casein. Hypothesis three: Consumption of 45 g/day of hemp protein with an added 5 g/day of HSP+ for 6 weeks will reduce 24 hr SBP compared to 50 g/day of HSP.

### STUDY DESIGN

An interventional, randomized, double-blind, crossover, phase II clinical trial is being conducted at the Clinical Nutrition Research Unit at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba. The trial will consist of 3

treatment periods of 6 weeks each, separated by 2- week washout periods. A summary of the proposed trial design is presented in Fig. 3.1. The Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) checklist is presented in Fig. 3.2 and Additional file 1. During each treatment period, participants need to come to the RCFFN twice a week on Tuesdays and Fridays to consume one treatment under supervision and pickup treatments for the next 2 or 3 days. Each treatment smoothie consists of frozen fruit (mango or pineapple), fruit juice (diet mango juice), sorbet, and 25 g of protein from treatment protein powder and the treatments will be consumed twice a day. HSP and the HSP+ powder have a green color and a stronger taste than casein protein powder, however, our team has identified that by using a combination of frozen fruit and sorbet in the fruit smoothies this color and taste can be well masked.

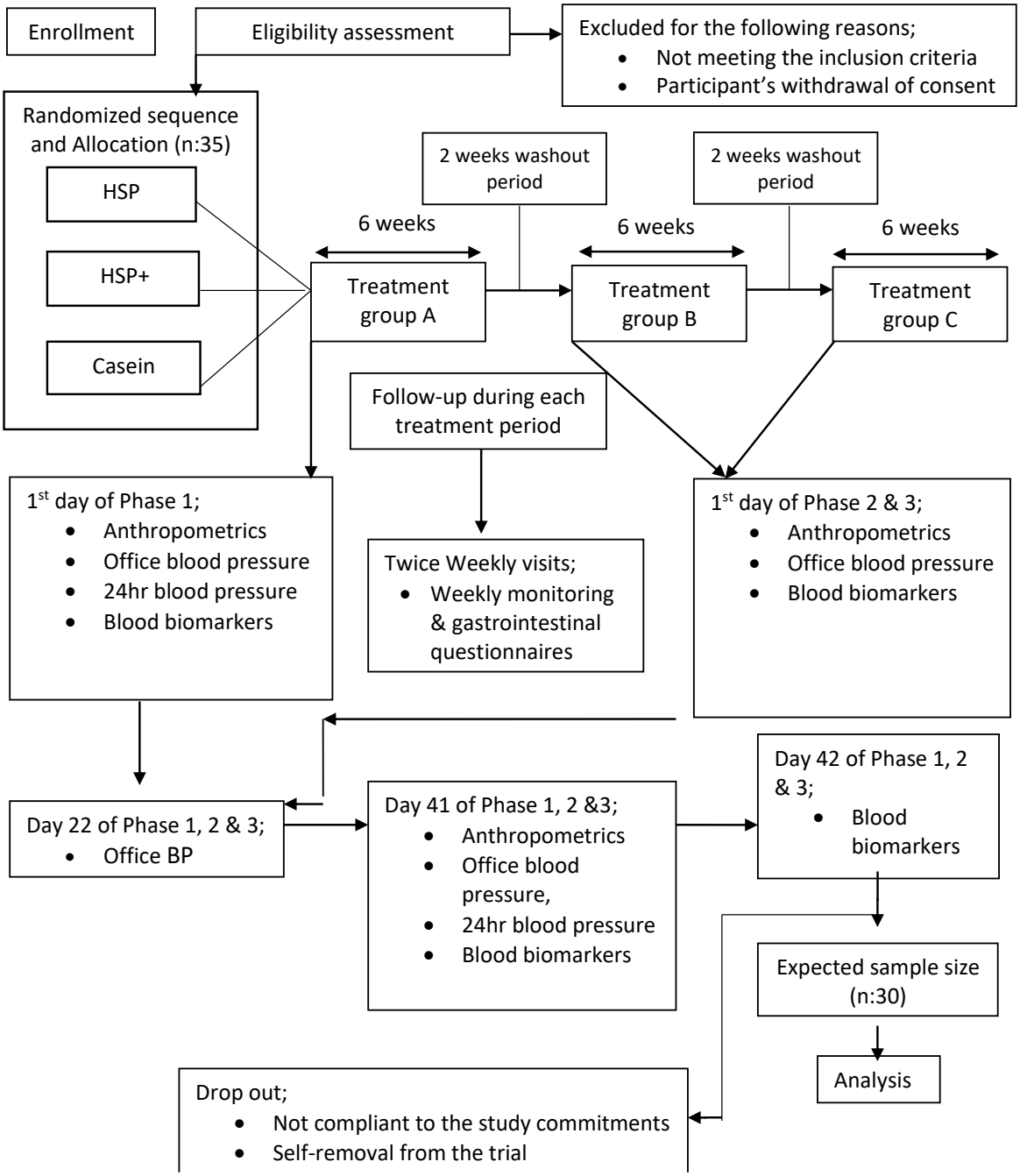
Participants will be instructed to consume <2 alcoholic beverages and no more than three 8 oz. cups of caffeine-containing beverages per day during the trial period. Participants will be instructed to perform their normal daily activities during the measurement day. Prolonged intense physical activity will be discouraged on measurement days and the days preceding measurement days. Participants will be strongly recommended to maintain consistency in their physical activities and dietary pattern during the trial period.

During the study period, body weight will be monitored as a change could occur due to the inclusion of extra protein in a free-living condition. All outcome variables will be adjusted for changes in body weight should significant changes occur between treatments. Gastrointestinal tolerability and weekly monitoring questionnaires will be completed by participants weekly during each intervention period.

**SAMPLE SIZE**

A total sample size of 30 participants was calculated as required to detect a change in 4 mm Hg in SBP with the proposed crossover design, using an estimated within-participant standard deviation of 5.45 mm Hg and a power of 0.80 and $\alpha$=0.05 (13). A recruitment goal of 35 participants has been set to account for the potential participant drop out.

**Figure 3.1.** Schematic Flow Diagram of the Trial Protocol




BP, blood pressure; HSP, Hemp seed protein; HSP$^{+}$, HSP plus HSP hydrolysate.

**Figure 3.2.** Representation of the Trial Enrolment, Interventions, and Analysis

| | **Study Period (01/05/2018 to 30/05/2020)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Enrolment** | **Allocation** | **Post-allocation** *(01/07/2018 to 29/02/2020)* | | | | | | | | | | | | **Close-out** |
| **TIMEPOINT**** | $-t_1$ *(01/05/2018 to 01/11/2018)* | **0** *(01/05/2018 to 01/11/2018)* | ***P1 D1*** | ***P1 D22*** | ***P1 D41*** | ***P1 D42*** | ***P2 D1*** | ***P2 D22*** | ***P2 D41*** | ***P2 D42*** | ***P3 D1*** | ***P3 D22*** | ***P3 D41*** | ***P3 D42*** | $t_x$ *(29/02/2020 to 30/05/2020)* |
| **ENROLMENT:** | | | | | | | | | | | | | | | |
| **Evaluating inclusion and exclusion criteria** | X | X | | | | | | | | | | | | | |
| **Informed consent** | X | | | | | | | | | | | | | | |
| **Demographic data** | X | X | | | | | | | | | | | | | |
| **Anthropometrics** | X | | | | | | | | | | | | | | |
| **Blood pressure** | X | | | | | | | | | | | | | | |
| **Medical history** | X | | | X | X | | | X | X | | | X | X | | |
| **Blood sample** (eligibility criteria evaluation) | X | | | | | | | | | | | | | | |
| **INTERVENTIONS*:** | | | ← | — | — | — | — | — | — | — | — | — | — | → | |
| ***Treatment group A*** | | | ← | — | — | → | | | | | | | | | |
| ***Treatment group B*** | | | | | | | ← | — | — | — | → | | | | |
| ***Treatment group C*** | | | | | | | | | | | ← | — | — | → | |
| **ASSESSMENTS:** | | | | | | | | | | | | | | | |
| ***24hrBP*** | | | X | | X | | | | X | | | | X | | |
| ***BP*** | | | X | X | X | | X | X | X | | X | X | X | | |
| ***Anthropometrics*** | | | X | | X | | X | | X | | X | | X | | |
| ***Blood Biomarkers,*** | | | X | | X | X | X | | X | X | X | | X | X | |
| ***Lab work*** | | | | | | | | | | | | | | | X |
| ***Data analysis and results report*** | | | | | | | | | | | | | | | X |

## STUDY SETTING

This study will be conducted at one site the RCFFN, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2 (Winnipeg is the capital and largest city of the province of Manitoba, with a population of approximately 775000 people, of primary European decent, but with a significant Indigenous and Filipino population). The site investigator is Dr. Rotimi Aluko at the RCFFN, University of Manitoba.

## STUDY POPULATION

Thirty-five participants will be enrolled in the study using advertisements in local media including social media, newspapers, and the official website of the Centre and on University Campuses, as well as our pre-existing database of participants. Potential participants will be initially screened over the phone using a telephone questionnaire where some brief questions regarding personal health information will be asked. If participants are determined as potentially eligible after telephone screening, they will be scheduled to come to the RCFFN in the morning to have their BP measured and undergo a blood screening where 20 ml fasting blood samples will be taken via venipuncture by a nurse to test for blood parameters related to exclusion criteria. If the potential participants meet all the inclusion criteria before they go for blood draw and not the exclusion criteria, the informed consent form will be provided by the study coordinator and the participant will sign the form and will be recruited to the study.

## INCLUSION CRITERIA

Hypertensive participants aged 18-75 years will be recruited, with a BMI between 18.5 and 40 $kg/m^2$. Participants must have SBP between 130 and 160 mmHg and DBP $\leq$ 110 mmHg. Additionally, participants must be willing to fast for 10-12 hr and abstain from alcohol two days before blood sampling and BP measurement. They must also abstain from coffee consumption for at least 14 hr and physical exercise for at least 4 hr before BP measurement. All women of child-bearing age will take a pregnancy test before commencement of the trial. The pregnancy test should be negative for women with child-bearing potential. All participants must be able and willing to give informed consent to participate in the trial before their inclusion.

## EXCLUSION CRITERIA

Participants will be excluded if they are undergoing or have changed the type or dosage of anti-

hypertensive drug treatment, regular high-dose NSAID treatment, cyclosporine, or tacrolimusin treatment for less than 3 months. Participants will be excluded if they have any dietary restrictions, which would prevent them from consuming the trial treatments. Participants must not have self-reported weight gain or loss greater than 5 kg in the past three months. Participants must be free of active cardiovascular disease including stroke, congestive heart failure, myocardial infarction, unstable angina pectoris, coronary artery bypass graft, percutaneous transluminal coronary angioplasty, temporal ischemic attacks, anemia, abnormal electrolytes, proteinuria, abnormal liver, kidney, thyroid function, bleeding/blood or psychiatric disorder. There will be some exceptions regarding some medications/supplements consumed by the participants. Those participants taking BP medications who have been stable on the medication for at least 3 months will be included in the study. Those taking medications (i.e., statins) or supplements (i.e., fish oils) for lipid-lowering reasons and who have been stable on such medications and supplements for at least 3 months will be included in the study. Participants with stabilized thyroid levels with treatment will be allowed to participate in the study.

Participants will be asked about current or historical diseases/conditions through verbal question and answer during screening. Participants will be excluded if they have clinically significant biochemistry defined as Sodium: <134 mmol/l, >148 mmol/l, fasting glucose: > 6.1 mmol/ l, or any other clinically significant abnormality in hematology and/or biochemistry at the investigator's discretion. Participants will be excluded if they have secondary hypertension, type 1 or type 2 diabetes, a history of cancer or malignancy in the last 5 years, or any metabolic disease, gastrointestinal disorder, or other clinically significant disease/disorder, which could interfere with the results of the study or safety of the participant or those with allergies or sensitivities to any of the ingredients in the study product. Participants will be excluded if they smoke tobacco/snuff/nicotine users, recreational drug users, or if they consume more than 14 alcoholic beverages a week. Women with child-bearing potential not using an acceptable method of birth control (i.e. implants, injectables, combined oral contraceptives, some intrauterine contraceptive devices (IUDs), sexual abstinence, or a vasectomized partner) or who are pregnant or plan to become pregnant during the trial period or women with breastfeeding will be excluded.

**CONDITIONS FOR WITHDRAWAL FROM THE TRIAL**

The following criteria are to be considered sufficient reason for discontinuing participation in the trial: non-compliance by the participant; developing condition(s) or consumption of supplements or medications as specified under exclusion criteria or changing the type or dosage of the specified medications; presenting an adverse event or any other medical situation where, in the opinion of the qualified investigator, the continuation of the trial treatment would compromise the participant's health; abusive behavior of participant towards trial staff; participants withdrawal of consent and self-removal from the trial for any reason. We will follow-up with study participants who have been withdrawn from the trial due to an adverse event until symptoms have resolved.

**RANDOMIZATION AND CONCEALMENT PROCEDURES**

Recruited eligible participants will be randomly assigned to one of the treatment sequences using a 3 by 3 Latin square design. The letters A, B, and C represent the three treatment groups, and the sequences are as follows: ABC, ACB, BAC, BCA, CAB, and CBA. The randomization procedure will be completed using a random number generator.

**BLINDING**

Because the casein powder, HSP and HSP+ have different visual appearances, treatment smoothies will be prepared by a kitchen staff member to reduce the differences in treatment appearance and given to clinical coordinators in a blinded fashion labeled A, B or C. Assignment of treatments to labels will be performed by a researcher independent of the investigators' groups. This researcher will keep a copy of the treatment coding, give a copy of the treatment coding to the assigned kitchen staff, and give a sealed copy of the coding to the principal investigator allowing participants, coordinators, and individuals performing the analyses and collecting outcome data to be blinded to the treatments. Participants will be instructed not to communicate the taste and texture of the smoothies with the research staff or other participants in an attempt to ensure double-blinding. The treatment coding will be broken after the conclusion of the statistical data analyses on the primary outcome. However, the code could be broken if there is an adverse event reported that requires identification of the treatment.

**SAMPLE COLLECTION**

Twelve-hour morning fasting (i.e., nothing to eat or drink except water 12 hr prior) blood samples will be collected on the first day of each phase (baseline), and days 41 and 42 of each treatment period of the trial (in total 9 times during the trial). These fasting blood samples (approximately 4 teaspoons or 20 ml) will be taken from the top of the forearm via venipuncture by a phlebotomist on each blood draw day for assessment of blood biochemical markers. Each blood test will take approximately 5 min. No alcoholic beverages are to be consumed within 48 hr before blood draws during the study periods. No food or caffeinated beverages consumption within 12 hr before blood draws during the study periods. Blood samples will be centrifuged at 1000 g for 20 min at 4 °C, aliquoted to yield serum, plasma, and RBC, and then stored at -80 °C until analyses.

**COMPLIANCE**

Compliance will be monitored through the visual affirmation by clinical coordinators that participants consumed the morning treatment smoothies on Tuesdays and Fridays. Participants will be required to return smoothie containers from the other day's smoothie treatments on the next visit. Also, participants need to fill out a treatment consumption checklist which includes the date and timing of each treatment consumption and return the checklist at the end of each treatment period. Missed smoothie consumption or return of smoothie containers will be recorded for each participant. Non- compliance will be defined as missing supervision for, or failing to return the empty smoothie containers from, 80% of the total smoothies per treatment period. Missing 2 consecutive measurement or blood sampling days will also be classified as non-compliance. Non-compliant participants will be asked to leave the trial. An intent-to-treat analysis will be performed to use the data from non-compliant participants.

**STUDY GROUPS**

Eligible participants will be assigned randomly to one of the following sequences: ABC, ACB, BAC, BCA, CAB, or CBA. So, all the participants will be provided with all 3 treatments in a cross-over design.

**STUDY PERIOD**

This study includes 3 phases of 6 weeks each, separated by 2 washout periods of 2 weeks. Participants will be involved in the study for 22 weeks.

## OUTCOMES AND MEASUREMENT TOOLS

The primary outcome of this trial will be 24 hr SBP, measured on the first day of the first phase and day 41 of each treatment phase using ambulatory BP monitors (ABPM, OnTrak, Spacelabs Healthcare Inc. WA, United States) (50, 51). Participants will be fitted with an ABPM to wear for a full day. Continuous and SBP and DBP measured over 24-hr will be recorded. Office BP will be determined using an automated oscillometric measurement device (Mobil-O-Graph, IEM, Stolberg, Germany) in an office setting at days 1 and 41 of each treatment phase (52, 53). Office BP will also be measured on day 22 to track the changes at the midpoint of each treatment phase. This measurement will take place in a quiet room while the participant is in a seated position and arm rested on an arm rest at heart level. Participants will be advised to rest quietly throughout the measurements. Oscillometric measurement will be performed 4 times at 2-minute intervals. The first measurement will be discarded, and the last three measurements will be averaged to determine office BP. The LCD of the oscillometric measurement device will be covered so that the participant is blinded to the BP values during the measurements. BP results will not be shared with participants until they have completed the trial.

## ANTHROPOMETRIC MEASUREMENTS

At days 1 and 41 of each treatment period, body weight and waist and hip circumferences will be measured following standardized procedures.

## BLOOD BIOCHEMICAL MEASUREMENTS

Plasma samples will be analyzed to determine ACE activity by a spectrophotometric method using FAPGG as substrate (25). Serum renin will be measured by commercial enzyme-linked immunosorbent assays (ELISA). NO concentrations will be determined in plasma using a colorimetric kit (Nitric oxide assay, Thermoscientific) (54).

## STATISTICAL ANALYSIS

Statistical analysis will be performed in an intent-to-treat fashion. In addition, a per-protocol analysis will also be performed to check for potential effect attenuation due to unequal

compliance or protocol deviations across treatments. Statistical analysis will be performed using SAS 9.4. The endpoint of treatments and baselines will be compared using a mixed model analysis of variance. Sequence and sex will be included as fixed factors, and participant will be included as a random factor, with participant repeated by period. Dunnett's test will be used to adjust for multiple comparisons with the endpoint of the baselines set as the control. Effects of treatment by time on measures repeated during treatments will be assessed using a mixed model analysis of variance with sequence, sex, and time as fixed factors and participants included as random factors, with participants repeated by period, with Tukey-Kramer adjustments for multiple comparisons. Model residuals will be visually inspected to assess independence, normal distribution, and constant variance. Data sets with residuals determined to violate these assumptions will be transformed before re-analysis. Results will be expressed as estimated least square means ± standard error of the mean (SEM) for all values. Statistical significance will be set at $p < 0.05$ for all analyses.

**RISKS AND BENEFITS**

No serious side effects or adverse events have been previously reported due to hemp or casein protein intake. Phlebotomy carries a potential health risk. Participants will be encouraged to report any adverse events as they happen, and participants will be prompted and asked every week about adverse events. Adverse events will be recorded through the "Adverse Event/Unanticipated Problems Form" provided by the Research Ethics Board (REB). All serious adverse events will be reported to the University of Manitoba REB who has approved the trial protocol. Study coordinators will follow-up with participants via email or phone after adverse events until symptoms have resolved. If the serious unexpected adverse reaction (SAEs) is fatal or life-threatening, the Therapeutics Products Directorate of Health Canada will be notified no later than seven days after the sponsor becomes aware of the information. If the serious unexpected adverse reaction is neither fatal nor life-threatening, the Natural and Non-prescription Health Products Directorate (NNHPD) will be notified immediately if possible, and no later than 15 days after the sponsor becomes aware of the information. Within eight days after having informed the NNHPD of a serious unexpected adverse reaction to the NHP, the sponsor will submit a report as complete as possible that includes an assessment of the importance and implication of any findings. The final report shall include relevant previous experience with the same or similar health products. The trial will be conducted following the

principles of good clinical practice.

**CONCOMITANT THERAPY**

All prescription and over-the-counter medications that are not deemed as falling into the exclusion criteria list, will be recorded at the screening visit by a general physician and will be monitored every week during the treatment periods. Should a medication be prescribed or started during the trial period, it will require qualified investigator approval.

**ANTICIPATED RESULTS AND CONCLUSION**

It is anticipated that the additional 50 g/day of dietary protein, whether from casein or HSP, or HSP+, will result in a reduction in BP compared to the baseline of the trial. Such results would support a simplified message that increasing dietary protein, by consuming 50 grams per day of protein through the use of protein powders, can help lower blood pressure. Such a recommendation is easier to explain and implement than recommendations where a specific macro-nutrient like carbohydrate or fat must be replaced by protein to have anti- hypertensive effects. It is also anticipated that the hemp protein treatments will lead to a greater reduction in BP compared to the casein, due to their increased arginine concentration. These results will be accompanied by increased blood NO concentrations on the hemp protein treatments. This finding would suggest that HSP, or protein sources with higher arginine, are appropriate choices of protein to select for individuals who may be looking to control their BP rather than lower arginine dietary protein sources.

The HSP+ can be predicted to lead to the greatest reduction in blood pressure. The HSP+ treatment is predicted to demonstrate the greatest reduction in ACE activity due to the ACE inhibitory effects of the bioactive peptides. If an additional benefit is seen with the addition of HSP bioactive peptide to the HSP, this finding would suggest that dietary protein and bioactive peptides lower BP through separate or synergistic mechanisms, and that a combination of increased dietary protein and bioactive peptides could be recommended for greater BP reduction over and above a simple increase in dietary protein intake. This proposed trial will directly address gaps in the scientific literature surrounding the anti-hypertensive properties of dietary protein. An increased understanding and demonstration of the anti-hypertensive properties of dietary protein and food protein-derived bioactive peptides is required before

greater recommendations of increased dietary protein can be made to individuals who are looking to control their BP without resorting to pharmaceutical agents.

**PARTICIPANT PRIVACY AND CONFIDENTIALITY OF DATA**

The following information will be collected during this project: name, address, date of birth, primary telephone number, and sex. This information is recorded (hard copy with scanned electronic back-up copy), which also contains the individual's written consent to participate in the project. Study records that contain identities will be treated as confidential following the Personal Health Information Act of Manitoba. These forms will be stored in a locked file cabinet in a locked office in the RCFFN and will never leave the site. The electronic back-ups will be on a single password-protected computer. The contact information will be entered into a separate contact Excel spreadsheet that contains no other personal/health information and will only be used by the study coordinator to inform/update study participants while the study is being carried out. This file is maintained on a single computer and will be password encrypted. The study data will be stored for 25 years after the completion of the study. All paper materials and samples related to the study will be stored in the locked filing cabinet in a locked office or locked freezers at the RCFFN under the supervision of Dr. Rotimi Aluko. Only the study coordinators and the principal investigator will have access to the samples. All study documents will bear an assigned participant code. If the results of the trial are published, the identity will remain confidential. All study data will be labeled with only the assigned participant code. This unique code will only be known to the participant and the research study staff. Documents of personal information linking the participants to their codes will be kept separately from any other records, also in a secure locked area. Due to the coding system, there will be no link between personal data and the sample data collected during analysis. All print materials will be destroyed using the contracted document destruction service at the University of Manitoba. All electronic files will be deleted in an encrypted format.

Health Canada may review and research records for auditing purposes. The University of Manitoba Biomedical REB may review research- related records for quality assurance purposes. Organizations that may also inspect/copy the research records for quality assurance and data analysis include groups such as Health Canada their representatives, and

other researcher groups who are performing meta-analysis or knowledge synthesis work who request access to the raw data. This review or use of raw data will not include personal information such as name, address, telephone number, and/or any other identifying information. Any data required to support the protocol can be supplied on request.

**DISCUSSION**

Unlike earlier trials which have compared carbohydrates to protein in terms of blood pressure, the currently proposed trial will compare different types of protein added to the participants' diet. In this instance, the participant may or may not replace existing calories in the diet with additional protein. Therefore, the potential exists that increased protein consumption will lead to a change in overall energy intake. An increase in energy intake could occur if the additional calories of the protein are not being compensated for by participants, or a decrease in energy intake could occur due to the satiating properties of protein compared to other macronutrients. These potential shifts in overall energy intake could result in changes in body weight, which could lead to BP changes. Therefore, outcome variables, such as BP, will be adjusted for any significant changes in body weight, which may occur during the treatment periods. Additionally, the mix of fat and carbohydrate being potentially replaced by protein in terms of energy intake may vary between participants. While this trial will not be able to determine exactly whether fat or carbohydrate energy is being replaced with protein, it will reflect the real-world impact of instructing patients and the general public to increase protein consumption by drinking protein smoothies, without specific counseling on replacing other foods.

As in all clinical trials with food-based treatments, potential differences in taste, texture, and smell can make the blinding of participants to the treatments difficult. Our research team has worked with HSP and has developed a smoothie recipe that is good at masking the type of protein powder from which it is made. However, if this blinding is not sufficient it may be possible for participants to identify the casein treatment versus the HSP and HSP+ treatments. The likelihood of participants being able to identify the HSP compared to the HSP+ treatment is low. Fortunately given the crossover design and the stringent monitoring of compliance in the proposed trial, it is unlikely that any un-blinding of the participants to the treatments, as described above, would be able to impact the results. All of the outcomes in this trial will be

objectively measured, therefore the impact of knowing if you are on a hemp-based treatment is unlikely to change your outcome measurements. Knowing you are on a particular treatment, for which your compliance is monitored and controlled, is unlikely to influence your BP measurement, especially given the fact that BP measures will not be shared with participants until they have completed the entire trial.

**TRIAL STATUS**

Recruiting. The date of starting recruitment for this study was 01 May 2018 and the approximate date when recruitment will be completed is 29 February 2020. The current protocol version number is 6.

**TRIAL MONITORING**

**TRIAL STEERING COMMITTEE (TSC) AND DATA MANAGEMENT TEAM**

Dr. Rotimi E. Aluko (Principal Investigator and project lead) and Maryam Samsamikor (clinical coordinator, responsible for recruitment and consent process), will administer the study. Any changes in the protocol, testing, or recruitment criteria will be communicated to the Bannatyne REB of the University of Manitoba through amendments. Also, all the amendments will be approved by the NNHPD branch of Health Canada. The REB will receive an annual progress report of the study and their team will supervise the process of conducting the research according to the protocol and quality of confidentiality where needed.

**PROTOCOL MODIFICATION**

Any changes in the protocol will be first approved by the Principal Investigator of the study. Then a detailed amendment will be sent to the University of Manitoba REB for conditional approval. The final approval from REB will be obtained after submitting the approval from NNHPD of Health Canada to the REB for the related changes. Before all approvals are granted, no changes in the procedure will be made. The University of Manitoba REB will audit the study and any deviations from the Protocol will be documented as a breach report. The clinical trial registry will be updated according to any changes made to the study protocol.

**AVAILABILITY OF DATA**

Biological specimens, such as blood will be kept in the freezer until further analysis. There will be no analysis other than what the participants will be aware of and will give consent for. The results of this study will not be shared as a data set, but non-identifying results will be posted on the World Wide Web for other researchers to use. Also, the results at the participant's level will be shared with them when available.

## ABBREVIATIONS

SBP: systolic blood pressure; DBP: diastolic blood pressure; HSP: hemp seed protein; HSP+: HSP; plus HSP hydrolysate; RAAS: renin-angiotensin-aldosterone system; THC: tetrahydrocannabinol; CI: confidence interval; ADMA: asymmetric dimethylarginine; SHRs: spontaneously hypertensive rats; ACE: angiotensin-converting enzyme; RCFFN: Richardson Centre for Functional Foods and Nutraceuticals; IUDs: intrauterine contraceptive devices; FBS: Fasting blood sugar; TC: total cholesterol; TG: Triglycerides; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; BUN: blood urea nitrogen; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; IR: insulin resistance; ELISA: enzyme-linked immunosorbent assays; NO: nitric oxide; SEM: standard error of the mean; SPIRIT: Standard Protocol Items: Recommendations for Interventional Trials; NNHPD: Natural and Non-prescription Health Products Directorate.

## DECLARATIONS

### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was reviewed and approved by the Bannatyne REB of the University of Manitoba on 19 December 2016 with the approval number HS20390 (B2016:125). Written informed consent forms will be obtained at the in-person screening day from all of the potential participants. Also, the present study was reviewed and approved by NNHPD of Health Canada on 20 February 2018 with the protocol number HEMP BP 001. In addition, this study was reviewed and approved by the Heart and Stroke Foundation of Canada as the original funding source (Grant #: G-16-00014211). This study is also registered on the publicly accessible Clinical Trial Registry Databank at Clinicaltrials.gov on 28 June 2018 under the number NCT03508895 (http://ClinicalTrials.gov).

### CONSENT FOR PUBLICATION

Not applicable

## AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study will be available from the corresponding author upon reasonable request following the completion of the trial.

## COMPETING INTERESTS

The authors declare that they have no competing interests.


## FUNDING

This study is funded by the Heart and Stroke Foundation of Canada and the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Canada. The sponsors played no part in the study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication.


## AUTHORS' CONTRIBUTIONS

DM and RB contributed to the general idea and design of the study. MS and RA took part in developing the final version of the protocol. MS contributed to recruitment, data collection, recordings, and reports as the clinical research coordinator. MS and RA contributed to getting approvals from NNHPD and the University of REB. All of the authors have read and approved the final version of the manuscript.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**TRANSITION STATEMENT 3**

In Chapter III by intricately detailing the protocol of our clinical trial, we now seamlessly transition to the findings and outcomes in Chapter IV. This pivotal chapter delves into the empirical exploration of the effects of HSP and its hydrolysate-derived bioactive peptide on 24-hrSBP and 24-hrDBP and crucial biomarkers associated with BP regulation. Chapter III served as our guidebook, outlining the methodologies and objectives that framed our clinical trial. As we step into Chapter IV, the focus shifts from theoretical constructs to real-world observations and measurable outcomes. This chapter unfolds the details of a double-blind, randomized, cross-over design trial involving 35 mildly hypertensive individuals. The exploration centers around the effects of consuming HSP and its hydrolysate on 24-hrSBP and DBP, offering valuable insights into their potential role in dietary hypertension management. The structured format of our trial, marked by 6-week treatment periods and 2-week washout intervals, allows for a comprehensive assessment of treatment effects. As the results will be presented, a reduction in 24-hrSBP and 24-hrDBP was observed after the consumption of HSP+ and HSP, compared to casein. The intricacies of these changes, along with the accompanying alterations in plasma biomarkers such as ACE activity, renin, and NO concentrations, are examined. The insights gained from this chapter not only contribute to the scientific understanding of hemp protein's impact on hypertension but also pave the way for informed discussions on its potential dietary applications in managing blood pressure.

# CHAPTER IV

## MANUSCRIPT 3

# EFFECTS OF WHOLE HEMP SEED PROTEIN AND HEMP SEED PROTEIN HYDROLYSATE CONSUMPTION IN HYPERTENSIVE ADULTS: A DOUBLE-BLIND RANDOMIZED CROSS-OVER STUDY

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Abbreviations:

ABPM, ambulatory blood pressure monitoring; ACE, angiotensin-converting enzyme; Arg, arginine; BP, blood pressure; FI, fluorescence intensity; GI, gastrointestinal; HSP, hemp seed protein; HSP+, hemp seed protein plus bioactive peptides; NO, nitric oxide; 24-hrSBP, 24-hour systolic blood pressure; 24-hrDBP, 24-hour diastolic blood pressure.

**ABSTRACT**

Background: The effects of consuming hemp seed protein (HSP) and its hydrolysate-derived bioactive peptide on blood pressure (BP) has not been investigated in humans.

Objective: This research aimed to investigate how consumption of HSP and its hydrolysate modulates 24-hr systolic and diastolic BP (24-hrSBP and 24-hrDBP) and plasma biomarkers of BP compared to casein.

Design: In a double-blind, randomized, cross-over design trial, 35 mildly hypertensive men and women with SBP between 130 and 160 mmHg, and DBP ≤110 mmHg were recruited. Participants were randomly assigned to varying sequences of three 6-week treatments, 50 g/d of casein, HSP, and HSP plus HSP-derived bioactive peptides (HSP+), separated by a 2-week washout period. Treatment effects were assessed by the linear mixed model with repeated measures.

Results: Consumption of HSP+ and HSP for 6 weeks, reduced the 24hrSBP and 24hrDBP compared to casein. After HSP+ consumption, 24hrSBP and 24hrDBP decreased from 135.06 and 79.98 mmHg to 128.08 ± 1.56 and 75.94 ± 1.38 mmHg respectively whereas these values were 133.49 ± 1.55 and 78.85 ± 1.37 after HSP consumption (P<.0001). The difference between HSP and HSP+ in their effects on 24hrSBP and 24hrDBP was -5.40 ± 0.64 and -2.90 ± 0.46, respectively (P<.0001). There were no differences between the HSP and HSP+ consumption in plasma ACE activity or renin and Nitric oxide (NO) concentrations. However, these two treatments were able to lower both ACE and renin activities and raise NO concentration in plasma compared with casein.

Conclusions: These results suggest that hemp protein consumption may have a role in the dietary management of hypertension, depending on the amount consumed and the type of protein they are replacing in the current diet. The additional hypotensive effect of the HSP+ suggests there may be a further application for HSP-derived peptides as antihypertensive agents. This trial was registered at clinicaltrials.gov as NCT03508895.

Keywords: hemp seed protein, plant protein, protein hydrolysate, bioactive peptides, ambulatory blood pressure, hypertension, angiotensin-converting enzyme, renin, antihypertensive properties

## INTRODUCTION

Hypertension is a chronic health issue described as blood pressure (BP) consistently above the normal values (SBP ≥130 and/or DBP ≥80 mmHg) (1,2). Globally, hypertension affects more than 30% of adults and causes more than 8.5 million deaths from vascular and renal diseases (3). Renin-angiotensin-aldosterone system (RAAS) is the primary hormonal system in body involved in BP regulation. Once renin is secreted from the kidneys into the blood, it converts liver angiotensinogen to angiotensin (Ang) I. Transformation of this physiologically inactive hormone to Ang II is catalyzed by angiotensin-converting enzyme (ACE) while passing through the lungs and kidneys (4,5). Ang II exerts various biological functions that conclusively result in vasoconstriction (6). Nitric oxide (NO) known as a vasodilation mediator is another powerful regulator of BP. Once produced by endothelial NO synthase (eNOS) from L-arginine in the vascular endothelium, NO decreases the vascular tone and increases the blood flow (7,8).

The pharmaceutical industry has developed BP medications in particular by utilizing ACE inhibition to blockade RAAS. ACE inhibitors such as enalapril, captopril, and lisinopril are the most commonly prescribed medications in the treatment of cardiovascular and renal diseases, and hypertension (9,10). Although pharmacologically effective, long-term use of these agents has been associated with adverse effects from cough to more serious complications like hyperkalemia and angioedema (11). The American Heart Association estimated that the national annual direct cost of hypertension in 2012-2013 was $47.3 billion where about half of this cost was for antihypertensive medications (12). Alternatives to taking medicine have been identified as desirable by many patients (13). Thus, innovative yet effective non-pharmacological approaches to reducing hypertension are of interest, particularly in patients considered to be at high risk of negative issues from combination therapy or those desiring to minimize their medication dose (14).

Non-pharmacological food-based interventions have gained increasing attention in ACE inhibition and subsequently hypertension prevention and control. The potential benefits of implementing nutrition interventions for hypertension may be cost-effective with minor or no adverse reactions (15,16). Dietary factors including greater consumption of protein have been strongly supported by epidemiological studies in terms of their ability to lower blood pressure (17). A cohort study of 272 men in five years revealed a negative correlation between plant

protein intake and BP (18). Hemp seed (Cannabis sativa L.) is an emerging source of plant protein that is rich in arginine (Arg) and may have hypotensive properties; therefore, the potential exists to utilize HSP for antihypertensive properties or from which to produce the hypotensive bioactive peptides (19,20).

Studies have demonstrated some bioactive peptides obtained through enzymatic hydrolysis from food protein sources such as HSP exert ACE and renin inhibitory properties (21). The hydrolysis of HSP could produce bioactive peptides which perform biological activities such as antihypertensive effects (9). In an animal study, oral administration of 200 mg/kg body weight of HSP hydrolysate (HPH) to spontaneously hypertensive rats (SHRs) lowered SBP by 30 mmHg and demonstrated ACE and renin inhibitory effects (22). Also, feeding growing SHRs with HPH prevented the expected increase in SBP compared to casein (~120 mmHg and 158 mmHg respectively) (23). The objective of this study was to investigate the ability of HSP and its hydrolysate-derived bioactive peptides on 24hrBP in a double-blind randomized cross-over trial.

## MATERIALS AND METHODS

### STUDY DESIGN

A randomized, double-blind, cross-over trial was designed with three 6-week treatment periods and 2-week washouts between periods during which the volunteers followed their regular diet. Methodological details of this study have been published (24). This trial was conducted at the Clinical Nutrition Research Unit of the Richardson Centre for Food Technology and Research (RCFTR), University of Manitoba. Participant randomization was completed in a 1:1 ratio by an external research assistant through sealed envelopes. A 3 × 3 Latin- square design followed by a random number generator was used to randomly assign the eligible participants to the following sequences: ABC, ACB, BAC, BCA, CAB, and CBA in which each = letter (A, B, and C) had been randomly assigned to one of the three treatments. The research participants and the researchers were unaware of which letter was assigned to which treatment. The trial was conducted in compliance with the principles of good clinical practice (GCP). The research protocol was reviewed and approved by the University of Manitoba's Biomedical Research Ethics Board in Winnipeg, Manitoba, Canada (protocol no. B2016:125). Also, the Non-prescription Health Products Directorate of Health Canada approved this study with the protocol

number HEMP BP 001. The trial was registered on the Registry Databank at clinicaltrials.gov as National Clinical Trial (NCT), ID: NCT03508895.

**STUDY POPULATION**

Thirty-five hypertensive volunteers aged 18-75 were screened and recruited from Winnipeg, Manitoba, Canada. News articles in Winnipeg Free Press newspaper and study posters were published and circulated and University of Manitoba e-mail lists were used to recruit for the study. Volunteers were initially screened over the phone using a brief screening questionnaire where they were asked about their general health and medical history. Then, potentially eligible participants were scheduled for an in-person screening process at the RCFTR to have their BP and blood samples taken. Before the in-person screening started, the participants were provided written informed consent forms by the research coordinator to sign. Then, while they were fasting, 20 ml of blood samples were taken by a phlebotomist via venipuncture to test blood biomarkers related to the exclusion criteria.

Individuals with SBP 130-160 mmHg and a DBP ≤ 110 mmHg and body mass index (BMI) of 18.5-40 kg/m2 were recruited. For those participants who were on medication, to be included in the study they needed to be on a stable type and dosage of medication for at least 3 months. Participants were excluded if their blood test results were reported as sodium < 134 mmol/l, > 148 mmol/l; fasting glucose > 6.1 mmol/l. Individuals with any clinically significant biochemistry abnormalities, secondary hypertension, active cardiovascular disease, diabetes, a history of cancer or malignancy in the last 5 years, and gastrointestinal (GI) disorder were also excluded. Smoking, consuming alcohol more than 14 drinks per week, people with child-bearing potential not using birth control, pregnancy, breastfeeding, and having weight change >± 5 kg in the past 3 months were also exclusion criteria. Finally, if determined as eligible, the study coordinator assigned the participants to interventions by opening a sealed envelop that contained a sequence of blinded treatments.

Both research coordinators and participants were blinded to the study treatments. The participants were instructed to avoid inconsistency in their physical activities and dietary patterns during the study and they were asked to perform their normal daily routines and abstain from prolonged strenuous exercise on measurement days. Also, they were asked to limit their alcohol and caffeine consumption to two or fewer alcoholic beverages and less than three 8-oz.

caffeinated beverages per day, respectively. The screening, recruitment, and the follow-up visits took 14 months starting from April 2018 until May 2019. After trial commencement, we expanded the range of eligible participants to include those with DBP ≤ 110 instead of 100 mmHg to improve the recruitment rate.

**STUDY INTERVENTION, TREATMENT PREPARATION, AND MASKING**

The protocol has been previously published (24). In this trial, participants consumed their regular diet plus 50 g/day of casein or HSP or 45 g HSP with an added 5 g of bioactive peptides (HSP+) for 6 weeks. The method used to prepare HSP hydrolysate (Fig. 4.1) was developed to simulate GI tract digestion (22). Table 4.1. demonstrates the amino acid profile of hemp seed hydrolysate adopted from Girgih et al. (2014) (23). The treatments were in the form of smoothies and each smoothie consisted of 25 g of protein from treatment powder mixed with frozen fruit, diet fruit juice, and sorbet, which were consumed twice a day (table 4.2). The treatments were consumed under supervision twice a week at RCFTR and the rest of the treatments were taken to be consumed at home. A member of the RCFTR metabolic kitchen staff was responsible for preparing the treatments according to the protocol and the treatments were served in single portions and identical stainless-steel cups and straws.

The HSP powder for the study was provided by Manitoba Harvest (HEMP Pro 70, soluble hemp protein concentrate, Manitoba, Canada) whereas the casein protein powder was purchased from Nutrablend Foods (New York, USA). Casein and HSP powder both had light tan color and they looked similar with the combination of frozen fruit and sorbet in the smoothies. A treatment consumption log was given to the participants to enter the date and time when they consume the smoothies. They were required to return the treatment containers on the next visit and the consumption checklist at the end of each treatment period to monitor for compliance. Returning the containers or being present for treatment consumption under supervision in at least 80% of the treatments per treatment period was defined as compliant. Also, a GI tolerability questionnaire was completed by participants weekly during each intervention period to evaluate the possible adverse events or complaints about tolerability.

**Figure 4.1.** Flow Diagram of the Hemp Seed Protein Hydrolysate Preparation

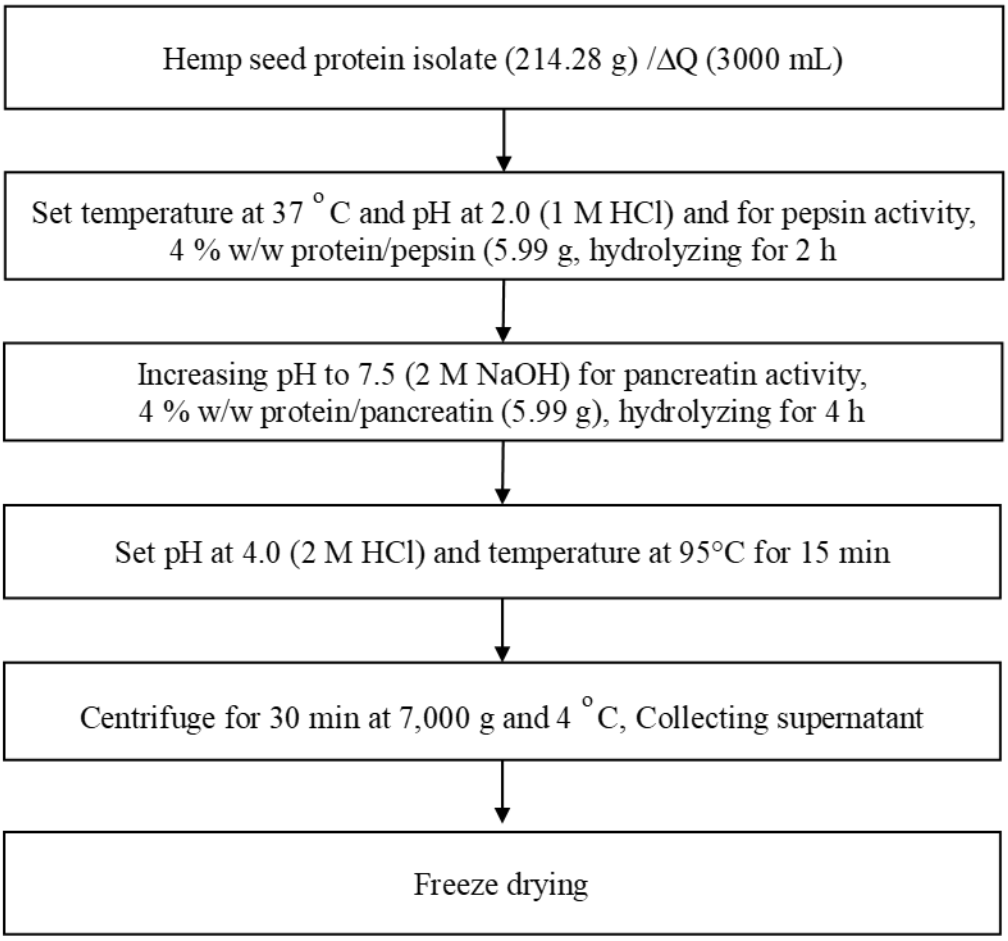


**Table 4.1** Amino acid composition of the hemp seed protein hydrolysate adopted from Girgih et al. (2014).

| Amino acid | Hemp seed hydrolysate |
|---|---|
| Asx | 10.79 |
| Thr | 3.70 |
| Ser | 5.43 |
| Glx | 18.12 |
| Pro | 4.72 |
| Gly | 4.50 |
| Ala | 3.95 |
| Cys | 1.20 |
| Val | 5.17 |
| Met | 2.03 |
| Ile | 4.01 |
| Leu | 6.78 |
| Tyr | 3.61 |
| Phe | 4.50 |
| His | 2.96 |
| Lys | 3.93 |
| Arg | 2.11 |
| Trp | 12.56 |

**Table 4.2.** Macronutrient Profile of Smoothies Per Portion

| | Protein powder (25 g) | Fruit juice (diet, 130 mL) | Pineapple (40 g) | Sorbet (125 mL) | Total |
|---|---|---|---|---|---|
| Energy (Kcal) | 120-170 | 5 | 20 | 80 | 330-334 |
| Fat, g | 1-6 | 0 | 0 | 0 | 1-6 |
| Carbohydrate, g | 2.5 | 1.3 | 5.1 | 20 | 28.9 |
| Protein, g | 25 | 0 | 0.2 | 0 | 25.2 |

## BLOOD SAMPLE COLLECTION

Approximately 20 ml samples of fasting blood were collected by a phlebotomist on the morning of day 1 and day 42 of each treatment period (6 time points in total). Other than treatment consumption, missing two consecutive sessions of blood sampling and measurement were considered non-compliance. Blood samples were drawn from the top of the forearm via venipuncture for the assessment of plasma biochemical markers. The participants were instructed to abstain from alcoholic beverages within 48 h prior to blood draws and food or caffeinated beverages within 12 h prior to blood draws during the trial phases. After blood collection, the samples were centrifuged at 1000 g for 20 min at 4 °C to separate and aliquot plasma samples, which were then stored at −80 °C for further analyses.

## OUTCOME MEASUREMENTS

The following outcomes were measured and there was no change to trial outcomes after the trial commencement.

## 24-HOUR SYSTOLIC AND DIASTOLIC BLOOD PRESSURE

The primary outcome of this study was 24-h SBP, it was measured on day one of the first treatment period and the last day of each period using ambulatory BP monitors (ABPM, OnTrak, Spacelabs Healthcare Inc., Snoqualmie, WA, USA) (25). Participants were fitted with an ABPM to wear for a full day and the continuous SBP and DBP measured over 24 h were recorded. Also, the participants were asked to record the time and the activity they were involved in each time the ABPM goes off in an ABPM log provided to them and drop the log off with ABPM after 24

h. The continuous SBP and DBP were measured every 30 min during the daytime and every 1 h during nighttime according to the participant's sleep routine. Both written and verbal ABPM instructions were provided to the participants to minimize measurement errors. The report of the results was transferred to a computer and read by sentinel software. Out of 36 measures, 27 readings (75% cut-off) were considered a successful wear period otherwise, the participant was asked to wear the ABPM for another 24 h to obtain the minimum acceptable number of readings (26).

**DETERMINATION OF PLASMA ACE ACTIVITY**

The spectrophotometric method with furanacryloyl-L-phenylalanylglycylglycine (FAPGG) as substrate was used (22) with minor modifications and the ACE enzyme (from rabbit lungs) was purchased from Sigma-Aldrich Canada Co (Oakville, ON, Canada) to determine the plasma activity of ACE. Concisely, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffering solution containing 0.3 M NaCl, pH 7.5) was mixed with 20 µL plasma or ACE (different enzyme concentrations were prepared; 0.0313, 0.0625, 0.125, 0.25, and 0.5 U/mL), and 200 µL of 50 mM Tris–HCl buffer. The absorption intensity was determined at 345 nm and was recorded for 2 min at 23 °C in a spectrophotometer (BioTek PowerWave XS Microplate Reader, CA, USA). The standard curve was defined using the results expressed as ΔA min-1 plotted against ACE enzyme concentration. Finally, linear regression model of the standard curve was used to calculate the plasma ACE activity (U/mL).

**DETERMINATION OF PLASMA RENIN CONCENTRATION**

A pre-established fluorometric technique was used with slight changes (27) and the renin inhibitor screening assay kit was purchased from Cayman Chemical (Michigan, USA) to quantify the plasma renin concentration. To prepare various concentrations (4.15, 8.3, 16.5, 33, 66, 132, and 250 µg protein/mL) renin was diluted with assay buffer containing 50 mM of Tris–HCl at pH 8.0 and 100 mM NaCl. A 96-well microplate was filled with 20 µL of renin substrate and 160 µL assay buffer. The chemical reaction was activated by adding 10 µL plasma or each diluted renin solution followed by 10 s of shaking and 15 min incubation at 37 °C in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). Afterward, fluorescence intensity (FI) was documented using emission spectra in the wavelength of 490 nm upon excitation at 340 nm and the results were presented as ΔFI min-1. The linear

regression from the plot of ΔFI min-1 versus renin concentrations was used to obtain the standard curve. The ΔFI min-1 obtained from the regression equation for each plasma sample was used to calculate plasma renin concentration (μg/mL).

**DETERMINATION OF PLASMA NO CONCENTRATION**

The plasma NO concentration was determined using nitrate/nitrite colorimetric assay kit (Cayman Chemical, Michigan, USA) according to the manufacturer's protocol. Briefly, nitrate standard concentrations were prepared by mixing nitrate standard with assay buffer (0, 5, 10, 15, 20, 25, 30, 35 μM). A 96-well plate was filled with the solutions in the following order: 80 μL of plasma (diluted 1:1 with assay buffer), 10 μL of enzyme cofactor mixture (containing 1.2 ml nitrate/nitrite assay buffer), and 10 μL of nitrate reductase mixture (containing 1.2 ml nitrate/nitrite assay buffer) then incubated in room temperature for 1 hr while covered by plate cover. Afterward, 50 μL of Griess reagent R1 and 50 μL of Griess reagent R2 were added to the wells, and the plate was incubated at room temperature for 10 min for color development. Then the absorbance was read at 550 nm in a spectrophotometer (BioTek PowerWave XS Microplate Reader, CA, USA). The plot of absorbance at 550 nm was made and the nitrate standard curve was used to determine NO products.

[Nitrate + Nitrite] (μM) = (A550 – y-intercept) (200 μL) × dilution

**SAMPLE SIZE AND STATISTICAL ANALYSIS**

A total sample size of 30 participants was determined to allow detecting of a clinically 4.0 mmHg change in the mean 24-hrSBP (primary outcome) with an estimated within-participant standard deviation of 5.45 mmHg. The power to show the significant difference (type 1 error (α) of 5%) in 24-hrSBP between groups was 0.80 (28,29). A total recruitment goal of 35 participants was set which allowed for potential participant dropouts to achieve the calculated sample size. Study outcomes were compared between treatments using endpoint values and statistical analysis was performed using a mixed-effects model with repeated measures in SAS (OnDemand for Academics) with treatment, period, and sequence as fixed factors and participant as a random effect in this model (30). Baseline values of the selected outcomes, period, sequence, and treatments were considered in the model as independent variables. There were no binary outcomes and no interim or adjusted analyses performed in this study. Results are presented as estimated least square means ± standard error of the mean (SEM) for all values. Statistical

significance was considered at $P < 0.05$ for all analyses. Pairwise comparisons of hemp treatments with casein were performed using Dunnett's test, and a Bonferroni correction was used to account for multiple comparisons between the hemp treatments.

## RESULTS

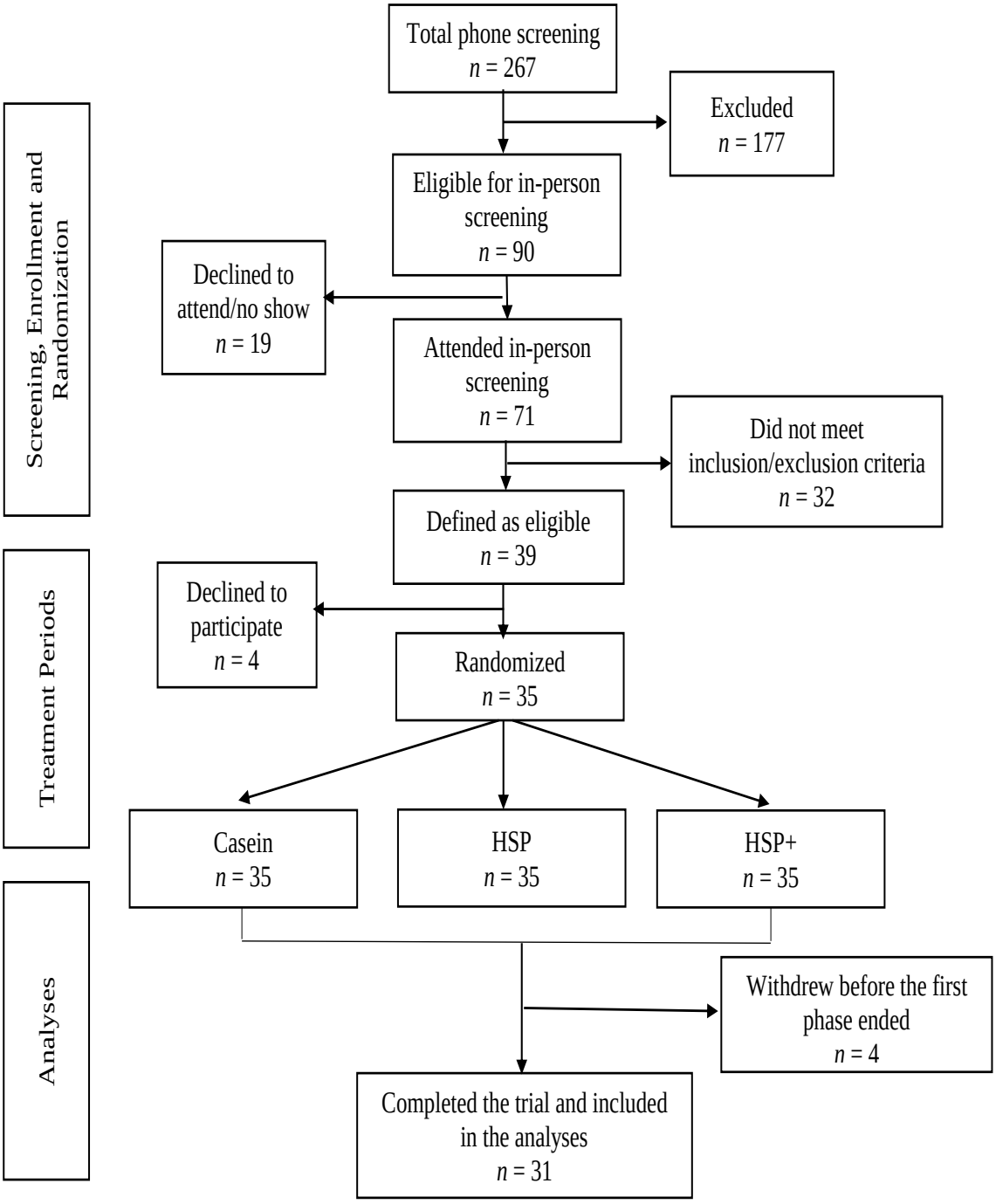
### BASELINE CHARACTERISTICS OF THE PARTICIPANTS

As shown in Fig. 4.2, a total of 35 participants were randomized to consume the treatments according to the assigned sequences. Four out of 35 participants withdrew from the trial owing to schedule conflicts, traveling, and personal reasons. These 4 participants dropped out before the end of the first period and were excluded from the analyses. The study was completed with a total of 31 participants included in the outcome analyses. The baseline characteristics of these 31 participants are represented in Table 4.3. At screening, the mean ± SD age was 61.09 ± 9.3 y, the BMI was 28.49 ± 4.9 kg/m2 and the waist circumference was 98.92 ± 12.6 cm. The office SBP was 145.61 ± 14.6 mmHg, and the office DBP was 89.91 ± 9.7 mmHg. Out of 31 participants, 11 (35.4%) of them were on antihypertensive medications.

### COMPARISON BETWEEN TREATMENT EFFECTS ON 24HRSBP AND DBP

As shown in Table 4.4, comparisons of after-treatment consumption revealed that HSP+ had the highest, whereas casein had the lowest hypotensive effects on both ambulatory SBP and DBP compared to the other two treatments ($P <.0001$). The highest reduction was related to the HSP+ which was able to decrease 24hr SBP by -8.87 mmHg compared to casein ($P <.0001$). A separate analysis was conducted on the participants who were on any type of antihypertensive medications and those who were not. The results of these subgroup analyses revealed the same pattern obtained from the analyses of total study samples ($P <.0001$). These results even showed greater hypotensive effects of HSP+ and HSP compared to casein in those who were not consuming antihypertensive medications. Also, period and sequence did not influence changes in 24hrSBD and DBP in these patients ($P > 0.05$).

**Figure 4.2.** Flow Chart of the Participants at Each Stage of the Study




HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides.

**Table 4.3.** Characteristics of Participants at Baseline

| | All subjects | Male | Female |
|---|---|---|---|
| No. (%) | 31 | 19 (61.2) | 12 (38.7) |
| Age, y | 61.09 ± 9.3 | 58.52 ± 8.9 | 65.08 ± 8.7 |
| BMI, kg/m2 | 28.49 ± 4.9 | 29.04 ± 4.7 | 27.61 ± 5.1 |
| WC, cm | 98.92 ± 12.6 | 103.24 ± 10.2 | 92.10 ± 13.57 |
| Office BP, mmHg<br>SBP<br>DBP | <br>145.61 ± 14.6<br>89.91 ± 9.7 | <br>145.71 ± 13.8<br>92.49 ± 8.7 | <br>145.44 ± 16.4<br>85.83 ± 10.2 |
| Antihypertensive medication use, No. (%) | 11 (35.4) | 7 (22.5) | 4 (12.9) |

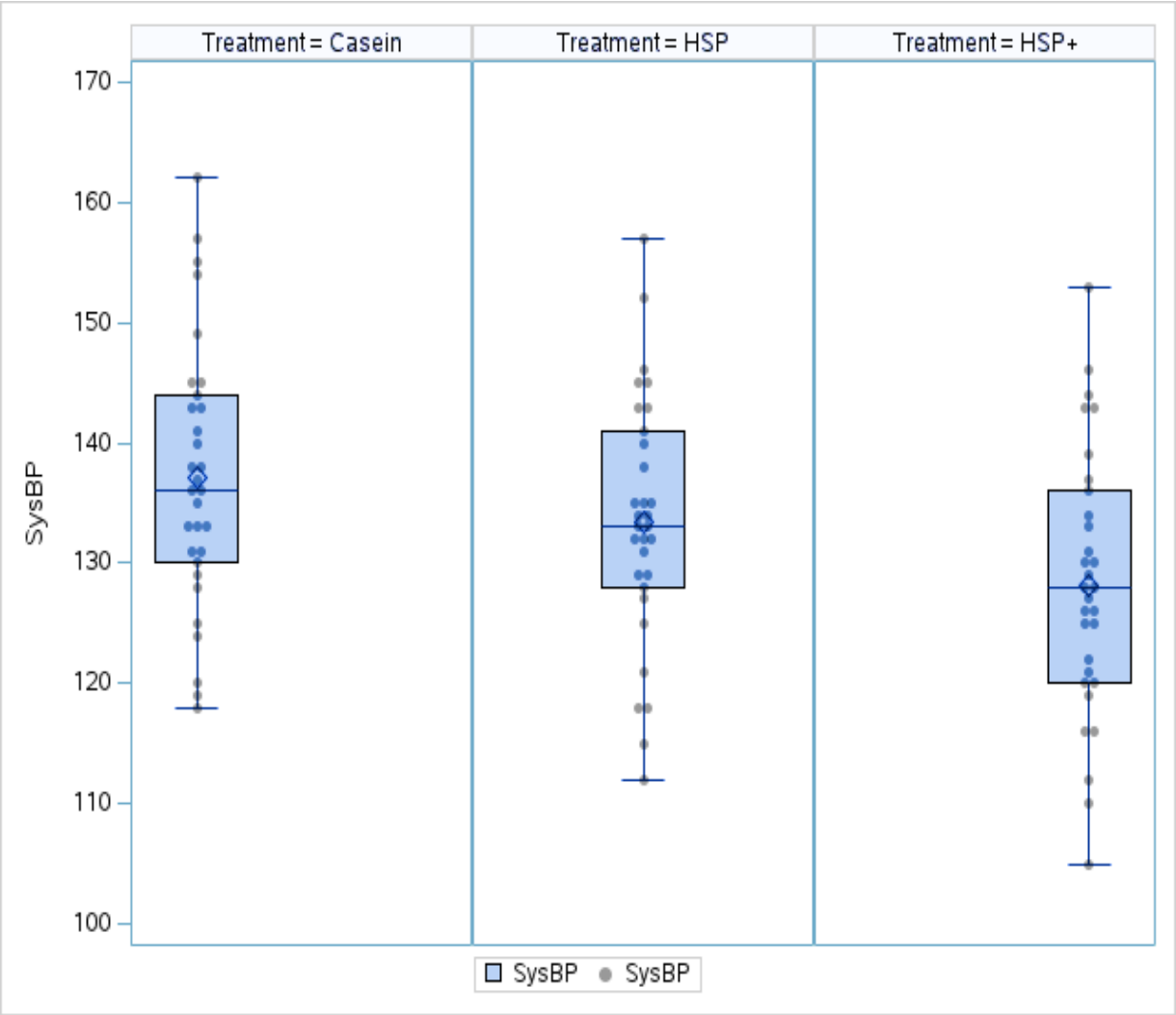
BMI, Body Mass Index; BP, blood pressure; cm, centimeter; DBP, diastolic blood pressure; kg/m2, kilograms to metres squared; mmHg, millimeters of mercury; SBP, systolic blood pressure; WC, waist circumference. Results are presented as mean + standard deviation.

**Table 4.4.** Comparison of the Effects of Each Pair of Treatments on 24 Systolic and Diastolic Blood Pressure

| | Δ HSP vs Casein | *P*[1] | Δ HSP$^+$ vs Casein | *P*[2] | Δ HSP$^+$ vs HSP | *P*[3] |
|---|---|---|---|---|---|---|
| Ambulatory BP (mm Hg) | | | | | | |
| Systolic | -3.46 ± 0.63 | <.0001 | -8.87 ± 0.67 | <.0001 | -5.40 ± 0.64 | <.0001 |
| Diastolic | -2.93 ± 0.45 | <.0001 | -5.84 ± 0.48 | <.0001 | -2.90 ± 0.46 | <.0001 |
| Ambulatory SBP (mm Hg) | | | | | | |
| On medication | -2.98 ± 1.05 | 0.009 | -6.54 ± 1.43 | <.0001 | -3.55 ± 1.42 | 0.025 |
| No medication | -3.89 ± 0.89 | <.0001 | -9.64 ± 0.93 | <.0001 | -5.75 ± 0.88 | <.0001 |
| Ambulatory DBP (mm Hg) | | | | | | |
| On medication | -2.70 ± 0.76 | 0.0008 | -3.84 ± 1.03 | 0.0004 | -1.14 ± 1.03 | 0.53 |
| No medication | -3.51 ± 0.62 | <.0001 | -6.52 ± 0.65 | <.0001 | -3.00 ± 0.61 | <.0001 |

BP, blood pressure; DBP, diastolic blood pressure; HSP, hemp seed protein; HSP$^+$, HSP plus HSP derived bioactive peptides; mmHg, millimeters of mercury; SBP, systolic blood pressure. All values are differences in estimated least-squares means ± SEM. P values were derived by using SAS Mixed Model. [1]P values HSP versus casein, [2]P values HSP$^+$ versus casein, [3]P values HSP$^+$ versus HSP.

**Figure 4.3.** Box Plot Analysis of 24 Systolic Blood Pressure Across Different Interventions



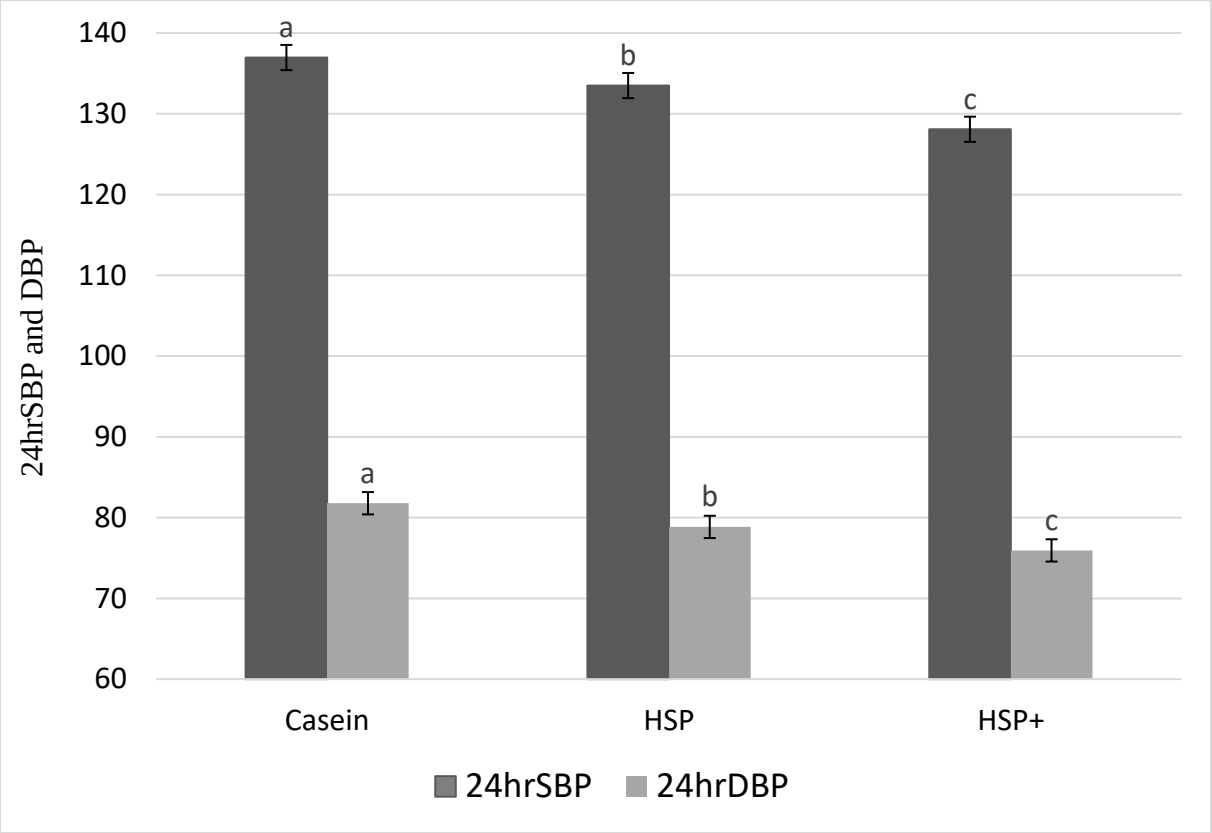

HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; SysBP, systolic blood pressure. The box plots represent estimated least-squares means ± SEM of SysBP after consumption of casein, HSP or HSP+ in 31 participants. Each dot represents the mean of 24-hr SysBP in each participant after consumption of each treatment. The results are derived by using SAS MIXED model.

Fig. 4.3 illustrates the mean 24hr systolic blood pressure after treatment consumption. Each dot represents the mean of 24hr SBP in each participant. After consumption of HSP+, 24hrSBP and DBP decreased to 128.08 ± 1.56 and 75.94 ± 1.38 mmHg, respectively. In comparison, these values were 136.96 ± 1.56 and 81.78 ± 1.38 after casein and 133.49 ± 1.55 and 78.85 ± 1.37 after HSP consumption. These results show that 6-week consumption of HSP+ led to the lowest ambulatory SBP and DBP among the three treatment groups (P <.0001) (Sup Fig 4.1). Fig. 4.4 illustrates the mean 24hr diastolic blood pressure after treatment consumption. Each dot represents the mean of 24hr DBP in each participant. Different treatment consumption effects on 24hr systolic and diastolic blood pressure are shown in Sup Fig 4.2 and 4.3. Each dot represents SBP or DBP in each participant read by ABPM during a 24hr period.

**PLASMA ACE ACTIVITY AND RENIN, AND NO CONCENTRATIONS**

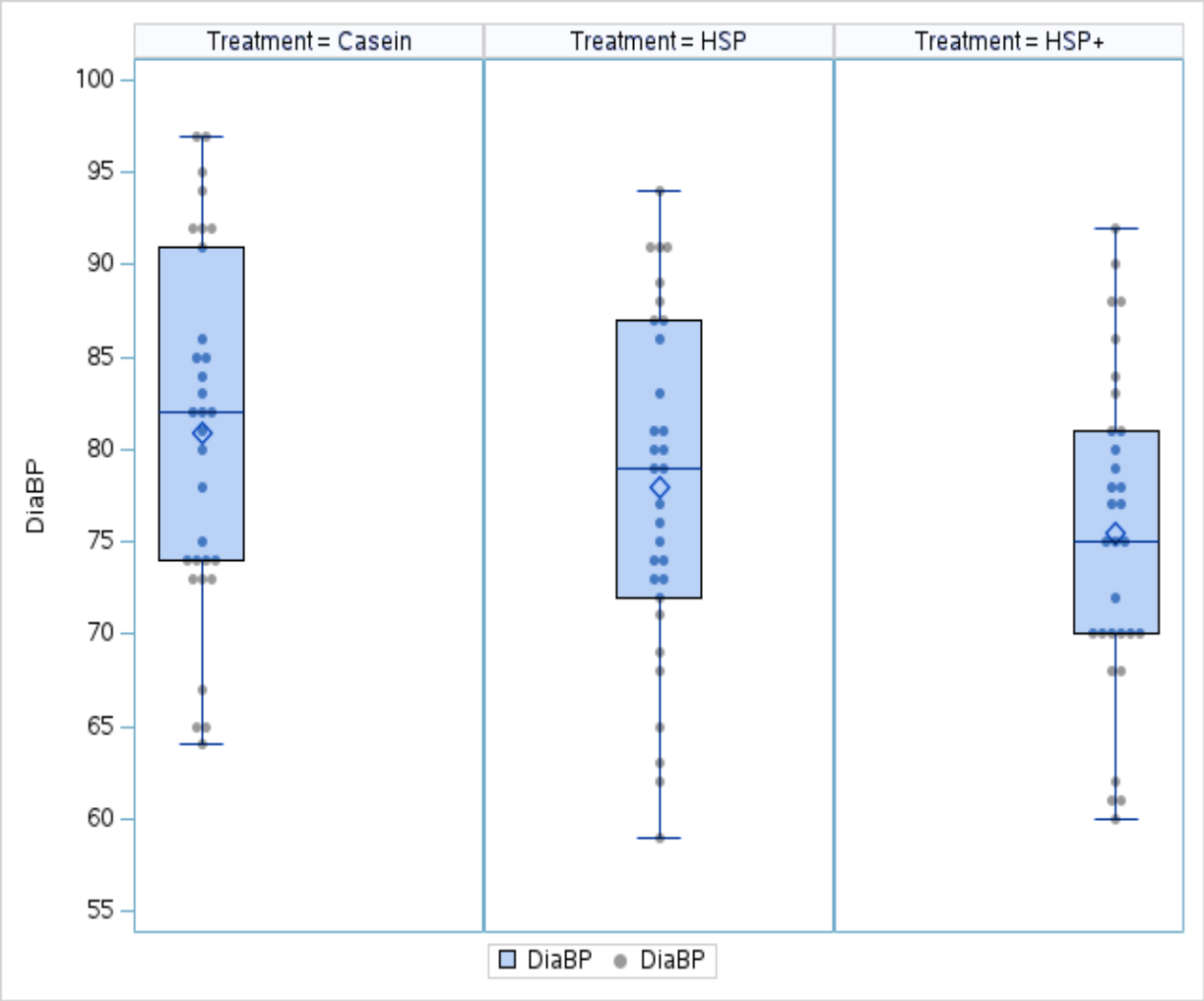
Table 4.5 shows the comparison of treatment effects relative to baseline. As represented HSP+ decreased the plasma activity of ACE compared with casein (-0.030 ± 0.006, P <0.0001); but not HSP. Similar to ACE activity, HSP+ led to a reduction in the plasma level of renin compared with casein (-0.010 ± 0.002, P = 0.0004), but not HSP. HSP+ also resulted in an increase in the plasma concentrations of NO, compared to casein, (6.29 ± 1.93, P = 0.003); but not HSP. The comparison between HSP and casein revealed that HSP was also able to lower plasma ACE activity and renin concentration and raise the plasma NO concentration (-0.026 ± 0.006, P = 0.0002 and -0.013 ± 0.002, P <.0001, and +4.27 ± 1.87, P = 0.047). Fig. 4.5 represents the endpoint values of ACE activity and renin, and NO concentrations after 6 weeks of treatment consumption. As shown in Fig. 4.5.a, the lowest ACE activity was observed after HSP+ consumption compared with casein (0.032 ± 0.004, P <.0001). As shown in Fig. 4.5.b, unlike ACE activity, the renin concentration was lower after consumption of HSP compared with casein (0.011 ± 0.001, P <.0001). Also, Fig. 4.5.c, shows NO had the highest and lowest concentration after HSP+ and casein consumptions respectively (21.89 ± 1.50, P <.0001 and 19.87 ± 1.45, P <.0001 and 15.59 ± 1.50, P <.0001 for HSP+, HSP, and casein).

**Supplemental Figure 4.1.** Effects of casein, HSP and HSP+ on 24hrSBP and DBP after 6-week treatment consumption.



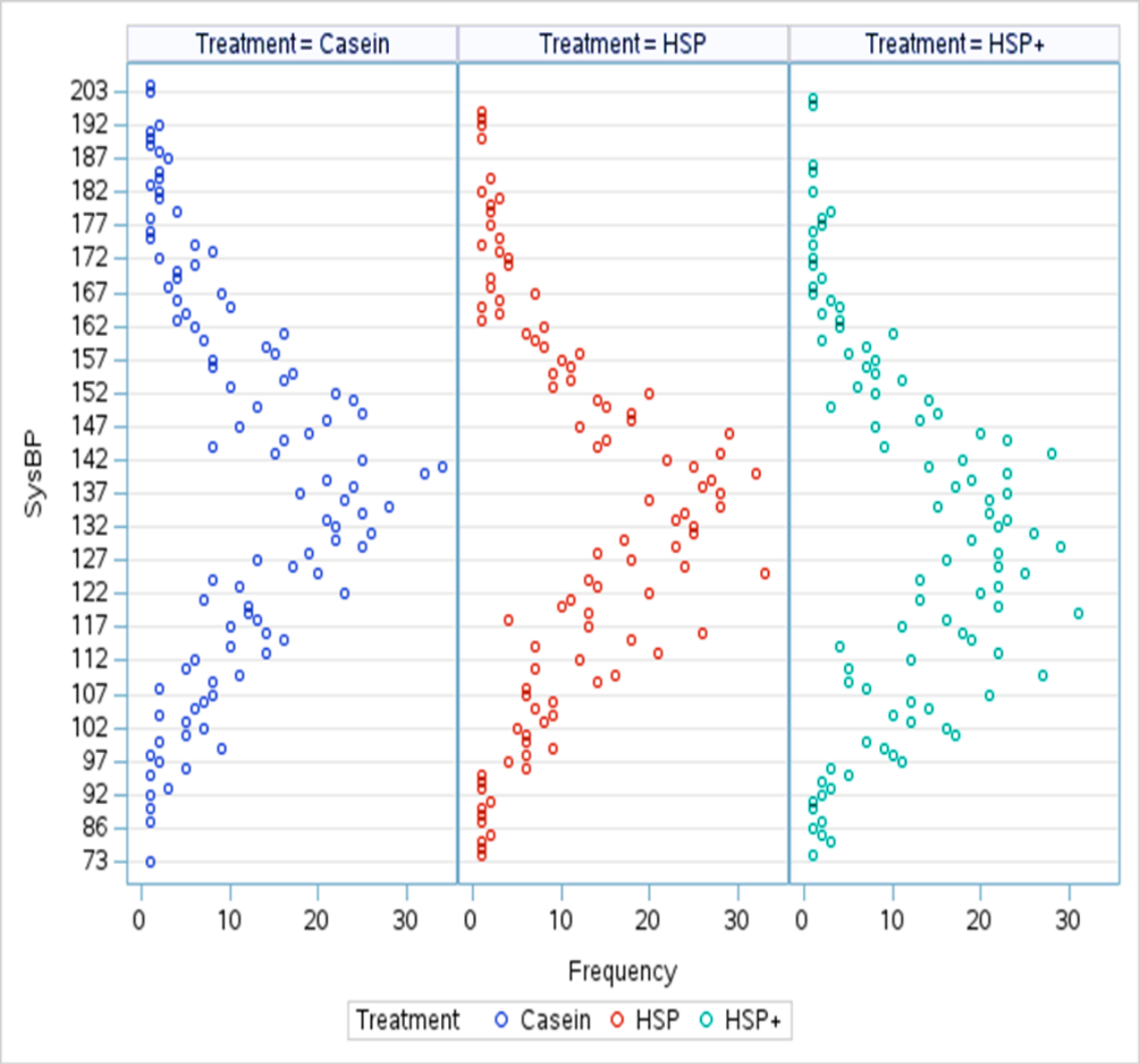

24hrDBP, 24-hour diastolic blood pressure; 24hrSBP, 24-hour systolic blood pressure; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides. The bars represent estimated least-squares means ± SEM of 24hrSBP and DBP derived by using SAS MIXED model. Bars with different superscript letters are significantly different.

**Figure 4.4.** Box Plot Analysis of 24 Diastolic Blood Pressure Across Different Interventions



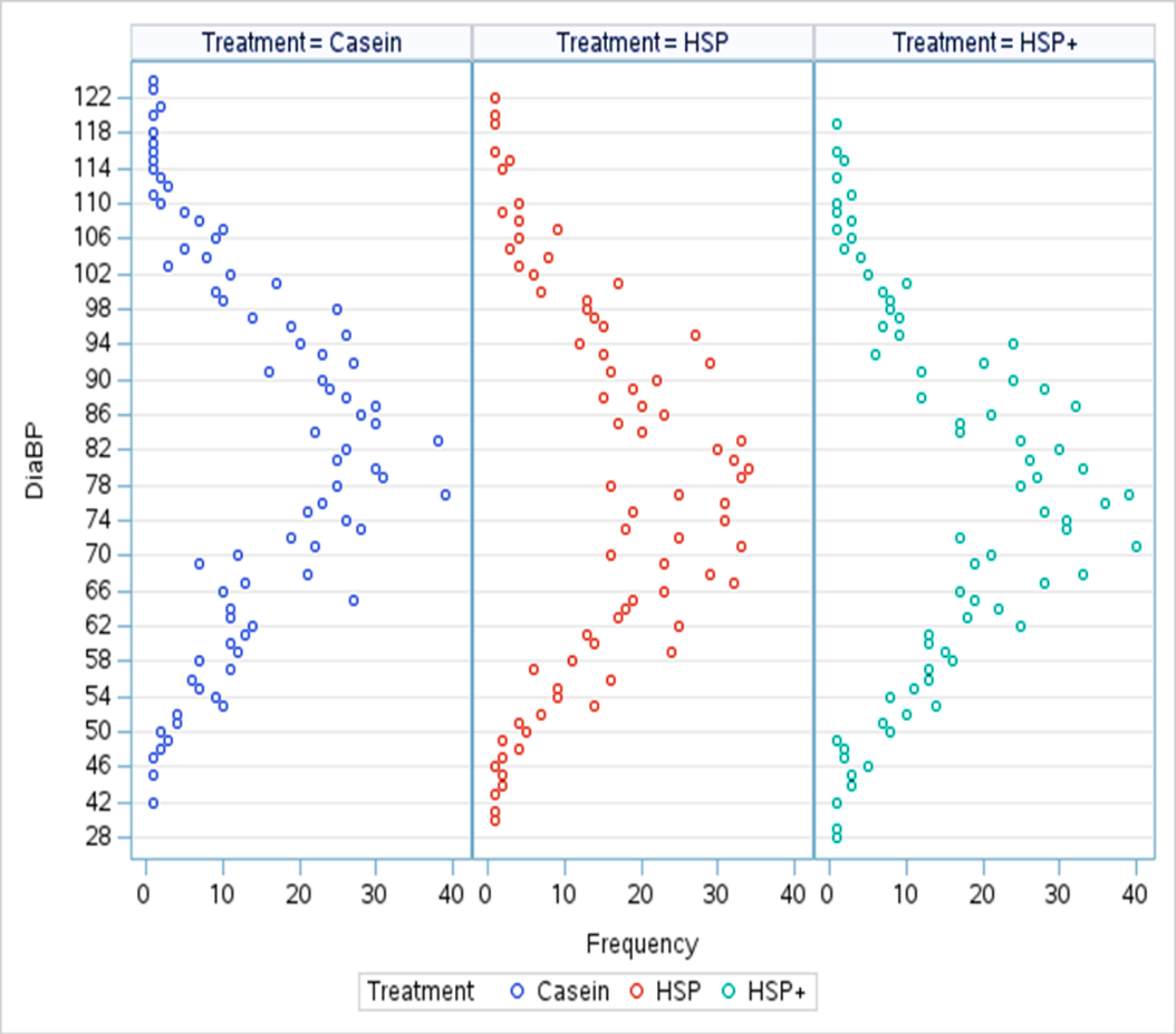

DiaBP, diastolic blood pressure; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides. The box plots represent estimated least-squares means ± SEM of DiaBP after consumption of casein, HSP or HSP+ in 31 participants. Each dot represents the mean of 24-hr DiaBP in each participant after consumption of each treatment. The results are derived by using SAS MIXED model.

**Supplemental Figure 4.2.** Distribution Dot Plot of 24 Systolic Blood Pressure After 6-week Consumption of Different Treatments




HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; SysBP, systolic blood pressure. Dots represent SysBP read by ABPM in 31 participants and X axis represents the frequency of each value repeated in different participants after consumption of each treatment.

**Supplemental Figure 4.3.** Distribution Dot Plot of 24 Diastolic Blood Pressure After 6-week Consumption of Different Treatments



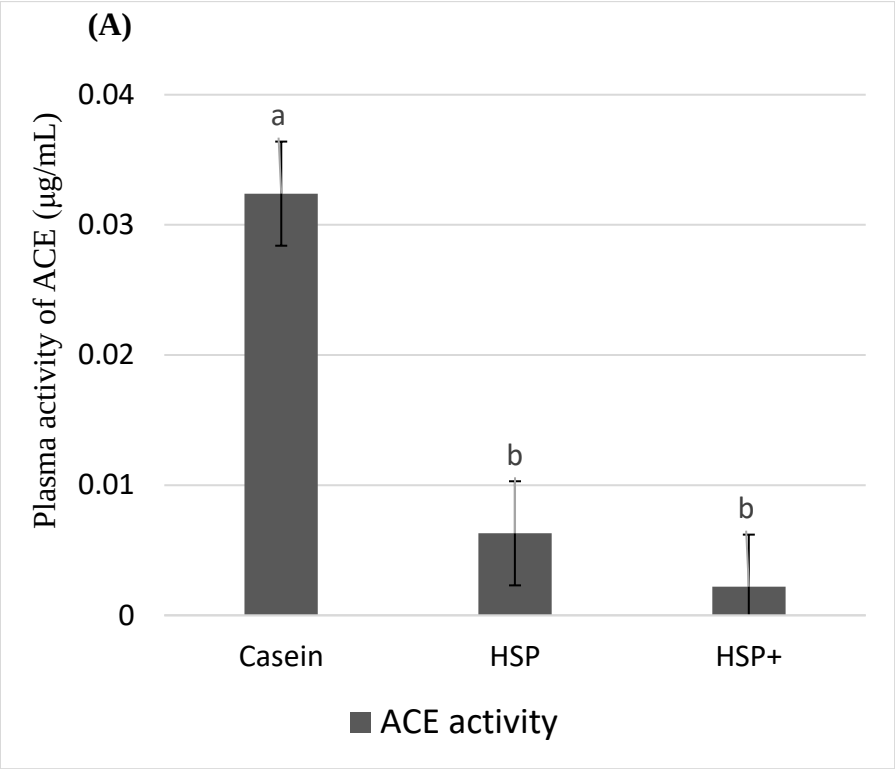

DiaBP, diastolic blood pressure; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides. Dots represent DiaBP read by ABPM in 31 participants and X axis represents the frequency of each value repeated in different participants after consumption of each treatment.

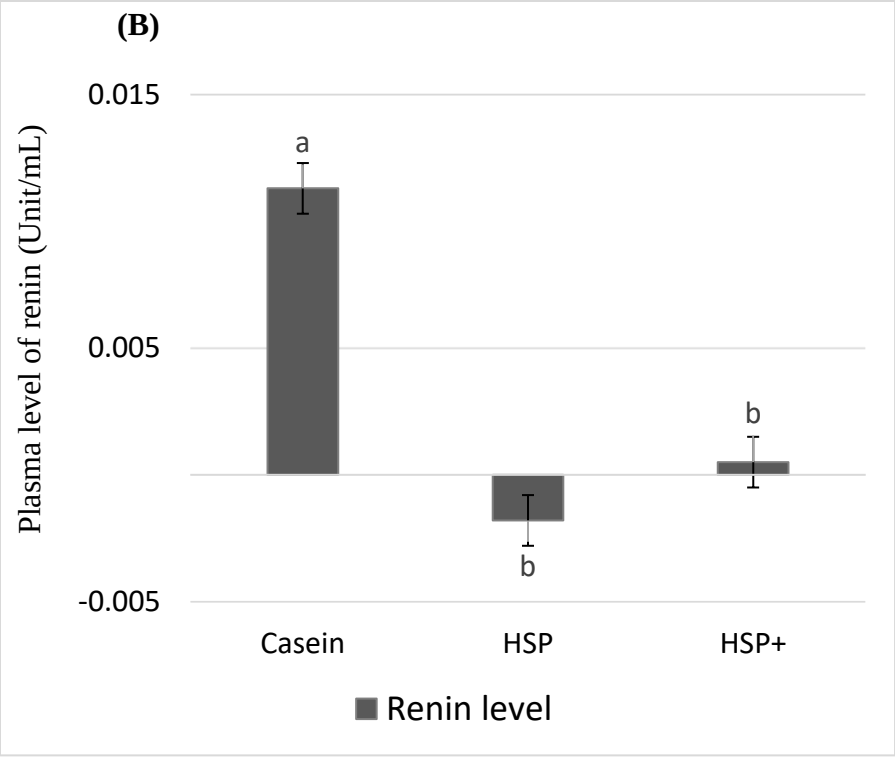
**Table 4.5.** Comparison of Treatment Effects on Plasma ACE, Renin, and NO Concentrations

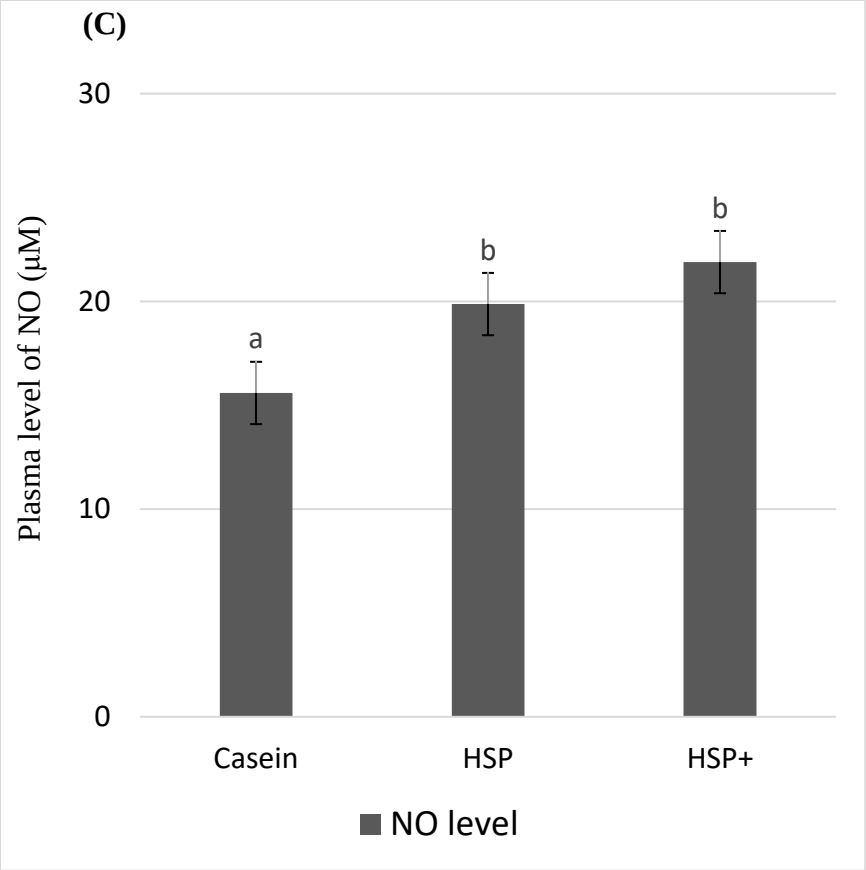
| | Δ HSP vs Casein | $P^1$ | Δ HSP$^+$ vs Casein | $P^2$ | Δ HSP$^+$ vs HSP | $P^3$ |
|---|---|---|---|---|---|---|
| ACE (μg/mL) | -0.026 ± 0.006 | 0.0002 | -0.030 ± 0.006 | <.0001 | -0.004 ± 0.006 | 1 |
| Renin (Unit/mL) | -0.013 ± 0.002 | <.0001 | -0.010 ± 0.002 | 0.0002 | 0.002 ± 0.002 | 0.37 |
| NO (μM) | 4.27 ± 1.87 | 0.047 | 6.29 ± 1.93 | 0.003 | 2.01 ± 1.88 | 0.57 |

ACE, angiotensin converting enzyme; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; NO, nitric oxide. All values are differences in estimated least-squares means ± SEM. P values were derived by using SAS Mixed Model. [1]P values HSP versus casein, [2]P values HSP$^+$ versus casein, [3]P values HSP$^+$ versus HSP.

**Figure 4.5.** Effects of casein, HSP and HSP+ consumption on Plasma ACE Activity and Renin and NO levels






ACE activity (A), renin (B), and NO (C) concentrations after treatment consumption. ACE, angiotensin converting enzyme; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; NO, nitric oxide. The bars represent estimated least-squares means ± SEM of plasma biomarkers derived by using SAS MIXED model. Bars with different superscript letters are significantly different.

## DISCUSSION

Our results demonstrated that consumption of 50 g/day of HSP or HSP+ for 6 weeks significantly reduced 24-hrSBP and DBP compared to casein. HSP+ exhibited even greater lowering effect on 24-hrBP compared to HSP. To date, a number of studies have investigated the effects of plant protein on BP. Although He et. al. (31) did not measure 24-hrBP, our results are consistent with their findings relating to the efficacy of plant protein to lower BP, they used soy protein in comparison with milk protein and carbohydrate control. Also, their findings did not show a difference between soy and milk protein in terms of the BP lowering effect. In another randomized clinical trial, He et al. reported that soybean protein isolate decreased both SBP and DBP compared to complex carbohydrate control (32). This trial used a parallel design and BP was measured in the office setting. Both HSP and soybean protein have a high Arg content that may contribute to some of their hypotensive properties.

Li et. al. (33) investigated bioactive peptides derived from pea protein hydrolysate in hypertensive patients in a randomized cross over trial. Consumption of 3 g/d pea protein hydrolysate, resulted in SBP reductions of 5 and 6 mmHg respectively after two and three weeks. Unlike our study, they did not measure 24-hrBP, and the study duration and the hydrolysate dosage were less than our trial. Also, they did not observe a hypotensive effect from pea protein isolate. In another randomized clinical trial by Ogawa et al., 12 weeks consumption of peptides from rice bran protein showed SBP lowering effects in patients with grade 1 hypertension. The tripeptide Leu-Arg-Ala was identified as the possible functional substance exerting the hypotensive effects (34). Kwak et. al. (35) compared black soy peptide supplementation with casein as the control in a randomized clinical trial. Unlike casein and consistent with our results, they showed that black soy peptides reduced both SBP and DBP after 8 weeks.

These findings are consistent with the study of Girgih et al. (21) who investigated BP lowering effects of HSP or HSP+ compared with casein in an animal experiment. They explored both the preventive and treatment effects of HSP and HSP+ in rats. Although each treatment period was for 4 weeks, their findings showed a greater hypotensive effect compared to our results, but even so, we were not anticipating similar results since animal studies usually do not translate into replications in human studies (36). Human trials investigating the effects of protein on BP have mostly used animal-based sources of protein such as whey and casein. Data presented by Fekete

et al. showed that in a randomized controlled trial, 8 weeks of whey protein (2 ×28 g) supplementation reduced 24-hrBP (-3.9 and -2.5 mmHg in SBP and DBP respectively) compared with maltodextrin as control. Also, whey protein was able to decrease day-time SBP and DBP compared to casein (37). It has been suggested that the inhibition of ACE could be the potential mechanism by which whey protein may affect BP (38).

Mollard et al. conducted an acute trial using hemp protein (40 and 20 grams) compared to soybean protein and carbohydrate control, but hemp protein did not show any impact at 15, 30, 45, and 60 min on office blood pressure (39). Other than the acute nature of this trial, inconsistent with our study they did not recruit from the hypertensive population. Generally, healthy and normotensive participants may not have the potential for possible BP lowering effects of hemp protein with a high Arg content.

Exploratory analysis revealed that the hypotensive effects of HSP and its hydrolysate are even higher in those participants who were not on any type of hypotensive medication. The greater antihypertensive effects in response to hemp protein consumption in these patients could be explained by the potential capacity of the pathways involved in BP regulation since these pathways may become saturated by antihypertensive medications.

The findings of our study add to the collective building of current literature on the hypotensive effects of HSP and its hydrolysate, which prior to this trial was based on in vitro and animal studies (22,23,40). The current study revealed the hypotensive impact of HSP+ compared with HSP and casein, however, we did not observe a significant difference in terms of plasma biomarkers of BP between HSP+ and HSP when compared together. Even though RCTs investigating the effects of HSP consumption on BP are lacking and so far, no clinical trial has evaluated the impacts of HSP-derived bioactive peptides consumption alone in hypertensive patients, our findings are predominantly consistent with other studies that reported a potential relationship between HSP and the bioactive peptides consumption and BP and its plasma biomarkers (23,41,42).

Our results showed that both HSP and HSP+ increased plasma NO concentration, but NO concentration was higher after the consumption of HSP+ compared with HSP. In recent years, increased attention has been generated toward amino acids with hypotensive potentials. Specific amino acids including cysteine, glutamic acid, tryptophan, and Arg have been particularly

studied for their hypotensive effects (43,44). HSP has a high content of Arg, but casein conversely is relatively low in Arg (45). Also, compared with HSP hydrolysate, HSP contains even greater amount of Arg (23). By including a treatment group consuming both HSP and HSP hydrolysate in our study, we aimed to explore whether the antihypertensive effects of HSP and its hydrolysate are additive or synergistic. The NO results suggest that the increasing effect of HSP and its hydrolysate on plasma NO concentration could be modulated through the same underlying mechanism; the conversion of Arg to NO.

This trial has several strengths including the cross-over design with the advantage of within-participant comparisons of response to treatment. Reducing much of the potential for an inter-individual variation in the study outcomes (30). This study has addressed a few contextual knowledge gaps in the association between individuals' protein intake and their BP. The most unique aspect of this study is that to our knowledge, this is the first clinical trial investigating the effects of HSP and its bioactive peptides in hypertensive patients. The ABPM devices used in the present study are considered to be the gold standard for measuring BP versus in-office and home measurements.

We also assessed any possible adverse effect of protein consumption on GI tract and most of the participants reported none of the GI symptoms. Only a few participants reported mild flatulence and abdominal discomfort in the first couple of weeks of treatment consumption, but the side effects did not last more than two weeks after starting the study. This trial also has some limitations that must be considered. Firstly, we were not able to observe the daily consumption of treatments, did not include a run-in period, and only had a 2-week wash-out period between treatments. Moreover, the extent to which disparities in dietary intake and physical activity patterns between treatments may have modified the BP or plasma biomarkers response to the study treatments in our trial remains speculative as we did not tightly control diet or exercise in participants in this free-living trial. Further studies will be needed to confirm the potential of HSP and its hydrolysate-derived bioactive peptides in BP reduction. Dose-response research is strongly encouraged in the future to investigate the highest dose of hemp seed bioactive peptides with the optimum hypotensive response.

## CONCLUSION

In conclusion, we found evidence that consumption of both HSP and HSP+ decreased 24-hrSBP and DBP compared with casein. Also, HSP+ demonstrated a greater lowering effect on 24-hrSBP and DBP compared with HSP. Similar to the hypotensive effects, HSP and HSP+ reduced plasma ACE activity and renin and increased NO concentrations when compared with casein. However, we didn't observe a difference between HSP and HSP+ in terms of their effects on plasma BP biomarkers. These results mostly align with the prior animal experiment that suggested a beneficial association between HSP and its hydrolysate intake and hypertension management. Further research particularly clinical trials are required to better understand how and to what extent HSP and HSP+ separately impact BP and, to determine the underlying mechanisms.

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We appreciate Dennis Joseph's technical assistance on this project. The authors' contributions were as follows—REA, DSM, and RCM: conceptualized the research and designed the research program; MS: prepared University of Manitoba Ethics and Health Canada documents and REA: submitted the documents for approvals; AMA: was involved in training MS to develop HSP hydrolysate production and MS prepared the HSP hydrolysate for the whole project; MS: was responsible for the screening, data collection, and clinical trial coordination; AMA: trained MS and MS: analyzed the study samples; MS: performed the statistical analyses and wrote the first draft of the manuscript; REA: was the Principal Investigator and administrative lead for the study; all authors: critically reviewed the manuscript, and approved the final manuscript. None of the authors reported a conflict of interest related to the trial.

## DATA AVAILABILITY

Deidentified data described in the manuscript, code book, and analytic code will be made available upon request pending application to and approval of the corresponding author.

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**TRANSITION STATEMENT 4**

In the preceding manuscript, we explored the potential antihypertensive effects of hemp seed protein and the bioactive peptides derived from hemp isolate in patients with hypertension. We employed specific gastrointestinal proteases including pepsin and pancreatin to cleave bonds in the protein structure and generate unique peptide fractions that had previously shown antihypertensive properties in an animal model. Our investigation discovered compelling findings that indicate both hemp seed protein and its hydrolysate exhibit promising characteristics in reducing BP through modulation of the renin-angiotensin system as demonstrated by a reduction in ACE and renin activities, along with increased NO levels when compared to casein. The bioactive peptides with greater antihypertensive effects than the whole hemp seed protein, exhibited significant potential for utilization in the development of functional foods targeting hypertension prevention. Building upon these findings and to better understand the molecular mechanisms involved in the BP-reducing effects of hemp seed bioactive peptides, we further investigated the potential antioxidant activities of these peptides and their relationship with antihypertensive properties. Therefore, the subsequent manuscript serves as a logical continuation of our research and aims to examine the production of reactive oxygen species and the body's antioxidant defenses and explore the link to the development and progression of hypertension. By exploring these facets, we anticipate discovering valuable knowledge about the potential role of hemp seed protein and its bioactive constituents in mitigating oxidative stress.

# CHAPTER V

## MANUSCRIPT 4

# HEMP SEED PROTEIN-DERIVED PEPTIDES WITH ANTIOXIDATIVE PROPERTIES IN INDIVIDUALS WITH HIGH BLOOD PRESSURE: A DOUBLE-BLIND RANDOMIZED CROSS-OVER STUDY

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**Abbreviations:**

AT-II, angiotensin II; CAT, catalase; DBP, diastolic blood pressure; ET-1, endothelin-1; FRAP, ferric reducing antioxidant power; GPX, glutathione peroxidase; HPH, HSP hydrolysates; HSP, hemp seed protein; $HSP^{+}$, hemp protein plus hydrolysate derived bioactive peptides; H2O2, hydrogen peroxide; ROOH, hydroperoxide; ˙OH, hydroxyl; NADPH, nicotinamide adenine dinucleotide phosphate; ORAC, oxygen radical absorbance capacity; ROO•, peroxyl; PTPs, plasma total peroxides; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSA, radical scavenging activity; SBP, systolic blood pressure; SOD, superoxide dismutase; $O_2^{\bullet-}$, superoxide.

**ABSTRACT**

**Background:** Human clinical trials investigating the antioxidant properties of hemp seed protein (HSP) and its bioactive peptides have not been conducted.

**Objective:** The purpose of this research was to assess how HSP and hemp seed hydrolysate-derived bioactive peptides alter the plasma oxidative stress biomarkers and improve the level of antioxidant enzymes in individuals who have hypertension.

**Design:** A 22-week double-blind, randomized, cross-over trial was conducted with 35 hypertensive participants with systolic and diastolic blood pressure of 130 - 160 and ≤110 mmHg, respectively. During each 6-week treatment phase, participants consumed 50 g of casein, HSP, or 45 g HSP plus 5 g HSP-derived bioactive peptides ($HSP^+$) daily according to their randomized sequences. During the 2-week washout periods, participants were on their regular diet. SAS Mixed model with repeated measures was used for treatment effect assessments.

**Results:** After 6 weeks of consumption of HSP and $HSP^+$, the plasma level of total peroxides (-0.012 ± 0.003 and -0.019 ± 0.004, $p < 0.05$ respectively) and reactive oxygen species (ROS) and reactive nitrogen species (RNS) (-459.62 ± 91.60 and -705.98 ± 112.56, $p < 0.05$ respectively) decreased compared to casein. Additionally, both HSP and $HSP^+$ raised catalase (CAT) levels in plasma when compared to casein (6.24 ± 1.94 and 8.07 ± 2.21, $p < 0.05$ respectively), but only $HSP^+$ was able to increase superoxide dismutase (SOD) compared to casein (0.296 ± 0.10, $p = 0.010$).

**Conclusions:** Our findings provide evidence that consumption of HSP and $HSP^+$ for 6 weeks significantly lowered the generation of ROS/RNS and plasma total peroxides (PTPs) compared to casein. Additionally, $HSP^+$ raised the SOD, and both $HSP^+$ and HSP increased CAT levels when compared to casein. The highest antioxidant activity between groups was observed after $HSP^+$ consumption. The results from this clinical trial suggest that hemp protein and bioactive peptides have the potential for increasing antioxidative activity This trial was registered at clinicaltrials.gov as NCT03508895.

**Keywords:** hemp seed protein, protein hydrolysate, bioactive peptides, oxidative stress, reactive oxygen species, reactive nitrogen species, plasma total peroxides, antioxidant enzymes, hypertension.

## INTRODUCTION

Hypertension as the primary risk factor for cardiovascular diseases plays a significant role in worldwide mortality and morbidity (1,2). Various biological mechanisms have been implicated in the pathophysiology of hypertension and oxidative stress has been identified as the unifying element among these factors that contributes to the initiation, progression, and severity of high blood pressure (3,4). Oxidative stress caused by an accumulation of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) leads to intracellular molecules damage and consequently cell death (5). Major ROS generated in biological systems include superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), hydroxyl ($^{\bullet}OH$), hydroperoxide (ROOH), and peroxyl ($ROO^{\bullet}$) radicals (6).

Several enzymatic and non-enzymatic sources of ROS are present in the endothelial cells of vascular tissue. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is in particular the major source of vascular superoxide when triggered by hormones, such as angiotensin II (AT-II), endothelin-1 (ET-1), aldosterone, and urotensin II (7,8). In order to maintain redox balance, cells need to regulate the generation and elimination of ROS. At physiological levels, ROS regulate vascular homeostasis by modulating vascular smooth muscle cell contraction, relaxation, and growth. However, under pathophysiological conditions, excessive intracellular levels of ROS generated through enzymatic sources in vascular cells result in the perturbations of homeostasis in the vascular wall (9,10).

Naturally, the body's antioxidant defense is responsible for maintaining the balance in oxidation-reduction (redox) reactions in living cells. The endogenous enzymatic defense, which is the first line of antioxidants includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Although these enzymes have a fundamental role in neutralizing free radicals, when the imbalance in the prooxidant-antioxidant generation in favor of the former occurs, the body's natural defense will be overwhelmed, and oxidative stress arises (11,12). Oxidative stress thus causes oxidation of lipids and proteins, which adversely alters cellular membranes and triggers a number of chronic diseases (13). Accordingly, the application of exogenous antioxidants is an effective strategy in defending against oxidative stress. Therefore, in recent years, an intensive search has ensued to find compounds with antioxidative activities that can supplement the body's natural defensive factors in the prevention of pathologic conditions (14).

Plant proteins have been identified to release antioxidative peptides under enzymatic hydrolysis or microbial fermentation (15). These peptides are mainly obtained from beans, nuts, seeds, grains, and leaves and have been demonstrated to show various antioxidative properties (16).

The seeds of the hemp plant *Cannabis sativa L* contain 25% protein. Hemp protein mainly stored as edestin and albumin, which are both highly digestible (17). Hemp seed is recognized as an emerging source of high-quality protein and bioactive peptides, however, utilization of this beneficial protein source in the food industry is limited and industrial production of hemp seed protein (HSP) derived bioactive peptides is yet to be developed (18). Extensive *in vivo* and *in vitro* research has been conducted with a focus on the functional properties of HSP including antioxidant and antihypertensive properties and attenuation of renal disease and cardiovascular impairment (19). Antioxidative properties in particular have been recognized as one of the primary attributes of hemp HSP and the bioactive derived from this protein source. Antioxidative properties of HSP are defined through numerous mechanisms such as preventing metal-catalyzed lipid peroxidation and increasing ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), radical scavenging, metal chelating, and antioxidant enzymes activities including SOD, CAT, GPX (20–32).

In several *in vitro* studies, antioxidant activities of HSP and hydrolysate derived from hemp seeds have demonstrated antioxidant activities, but orally administered HSP in *in vivo* trials are limited. Also, animal feeding trials assessing the antioxidant activities of HSP hydrolysates are rare and to our knowledge, no clinical trial has investigated this effect so far (33). Thus, the purpose of this clinical trial was to investigate the role of HSP and its bioactive peptides in suppressing oxidative stress and boosting the natural antioxidant defense in participants who have hypertension using a double-blind cross-over design.

## MATERIALS AND METHODS

### STUDY PARTICIPANTS

Volunteers were recruited from the general population of Winnipeg, Manitoba, Canada by using newspaper advertisements and circulating the study posters through the University of Manitoba email lists. After a phone screening regarding the participant's general health and medical history, those who were identified as potentially eligible were asked to attend an in-person screening.

The primary inclusion criteria were systolic blood pressure (SBP) between 130 and 160 mmHg and diastolic blood pressure (DBP) ≤ 110 mmHg. Before the screening process started, the PARTICIPANTs were provided written informed consent to sign. Participants were aged 18–75, had a BMI of 18.5-40 kg/m$^2$, were non-diabetic and had no active cardiovascular disease, history of cancer in the last 5 years, and gastrointestinal disorder, and had not changed their blood pressure (BP) medications or nutritional supplements at least three months before the start of the trial. Smoking, drinking alcoholic beverages more than 14 drinks per week, pregnancy, lactating, and major weight change (>± 5 kg) in the last 3 months were considered as other exclusion criteria.

**STUDY DESIGN**

This research is a part of a larger clinical trial aimed at investigating the effects of HSP and its hydrolysate on 24-hr systolic and diastolic blood pressure. A randomized, controlled, double-blind, cross-over designed trial was performed for 22 weeks during which the participants consumed each interventional treatment for 6 weeks separated by washout periods of 2 weeks where no treatment was consumed. The screening, recruitment, and subsequent follow-up visits spanned a period of 14 months, starting in April 2018 and concluding in May 2019. Details on the methodological process of this trial have been previously published (34).

The study was preceded by a phone and in-person screening process where the volunteers were assessed for their eligibility and were randomized if they were defined as eligible after the screening process. The letters A, B, and C were randomly assigned to the treatments and six different sequences were generated followed by a random number generator assigning the participants to these sequences.

After randomization by a research assistant out of the research team, the participants were asked to attend their first session at the Clinical Nutrition Research Unit of the Richardson Centre for Food Technology and Research (RCFTR), located at the University of Manitoba and the baseline measurements were performed, and blood samples were collected. During the study periods, the participants consumed the treatments based on their assigned sequences whereas in the washout periods they were instructed to follow their regular diet.

The trial was initially approved by the University of Manitoba Biomedical Research Ethics Committee in Winnipeg, Manitoba, Canada (protocol no. B2016:125) and Non-prescription

Health Products Directorate of Health Canada (protocol number HEMP BP 001) and is registered at clinicaltrials.gov as National Clinical Trial (NCT), ID: NCT03508895. The procedures followed in this study were in accordance with the University of Manitoba guidelines and the principles of good clinical practice (GCP).

**STUDY INTERVENTION, TREATMENT PREPARATION, AND MASKING**

During the 22-week intervention period, participants consumed their normal diet plus 2 cups of treatment smoothies per day one in the morning and one in the evening, which provided 50 g of casein or HSP or 45 g hemp protein plus 5 g of bioactive peptides ($HSP^{+}$) for 6 weeks. The method was adopted to generate HSP hydrolysates was previously developed in our research group to mimic human digestive tract conditions (35).

Manitoba Harvest provided HSP (HEMP Pro 70, soluble hemp protein concentrate, Manitoba, Canada) and casein protein powder was from Nutrablend Foods (New York, USA). Both types of protein powder had light tan color and were mixed with frozen fruit, diet fruit juice, and sorbet to make treatments in the form of smoothies.

Study treatments were prepared in the RCFTR kitchen in similar portion size and appearance, and served to the participants at RCFTR twice a week while the smoothies for the rest of the days were consumed at home. Participants were advised to not communicate about the taste and color of the treatment with other participants and the study staff. Also, they were required to complete a treatment consumption log whenever they had the treatments and mention the date to be reviewed for their compliance. Compliance was defined as attending in more than 80% of in-person treatment consumptions per phase or returning empty treatment containers.

**BLOOD SAMPLE COLLECTION**

Blood samples were taken by a phlebotomist on the first and last day of each treatment period where the participants were asked to abstain from food for 12 hours. Following blood collection, the tubes were centrifuged (at 1000 g for 20 min at 4 °C) and then plasma was separated and aliquoted to be stored at -80 °C. After the completion of the feeding trial, all samples were analyzed for determination of ROS, plasma total peroxides (PTPs), SOD, and CAT in the plasma.

**OUTCOME MEASUREMENTS**

**PLASMA ROS/RNS ASSAY**

The OxiSelec ROS/RNS assay kit was used for measuring total ROS/RNS free radicals activity according to the manufacturer's instructions. The assays start with adding 50 µL of either samples or $H_2O_2$ standards to a 96-well fluorescence plate. Then 50 µL of catalyst was added to each well, mixed, and incubated for 5 min at room temperature. The reaction starts after adding 100 µL of DCFH solution to each well and left at room temperature where the plate is protected from light. After incubation for 30 min, the plate was placed in the microplate reader and the fluorescence read at 480 nm excitation and 530 nm emission wavelengths.

**PLASMA TOTAL PEROXIDES ASSAY**

The level of total peroxides in plasma was measured using a previously developed method (36) with a few modifications. A 1.5 mL aliquot of 0.1 M sodium phosphate buffer (pH 7.0) was mixed with 1 mL of plasma sample whereas the blank contained 1 mL of buffer with 1.5 mL of buffer solution. After warming up the sample mixture and blank in a water bath for 2-3 min to reach 60°C, a 0.1 mL aliquot was mix with 4.7 mL ethanol in water solution (75% v/v), 100 µL ammonium thiocyanate (30%, w/v), and 100 µL $FeCl_2$ (0.02 M) in HCl (1 M). In a clear 96-well microplate, 0.2 mL of this mixture incubated at room temperature for 3 min before placing it in the microplate reader to measure absorbance at 500 nm. The absorbance value was used to calculate the total plasma level of peroxides.

**PLASMA SUPEROXIDE DISMUTASE ASSAY**

The SOD OxiSelect assay kit was purchased from Cell Biolabs Inc. (San Diego, CA, USA) and the manufacturer's instructions were followed to analyze the samples. Briefly, 15 µL of xanthine oxidase solution, was mixed with 2.235 mL of 10% assay buffer (diluted with double distilled water), and thereafter, chromogen solution, assay buffer, and double distilled water in order to prepare the master mix. Then 10 µL plasma or buffer (for blank) was added to a clear 96-well microplate followed by adding 15 µL of master mix and 10 µL of 10% xanthine oxidase solution. The mixture was incubated at 37°C for 1 h in a water bath. The absorbance was read at 490 nm and SOD activity determined based on the inhibition of chromogen reduction when in exposure to the superoxide onions produced by xanthine.

**PLASMA CATALASE ASSAY**

The CAT assay kit was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). The steps were according to the manufacturer's manual. Briefly, in a 96-well black microplate, the followings were added: 25 µL of standard solutions (containing 10% assay buffer, diluted with double distilled water plus different concentrations of CAT standards) or plasma samples and 25 µL of $H_2O_2$ (40 25 µM) and incubated at room temperature for 30 min. Thereafter, 50 µL of ADHP/HRP solution was added to all wells and incubated for another 30 min at 37°C in a water bath. Finally, the fluorescence intensity was determined at excitation and emission wavelengths of 550 and 590 nm, respectively in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA).

**SAMPLE SIZE AND STATISTICAL ANALYSES**

The sample size was calculated based on the primary outcome that was 24-hour systolic blood pressure (24-hrSBP). The secondary outcomes, which include redox biomarkers were not used directly in the sample size calculation. We determined a total sample size of 30 participants to enable the detection of a clinically significant 4.0 mmHg change in the mean 24-hrSBP, considering an estimated within-participant standard deviation of 5.45 mmHg. Thirty-five participants were recruited to reach a total of 30 participants considering a dropout rate of 15%. The calculated power and type 1 error (α) were 0.80 and 0.05, respectively. Thirty-one participants completed the study, and their data were used for data analyses. The effects of treatments on research outcomes were compared by using endpoint values and data was analyzed using SAS MIXED procedures with repeated measures (OnDemand for Academics). Treatment, period, and sequence were considered as fixed factors, and the participant as a random effect in this model (37). Independent variables included period, sequence, treatments, and baseline values of the selected outcomes. Dunnett's test was performed to compare hemp treatments with casein, and to adjust for multiple comparisons between the different hemp treatments, a Bonferroni correction was applied. Statistics are presented as estimated least square means ± SEM and statistical significance was set at $p < 0.05$ for all analyses.

**RESULTS**

This trial recruited 35 participants and four individuals withdrew from the study due to personal reasons. These participants were excluded from analysis. Outcome analyses were conducted on a total of 31 participants. Table 5.1 shows the characteristics of these participants at baseline.

**Table 5.1.** Baseline Characteristics of Study Participants

| | All subjects | Male | Female |
|---|---|---|---|
| **No. (%)** | 31 | 19 (61.2) | 12 (38.7) |
| **Age, y** | 61.09 ± 9.3 | 58.52 ± 8.9 | 65.08 ± 8.7 |
| **BMI, kg/m2** | 28.49 ± 4.9 | 29.04 ± 4.7 | 27.61 ± 5.1 |
| **WC, cm** | 98.92 ± 12.6 | 103.24 ± 10.2 | 92.10 ± 13.57 |

BMI, Body Mass Index; WC, waist circumference. Values are presented as mean + standard deviation.

## COMPARISONS BETWEEN TREATMENTS IN PLASMA OXIDATIVE/ ANTIOXIDANT BIOMARKERS

Table 5.2 represents the differences between treatments in terms of their effect on oxidative/antioxidant biomarkers in plasma. As indicated in the middle column, comparisons between $HSP^+$ and casein revealed that the level of ROS/RNS and PTPs were significantly lower whereas the level of SOD and CAT were both higher when the participants were fed with $HSP^+$ compared to casein ($p < 0.05$). Similar to $HSP^+$, HSP was able to reduce the level of ROS/RNS and PTPs and raise SOD and CAT levels compared to casein, however, the difference between HSP and casein in terms of their increasing effect on CAT was not significant ($p > 0.05$). The comparison between HSP and $HSP^+$ revealed that $HSP^+$ was also able to show a greater lowering effect on ROS/RNS and PTPs and an increasing effect on SOD and CAT levels in plasma compared to HSP, but the difference was only significant for ROS/RNS levels (-246.36 ± 106.16, $p = 0.04$).

## PLASMA ROS/RNS AND PTPS LEVELS

Fig. 5.1 represents the ROS/RNS levels after 6 weeks of treatment consumption. As shown in Fig. 5.1, the lowest ROS/RNS level was observed after $HSP^+$ consumption (503.58 ± 84.83 $p$ <0.0001) compared with other treatments. Also, the plasma level of ROS/RNS was lower after consumption of HSP (749.94 ± 76.57, $p$ <0.0001) compared to casein (1209.56 ± 79.63, $p$ <0.0001). As shown in Fig. 5.2, the same pattern shown for ROS/RNS levels was observed in the level of PTPs. Thus, PTPs level was the highest after casein and the lowest after $HSP^+$ consumption for 6 weeks. PTPs values after casein, HSP and $HSP^+$ consumption were 0.073 ± 0.003, 0.060 ± 0.002, and 0.053 ± 0.003 ($p$ <0.0001) respectively.

## PLASMA SOD AND CAT LEVELS

Fig. 5.3 illustrates SOD levels after 6-week treatment consumption. In contrast to ROS/RNS and PTPs levels, the comparisons between the values showed that the greatest SOD level was obtained after $HSP^+$ consumption followed by HSP and casein. As indicated in the bar chart, the plasma SOD level was 0.63 ± 0.08 for $HSP^+$ where this amount was 0.43 ± 0.08 and 0.34 ± 0.08 for HSP and casein respectively ($p$ <0.0001).

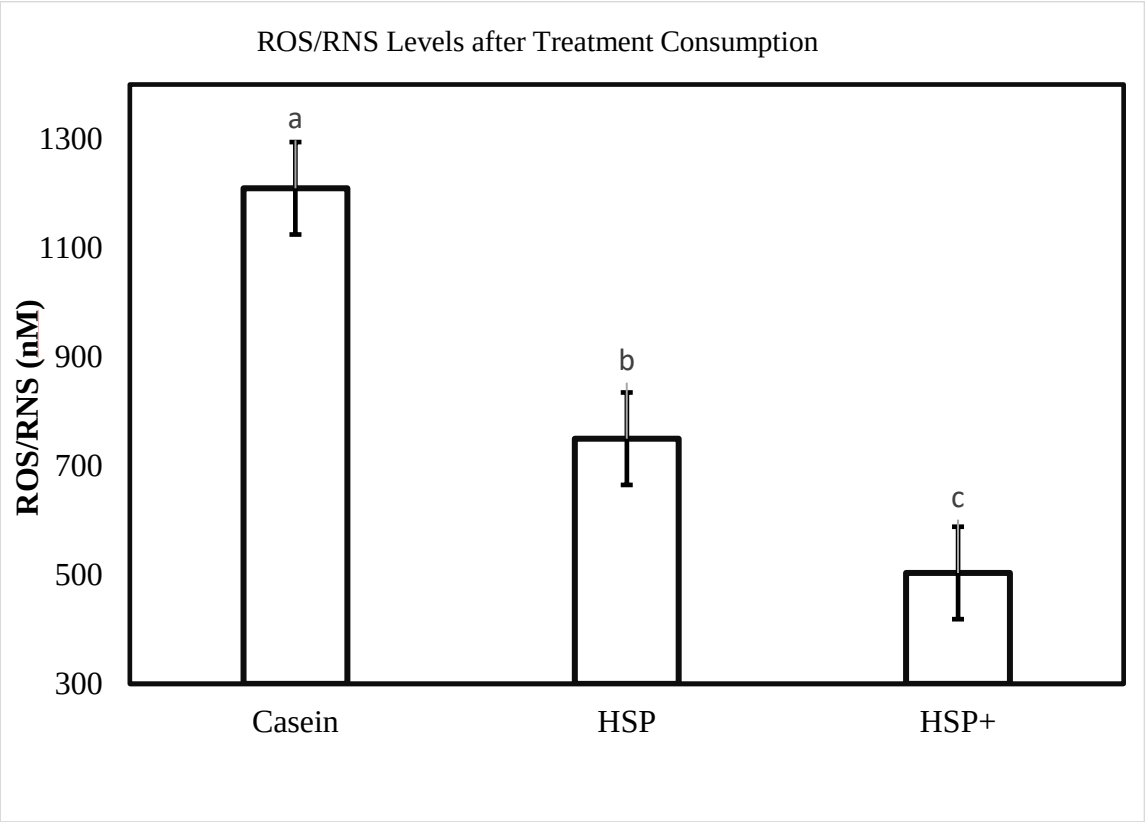
Fig. 5.4 represents the plasma CAT levels after the treatment consumption for 6 weeks. Similar to, SOD, $HSP^+$ led to the highest CAT level (418.26 ± 1.83) whereas HSP and casein consumption were not able to raise the CAT levels as $HSP^+$ did (416.43 ± 1.69 and 410.19 ± 1.73, $p$ <0.0001 respectively for HSP and casein).

**Table 5.2.** Differences Between Treatment Effects on Plasma Oxidative/Antioxidant Biomarkers

| | Δ HSP vs Casein | $P^1$ | Δ HSP+ vs Casein | $P^2$ | Δ HSP+ vs HSP | $P^3$ |
|---|---|---|---|---|---|---|
| **SOD (U/µL)** | 0.093 ± 0.10 | 0.55 | 0.296 ± 0.10 | 0.01 | 0.202 ± 0.10 | 0.11 |
| **CAT (mU/mL)** | 6.24 ± 1.94 | 0.004 | 8.07 ± 2.21 | 0.001 | 1.82 ± 2.1 | 0.78 |
| **PTPs (µg/mL)** | -0.012 ± 0.003 | 0.004 | -0.019 ± 0.004 | 0.0002 | -0.007 ± 0.004 | 0.22 |
| **ROS/RNS (nM)** | -459.62 ± 91.60 | <.0001 | -705.98 ± 112.56 | <.0001 | -246.36 ± 106.16 | 0.04 |

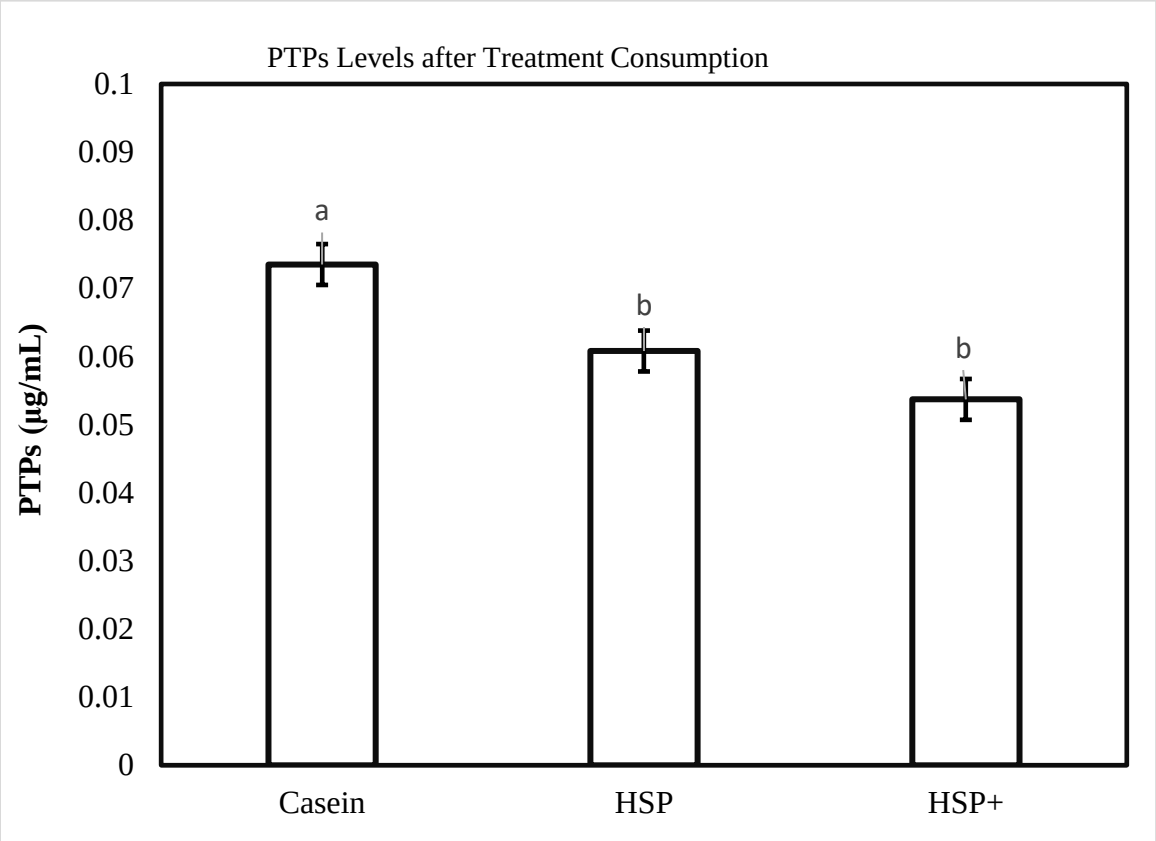
CAT, catalase; HSP, hemp seed protein; $HSP^+$, HSP plus HSP derived bioactive peptides; PTPs, plasma total peroxides; ROS/RNS, reactive oxygen species/reactive nitrogen species; SOD, superoxide dismutase. All values are differences in estimated least-squares means ± SEM. P values were derived by using SAS Mixed Model. [1]P values HSP versus casein, [2]P values $HSP^+$ versus casein, [3]P values $HSP^+$ versus HSP.

**Figure 5.1.** Plasma ROS/RNS levels (nM) after consumption of casein, HSP and HSP$^{+}$ for 6 weeks



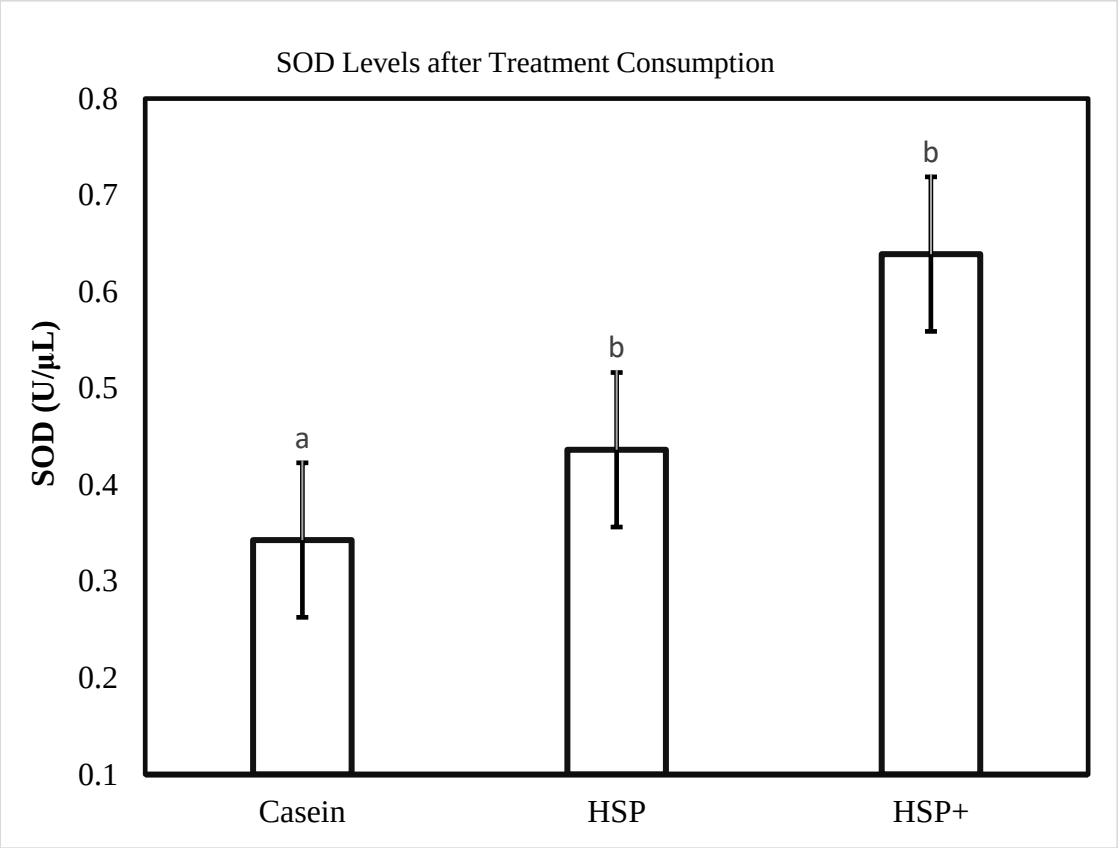

HSP, hemp seed protein; HSP$^{+}$, HSP plus HSP derived bioactive peptides; ROS/RNS, reactive oxygen/nitrogen species. Values are presented as estimated least-squares means ± SEM by using SAS Mixed Model. Bars with different superscript letters are significantly different.

**Figure 5.2.** PTP levels (μg/mL) after consumption of casein, HSP and HSP+ for 6 weeks



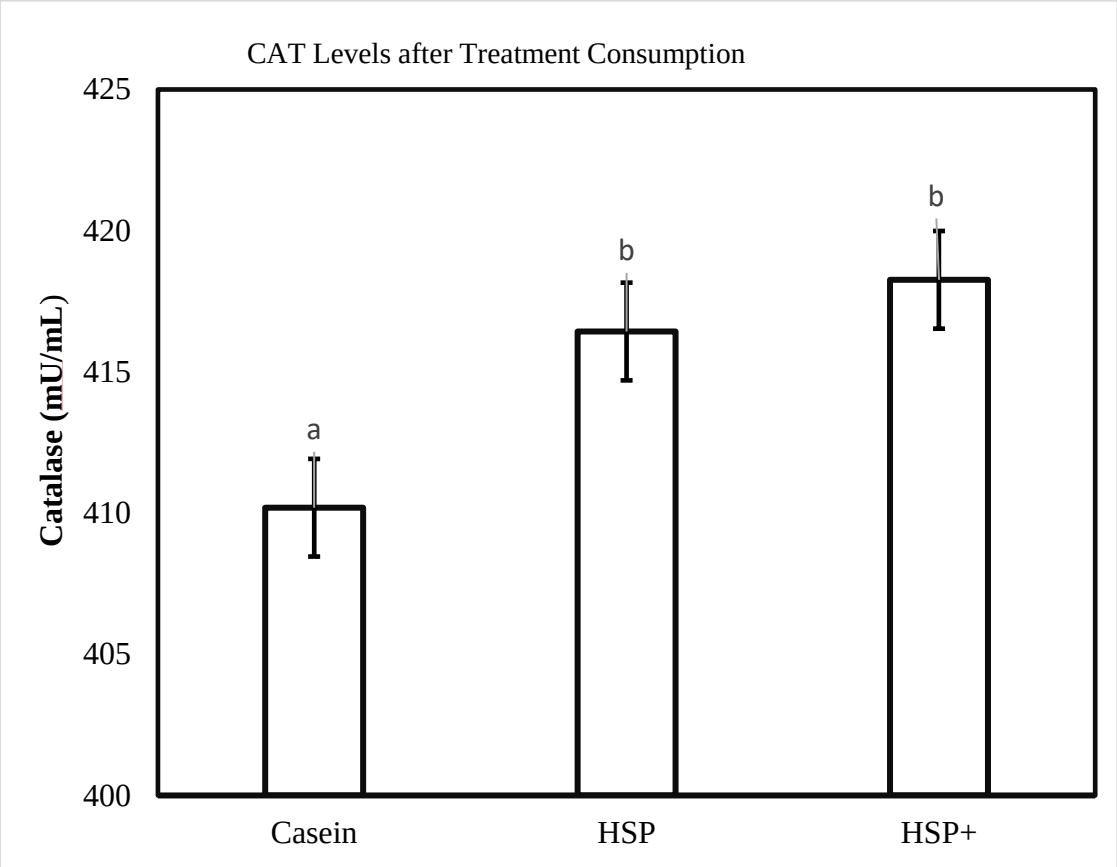

HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; PTPs, Plasma total peroxides. Values are presented as estimated least-squares means ± SEM by using SAS Mixed Model. Bars with different superscript letters are significantly different.

**Figure 5.3.** Plasma SOD levels (U/μL) after consumption of casein, HSP and HSP+ for 6 weeks




HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; SOD, superoxide dismutase. Values are presented as estimated least-squares means ± SEM by using SAS Mixed Model. Bars with different superscript letters are significantly different.

**Figure 5.4.** Plasma CAT levels (mU/mL) after consumption of casein, HSP and HSP+ for 6 weeks




CAT, catalase; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides. Values are presented as estimated least-squares means ± SEM by using SAS Mixed Model. Bars with different superscript letters are significantly different.

## DISCUSSION

In the current clinical trial, we have highlighted the antioxidant effects of HSP, and its bioactive peptides compared to casein protein in individuals who have hypertension. There is accumulating evidence confirming the association between oxidative stress and high blood pressure (3,7,38). In light of the current knowledge about redox signaling pathways in hypertension, investigating the potential therapeutic agents from natural sources with antioxidant activities to control high blood pressure is on the rise (39). Despite several published animal studies indicating the beneficial effects of protein from hemp seeds against oxidative stress, the clinical impact in humans remains elusive.

In a recently published review paper on the health-beneficial effects of HSP hydrolysates, the authors did not find any clinical trial conducted in the literature and the findings were mainly reported based on the existing *in vitro* trials (33). It has been previously shown that HSP-derived peptides have the ability to attenuate hypertension development when they were orally administered to SHRs. Moreover, the antihypertensive effects of these peptides were aligned with their antioxidative properties, which supports the claim that oxidative stress is a fundamental mechanism for the development of hypertension (21,40). Although the models applied to assess antioxidant activities *in vitro* and *in vivo* studies and the conditions under which the bioactive peptides are produced are diverse, the results have been mainly consistent between various studies. However, the results from cell or animal experiments are not powerful predictors of human clinical trials and it is difficult to compare their results.

As the first clinical trial assessing the antioxidative properties of HSP and its bioactive peptides, we measured the level of ROS and PTPs as oxidative stress indicators and determined SOD and CAT levels as free radical scavenging enzymes to examine the potential antioxidative impact of HSP and its hydrolysates in hypertensive participants. Considering the combination of both markers of oxidative damage and antioxidants can help us better interpret the overall oxidation state of the participants after treatment consumption (41). The results from this feeding trial showed that the level of ROS and PTPs generated in participants were higher when they were fed with the casein protein compared to when they consumed the treatments containing HSP and its hydrolysates for 6 weeks. Hence, the SOD level was higher after $HSP^{+}$ consumption and the

CAT level was higher after consumption of both HSP and $HSP^+$ when compared with casein protein.

Higher ROS/RNS levels in casein-fed participants compared to when they were fed with HSP or $HSP^+$ could be as a result of lower radical scavenging activity (RSA) of casein in these participants. In alignment with our results, in an *in vitro* study of HSP hydrolysates (HSH) in $H_2O_2$-treated $HepG_2$ cells, the highest concentration of HPH showed the greatest inhibitory effect on ROS generation in these cells ($p < 0.05$) (26). Hence, in our trial, PTPs were significantly higher in casein-fed participants but the difference between HSP and $HSP^+$ was not significant. Previously, the bioactive peptides from hemp seed had demonstrated higher efficacy in reducing the PTPs in SHRs (21). Faster absorption, less transit time from the gastrointestinal tract (GIT), and higher bioavailability of peptides, as well as greater contents of aromatic and hydrophobic amino acids, compared to HSP, were suggested as underlying factors responsible for the greater effectiveness of the bioactive peptides in neutralizing PTPs.

When ingested, protein molecules and bioactive peptides undergo the action of digestive enzymes in the GIT. It is important to consider bioactive peptides' resistance against these enzymes to be able to pass through the GIT barrier and reach the target organs where they can exert their beneficial impacts. Additionally, distribution and metabolism inside the living body are necessary to be evaluated when their functional properties are being assessed. These variables are the major limiting factors when comparing *in vitro* conditions with *in vivo,* especially in clinical trials under free-living conditions (42). Other than that, the composition and amino acid sequence of bioactive peptides play an important role in their functional properties. For instance, it was shown that peptides with 5-16 amino acids have exhibited antioxidant activities (43).

However, only $HSP^+$-fed participants had significantly higher SOD activity, and CAT activity significantly increased after consumption of both HSP and $HSP^+$ when compared to casein. SOD is the primary line of defense that protects the body against the toxicity of peroxides. SOD catalyzes the conversion of $O_2^{\bullet-}$ to molecular oxygen ($O_2$) and a highly reactive molecule, $H_2O_2$ (12). Afterward, in a two-step reaction, CAT decomposes two $H_2O_2$ molecules into two molecules of water and one molecule of oxygen that can be further utilized in other metabolic

reactions (44). CAT has a prominent role in defending the body against ROS since the reactions catalyzed by CAT do not produce extra free radicals.

Higher activities of SOD and CAT were observed in a dose-dependent manner in $HepG_2$ cells when they were pre-incubated with different concentrations of HPH (26). The authors concluded that HPH could activate endogenous antioxidant enzymes and thus protect $HepG_2$ cells from $H_2O_2$-induced oxidative stress damage. Although this research was similar to our study in terms of using HPH as a treatment, in contrast to our trial they did not include HSP as a separate treatment to compare the results. Our results are supported by a previous animal feeding trial investigating the scavenging activity of HSP and its hydrolysates against peroxide radicals (21). In that research, adult SHRs exhibited a diminished antioxidant defense when they were fed with the casein compared to HSP and especially the HPH diet. Their observation demonstrated the ability of peptides in promoting antioxidant capacity by increasing the level of SOD and CAT in addition to attenuating total peroxide levels in plasma. The method under which the bioactive peptides were generated in this research was similar to the procedures we followed.

While the level of SOD and CAT in $HSP^+$-fed participants were significantly higher than the casein group, these differences were not different between HSP and $HSP^+$, however, in both cases, $HSP^+$-fed participants showed higher SOD and CAT activity than HSP did. The findings related to CAT levels in the comparison between HSP and HSP+ were consistent with the observed levels of PTPs. The fact that HSP+ failed to significantly increase CAT activity compared to HSP could have contributed to the results indicating that HSP+ did not show a significant reduction in PTPs compared to HSP. This suggests that CAT plays a crucial role in catalyzing the decomposition of H2O2, a simple peroxide found in the plasma, which may be associated with higher PTPs levels in HSP+ cases (45).

This trial possesses several strengths. Particularly noteworthy is that, to the best of our knowledge, this is the first clinical trial investigating the effects of HSP and its bioactive peptides in humans. The study's internal validity was strengthened by employing a randomized cross-over design, which also ensured equitable representation of the treatment groups. However, some limitations must be considered. Ensuring strict compliance with the assigned interventions throughout the study was challenging, as we were not able to monitor the treatment consumption on a daily basis. Also, due to the short washout period, there is a possibility of some carryover

effects from one intervention to another, which could influence the results. Moreover, since the study involves hypertensive participants, the results may not be fully generalizable to the broader population and long-term effects of the interventions remain unclear.

## CONCLUSION

The antioxidant activity of HSP and $HSP^+$ were compared to casein in hypertensive participants. $HSP^+$ exerted the highest protective effect against oxidative stress damage through attenuating ROS/RNS and PTPs production and raising antioxidant enzymes including SOD and CAT. These findings suggest that the hemp seed hydrolysate peptides with antioxidant potential could be utilized as an invaluable source of natural antioxidants in the manufacture of food designed for high blood pressure management.

## ACKNOWLEDGMENTS AND AUTHOR CONTRIBUTIONS

We acknowledge RCFTR for the financial support and Manitoba Harvest for providing HSP powder for this study. We appreciate the RCFTR staff in the Clinical Nutrition Unit for their great collaboration, Dennis Joseph for his excellent technical assistance, and the metabolic kitchen staff for preparing the treatments in this research. Lastly, we thank the participants of this trial for their contribution to knowledge generation. The authors' contributions were as follows— REA, DSM, and RCM: conceived and designed the trial; DSM and REA: obtained the funding and provided the materials; MS: prepared University of Manitoba Ethics and Health Canada documents and REA: submitted the documents for approvals; AMA: was involved in training MS to develop HSP hydrolysate production and MS prepared the HSP hydrolysate for the whole project; MS: conducted the screening, data collection, and coordinated the clinical trial; AMA: trained MS and MS: prepared samples for experiments and conducted the analyses of the samples; MS: conducted the statistical analyses and wrote the first manuscript draft; REA: was the Principal Investigator and administrative lead for the study; all authors: critically reviewed the manuscript and approved the final manuscript.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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## TRANSITION STATEMENT 5

The findings presented in the former manuscript provided the evidence demonstrating antioxidant potential of hemp protein peptides. Our focus revolved around investigating the potential of hemp peptides to reduce reactive oxygen species and plasma total peroxides as prominent underlying components associated with oxidative stress. To deepen our understanding of the antioxidant and blood pressure-reducing properties of hemp seed bioactive peptides, the next study aims to investigate the association between hemp peptide consumption and the plasma levels of polyunsaturated fatty acid (PUFA)-derived oxylipins. Oxylipins, known for their involvement in oxidative and inflammatory mechanisms have demonstrated the ability to promote vascular dysfunction and hypertension. However, some oxylipins have the potential to stimulate vasodilation and promote cardioprotective and blood pressure-reducing effects. The type of oxylipin function is determined by several factors including their chemical structure, the parent fatty acids, and the enzymes involved in their production. By exploring the plasma level of oxylipins after consumption of treatments, we seek to gain a more comprehensive knowledge of hemp seed protein bioactive peptides and their potential diverse physiological implications. Investigating the impacts of these peptides on various oxylipins and comparing the results with our previous findings, could enhance better understanding of the specific pathways and mechanisms by which hemp peptides exert their physiological impacts. This study endeavored to broaden comprehension of the interrelationships between hemp seed protein bioactive peptides consumption, oxylipin levels, and the observed blood pressure-reducing, and antioxidant properties of these peptides as well as the possible collective impact of these components.

# CHAPTER VI

## MANUSCRIPT 5

# OXYLIPIN PROFILE ANALYSIS IN HYPERTENSIVE PATIENTS AFTER HEMP SEED PROTEIN CONSUMPTION: A DOUBLE-BLIND RANDOMIZED CROSS-OVER STUDY

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**ABSTRACT**

**Background:** Oxylipins are bioactive lipids formed through the enzymatic or non-enzymatic oxidation of polyunsaturated fatty acids. Various types of oxylipins are involved in the regulation of blood pressure and related physiological processes. Research on how hemp seed protein (HSP) and its bioactive peptides modulate plasma oxylipins in humans is lacking.

**Objective:** This research assessed how consumption of HSP, and its hydrolysate alter oxylipin profile in comparison to casein.

**Design:** Mildly hypertensive men and women with systolic blood pressure of 130-160 mmHg, and diastolic blood pressure ≤110 mmHg were provided 50 g/d of casein, HSP, and HSP plus HSP derived bioactive peptides (HSP+) for 6 weeks with each phase separated by a 2-week washout period using a double-blind, randomized, cross-over design trial. Treatment effects on plasma oxylipin profile were assessed by the linear mixed model with repeated measures.

**Results:** Epoxy ALA and DHA-derived oxylipins, 12,13 EpODE, 19,20 EpDoPE, and 16,17 EpDoPE and its diol metabolite 16,17 DiHDoPE showed the highest plasma concentrations after 6 weeks consumption of HSP+ compared to HSP or casein. In addition, a similar pattern was observed for hydroxy-DHA and EPA oxylipins including 11 and 20-HDoHE and 12-HEPE as they reached the highest levels after HSP+ consumption compared to the other two treatments. In contrast, the highest concentration of 9-oxoODE and 20-cooh-AA derived from linoleic acid was obtained following the consumption of casein.

**Conclusions:** Our findings suggest that hemp protein plus bioactive peptides consumption may have a role in the regulation of blood pressure through the modulation of epoxy oxylipins that are involved in maintaining vascular functions. This trial was registered at clinicaltrials.gov as NCT03508895.

**Keywords:** hemp seed protein, protein hydrolysate, bioactive peptides, hypertension, oxylipin profile, epoxy oxylipins, n-3 derived oxylipins.

## Introduction

The membrane of vascular muscle cells plays a vital role in the regulation of vascular tone by the production of endothelium-derived relaxing factors such as nitric oxide (NO) and prostaglandins. However, oxidative stress and inflammatory responses through the synthesis of reactive oxygen species (ROS), interleukins IL-1B and IL-6, lipopolysaccharides (LPS), and, tumor necrosis factor (TNF-α) can result in endothelial dysfunction, and vascular remodeling leading to hypertension (1,2). Therefore, the factors that inhibit the production and thus the level of inflammatory mediators might contribute to hypertension prevention and treatment (3).

Oxylipins are bioactive lipid metabolites generated from the oxidation of polyunsaturated fatty acids (PUFAs) through different pathways including cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) (4,5). Although oxylipins are involved in diverse physiological processes, their role in the initiation and progression of hypertension still needs to be elucidated in order to obtain an early diagnosis and effective therapy (6). Oxylipins regulate inflammatory and oxidative pathways that are both identified as a major underlying cause of hypertension (7). Also, oxylipins may exhibit vasodilatory or vasoconstriction impacts depending on the oxidative pathways and PUFA precursors (omega-3 or omega-6), by which they are produced (8).

Prostaglandins (PGs), such as prostacyclin (PGI2), PGD2, and PGE2 and thromboxanes (TXs) and leukotrienes (LTs) are arachidonic acid derivatives collectively known as eicosanoids and synthesized by the catalytic activity of COX (the former) and LOX (the latter) (9). Eicosanoids possess complex functions in regulation of inflammatory processes, smooth muscle contraction and various organ functions. While PGs are mainly involved in vasodilatory activities, TXs are known as vasoconstrictive oxylipins. For example, PGI2 is known for its vasodilatory properties however, in certain conditions, when PGI2 levels are reduced, its vasoconstrictive counterpart, TxA2, can dominate, resulting in an imbalance that may contribute to hypertension. In a healthy individual, the balance between PGI2 and TxA2 maintains normal blood pressure. However, in certain diseases, such as atherosclerosis, this balance may be disrupted, contributing to an increased risk of high blood pressure (10).

One group of eicosanoids that often exhibit vasodilatory effects are the epoxyeicosatrienoic acids (EpETrEs). EpETrEs act on endothelial blood vessel cells and interfere with angiotensin-II

actions and stimulate the release of NO leading to decrease in blood pressure. This mechanism is essential for maintaining proper blood flow and reducing vascular resistance (11,12). It has been shown that reduced levels of arachidonic acid (AA) in the lipidomic profile of hypertensive patients compared to normotensive subjects might be associated with increased membrane fatty acid mobilization (13). Furthermore, epoxy fatty acids originating from EPA (epoxy-eicosatetraenoic acid or EpETE), and DHA (epoxydocosapentaenoic acid or EpDoPE), are acknowledged for their ability to regulate blood pressure. The vasodilatory impact of EpETE may surpass that of EpETrE in certain vascular beds. EpETE demonstrates the ability to inhibit Ca2+ and isoproterenol-induced contractility in neonatal cardiomyocytes, indicating potential antiarrhythmic effects. On the other hand, EpDPE replicates the anti-inflammatory, vasodilatory, and anticancer effects observed with EpETE (5).

Hemp seed protein (HSP) and its bioactive peptides derived through enzymatic proteolysis have been investigated for their potential antioxidant and antihypertensive properties (14–16). However, there is limited literature on the health benefits of HSP and particularly HSP-derived bioactive peptides in comparison to other plant proteins due to the banned cultivation of hemp crops until 1998 (17). The anti-inflammatory and antioxidant actions exerted by the HSP hydrolysates have been shown to be related to the peptides produced under various hydrolysis conditions such as proteases used and the degree of hydrolysis (18). For instance, bioactive peptides generated from HSP by the activity of alcalase and flavourzyme demonstrated in vitro anti-inflammatory effects by down-regulating the expression levels of inflammatory cytokines TNF-α, IL-1β, and IL-6 and up-regulating the gene expression of the anti-inflammatory cytokine IL-10 (19).

Hence, the objectives of this study were to first determine and quantify the plasma oxylipin profile of hypertensive participants and second to compare changes in oxylipin concentrations after casein, HSP, and HSP derived bioactive peptide consumption.

## Materials and Methods

### Study participants

The study was advertised through newspaper and posters and University of Manitoba e-mail lists in Winnipeg, Manitoba, Canada. The volunteers aged 18-75 were screened and enrolled at the Clinical Nutrition Research Unit of the Richardson Centre for Food Technology and Research

(RCFTR), University of Manitoba. The participants had systolic blood pressure (SBP) and diastolic blood pressure (DBP) of 130-160 mmHg and ≤ 110 mmHg respectively and body mass index (BMI) of 18.5-40 kg/m$^2$. Those with any biochemical imbalances, cardiovascular disease, gastrointestinal (GI) disorder, diabetes, and a history of cancer were excluded from the study. The research coordinator allocated the eligible participants to the sequence of treatments by opening a sealed envelope where both coordinator and participants were blinded to the treatments. In total 20 participants completed the study and their results were used for data analysis.

**Study design**

This study was a randomized, double-blind, cross-over trial with three 6-week intervention periods for each phase and 2-week washout periods between cross-over phases. The details of the research methodology have been previously published (20). The randomization process of participants was conducted by an external research assistant using sealed envelopes. Letters A, B, and C were randomly assigned to the study treatments and then participants were randomly allocated to one of the six sequences (ABC, ACB, BAC, BCA, CAB, and CBA). The study was approved by the University of Manitoba's Biomedical Research Ethics Board and Health Canada and was registered at clinicaltrials.gov as National Clinical Trial (NCT), ID: NCT03508895.

**Study intervention**

Study treatments contained 50 g/day of casein or HSP or 45 g of HSP with an added 5 g of bioactive peptides (HSP+) and were consumed by the participants on top of their regular diet. The HSP hydrolysate was prepared using gastrointestinal tract enzymes including pepsin and pancreatin using a method previously developed (21). The treatments were prepared similarly and served in the form of a smoothie in combination with frozen fruit, diet fruit juice, and sorbet (table 6.1). The treatments were consumed under biweekly supervision at the RCFTR and home. Compliant was defined as consuming more than 80% of treatments per phase.

**Blood sample collection**

At days 1 and 42 of each treatment period (6 times in total), 20 ml of fasting blood samples were taken by venipuncture for plasma oxylipin analysis. Immediately after blood collection, the samples were centrifuged at 1000 g for 20 min at 4 °C, and plasma samples were separated, aliquoted, and then stored at -80 °C until further analyses.

**Oxylipin analysis**

Free oxylipins were analyzed as previously described (22,23). Briefly, to 300 µL of plasma samples were added 1 mL pH 3 water, 15 µL antioxidant cocktail, 220 µL methanol, and 100 µL deuterated internal standards (Cayman Chemical, MI). Samples were acidified to pH<3.0 with HCl and centrifuged for 5 min at 14000 xg. Supernatants were then loaded on the Strata-X SPE columns (Phenomenex, CA) preconditioned with 3 mL methanol and pH 3 water, columns were washed, and free oxylipins were eluted in 100% methanol. After drying, samples were re-suspended in the mobile phase for oxylipin analysis by HPLC/MS/MS using a Luna 5µm C18 column (100Å 250 × 2.0 mm, Phenomenex, CA) on a Shimadzu Nexera XR HPLC, coupled to an ABSciex QTRAP 6500 MS operating in negative mode with electrospray ionization. Oxylipins were separated with the following gradient: 100% solvent A between 0 and 1 min, and then solvent B (acetonitrile/isopropyl alcohol, 50/50, v/v) was increased linearly to 25% at 3 min, to 45% at 11 min, to 60% at 13 min, to 75% at 18 min, and to 90% at 18.5 min. Solvent B was then held at 90% until minute 20, dropped to 0% by 21 min, and held until 25 min. Quantification of oxylipins was performed using stable isotope dilution. The level of quantification was set at >5 times above baseline. Further details of all oxylipins scanned, mass transitions, internal standards, standard curve slopes and retention times are provided in (23,24).

**Statistical analysis**

The effects of three interventions on plasma oxylipins were compared by performing a mixed-effects model with repeated measures in SAS (OnDemand for Academics). Results are presented as estimated least square means ± standard error of the mean (SEM) for all values. $P < 0.05$ was considered statistically significant for all analyses. Dunnett's test was applied for pairwise comparisons of hemp treatments with casein, and multiple comparisons between the hemp treatments were assessed by Bonferroni correction.

**Results**

**Baseline characteristics of study participants**

A total of 22 participants were recruited and randomized to start the study and two of them withdrew during the first phase of the trial because of personal issues and traveling. The study was completed with 20 participants and their data was used for the analyses. The baseline characteristics of these participants are shown in Table 6.2. As represented, 60% (12) of the

participants were male and 40% (8) were female. In the beginning, the mean ± SD age was 58.09 ± 8.7 y, BMI was 27.91 ± 5.1 kg/$m^{2,}$ and office SBP and DBP were 144.21 ± 12.9 and 90.48 ± 8.9 mmHg respectively. Baseline age, weight and height were different between male and female participants but, there were no differences in BMI, SBP and DBP. Also, none of the study participants were on any type of antihypertensive medication.

**Table 6.1.** Composition of the Intervention Products Per Portion (1 Smoothie)

| | **Protein powder** | | **Fruit juice** | **Pineapple** | **Sorbet** | **Total** | |
|---|---|---|---|---|---|---|---|
| | hemp | casein | | | | hemp | casein |
| **Amount (g or mL)** | 39 | 35 | 130 | 40 | 125 | 334 | 330 |
| **Calories (kcal)** | 170 | 120 | 5 | 20 | 80 | 275 | 225 |
| **Protein (g)** | 25 | 25 | 0 | 0.2 | 0 | 25.2 | 25.2 |
| **CHO (g)** | 2.6 | 2.5 | 1.3 | 5 | 20 | 28.9 | 28.7 |
| **Fat (g)** | 6.6 | < 1 | 0 | 0 | 0 | 6.6 | < 1 |
| **n-6 fatty acids (g)** | 4.2 | < 0.8 | 0 | 0 | 0 | 4.2 | < 0.8 |
| **LA (g)** | 4 | < 0.001 | 0 | 0 | 0 | 4 | < 0.001 |
| **AA(g)** | < 0.001 | < 0.001 | 0 | 0 | 0 | < 0.001 | < 0.001 |
| **n-3 fatty acids (g)** | 1.3 | < 0.8 | 0 | 0 | 0 | 1.3 | < 0.8 |
| **ALA (g)** | 1.2 | < 0.001 | 0 | 0 | 0 | 1.2 | < 0.001 |
| **EPA (g)** | < 0.001 | < 0.001 | 0 | 0 | 0 | < 0.001 | < 0.001 |
| **DHA (g)** | < 0.001 | < 0.001 | 0 | 0 | 0 | < 0.001 | < 0.001 |

AA, arachidonic; ALA, alfa linolenic acid; CHO, Carbohydrate; DHA, docosahexaenoic; EPA, eicosapentaenoic; LA, linoleic acid.

**Table 6.2.** Baseline Characteristics of Study Participants

| | All subjects | Male | Female | *P*[1] |
|---|---|---|---|---|
| No. (%) | 20 | 12 (60) | 8 (40) | |
| Age (y) | 58.09 ± 8.7 | 55.16 ± 8.5 | 64.5 ± 5.3 | 0.010 |
| Weight (kg) | 81.90 ± 17.9 | 90.62 ± 15.7 | 68.82 ± 12.2 | 0.004 |
| Height (cm) | 170.95 ± 10.1 | 177.75 ± 6.1 | 160.75 ± 5.3 | 0.000 |
| BMI (kg/m2) | 27.91 ± 5.1 | 28.78 ± 5.4 | 26.59 ± 4.4 | 0.363 |
| Office BP (mmHg) | | | | |
| SBP | 144.21 ± 12.9 | 140.11 ± 10.2 | 150.37 ± 14.1 | 0.122 |
| DBP | 90.48 ± 8.9 | 92.33 ± 7.0 | 87.7 ± 10.6 | 0.332 |

BMI, Body Mass Index; BP, blood pressure; DBP, diastolic blood pressure; SBP, systolic blood pressure.
[1]P values for male versus female.

**Oxylipin distribution in plasma samples**

Out of 162 oxylipins scanned, 77 were quantified in the plasma samples. More than half of the oxylipins were derived from n-6 PUFA; approximately two-thirds of these were formed from AA and one-fourth from linoleic acid (LA). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)-derived oxylipins formed less than half and one-third of the remaining n-3 oxylipins respectively. In addition, one-fourth of n-3 PUFA-derived oxylipins were formed from α-linolenic acid (ALA) as shown in Fig. 6.1. Among all PUFA precursors, LA-derived oxylipins constituted the highest mean concentration among all oxylipins and 9-hydroxy-octadecadienoic acid (9-HODE) was the most abundant oxylipin quantified in the plasma samples (table 6.3).

Fig. 6.2. illustrates the proportion based on mean concentration of oxylipins derived from each fatty acid after consumption of casein, HSP, and HSP+. Approximately four-fifths of the oxylipins were derived from n-6 PUFA; two-thirds of these were formed from LA. ALA-derived oxylipins constituted about half of the remaining n-3 PUFA-derived oxylipins, one-fifth were generated from DHA, 10% from EPA, and one-fourth were from other n-3 PUFA-derived oxylipins (Fig. 6.2).

Different treatments were provided to the participants for 6 weeks each. The effects of hemp protein and its bioactive peptides compared to casein on plasma oxylipins were investigated. Changes in mean ambulatory systolic and diastolic blood pressure after consumption of casein, HSP, and HSP+ is shown in fig. 6.3.

**Effect of dietary protein on plasma n-3 PUFA-derived oxylipins**

Although total n-3 PUFA-derived oxylipins did not differ when treatments were compared together, the amount of 7 out of 32 n-3 oxylipins was significantly different after consumption of casein, HSP, and HSP+ (Fig. 6.4). Out of these 7 oxylipins, 5 of which were derived from DHA, 16,17-epoxy-docosapentaenoic acid (16,17-EpDoPE), 19,20-epoxy-docosapentaenoic acid (19,20-EpDoPE) and 16,17-dihydroxy-docosapentaenoic acid (16,17-DiHDoPE) had the highest levels after consumption of HSP+ when compared to casein or HSP. Similarly, both 11-hydroxy-docosahexaenoic acid (11-HDoHE) and 20-hydroxy-docosahexaenoic acid (20-HDoHE) had the highest concentrations after HSP+ intake when compared to the other treatment groups. Moreover, the same trend was observed in the level of EPA-derived 12-hydroxy-eicosapentaenoic acid (12-HEPE) and ALA-derived 12,13-epoxy-octadecadienoic acid (12,13-EpODE) where the highest concentrations were obtained after HSP+ consumption ($P < 0.05$).

**Effect of dietary protein on plasma n-6 PUFA-derived oxylipins**

The difference between treatment effects on n-6 PUFA-derived oxylipins was minimal. Also, similar to n-3 derived oxylipins, total n-6 PUFA derived oxylipins did not differ between the 3 treatments, and a difference between treatments was observed in only 2 out of 44 quantified n-6 oxylipins (Fig. 6.4). The first oxylipin was 20-carboxy-arachidonic acid (20-cooh-AA), which is derived from AA. The highest concentration of 20-cooh-AA was observed after the consuming casein whereas the lowest was after HSP+ intake ($P < 0.05$). Also, the level of 9-Oxo-octadecadienoic acid (9-oxoODE) derived from LA was the highest after casein compared to HSP consumption ($P < 0.05$). There was no difference between treatment effects on dihomo-gamma-linolenic acid (DGLA), gamma-linolenic acid (GLA) or ardenic acid (ADA) derived oxylipins.

**Discussion**

In a research conducted on hypertensive rats, hemp seed and bioactive peptides derived from its hydrolysate resulted in systolic and diastolic blood pressure reductions (15). Understanding the

underlying physiological mechanism responsible for the observed impact is crucial in establishing potentially effective antihypertensive strategies. Our study was a novel research that revealed consumption of hemp seed protein bioactive peptides may reduce blood pressure by altering plasma oxylipins, which play a crucial role in maintaining vascular tone, blood pressure, and overall cardiovascular health in patients with high blood pressure. Among the oxylipins that are known to impact blood pressure, epoxy oxylipins, and in particular those derived from DHA were the most distinct metabolites altered in response to HSP+ consumption.

Firstly, we found that the concentration of two CYP-derived omega-3 PUFA oxylipins, namely 16,17-EpDoPE and 19,20-EpDoPE, which are DHA-derived epoxides, was significantly higher after consuming HSP+ when compared to casein and HSP respectively. Oxylipins produced through the oxidation of PUFAs such as EpDoPEs have potent vascular effects shown to be relevant to human health and contribute to the antihypertensive actions of DHA (25,26). Particularly, 19,20-EpDoPE has been shown to act as a potent vasodilator and antiarrhythmic molecule and it is suggested that this vasodilatory effect is likely mediated via increases in nitric oxide (NO) signaling and potassium channel activation in vascular smooth muscle cells (27,28). Our data also indicate that the highest level of 12,13-EpODE, an ALA-derived oxylipin metabolized by CYP epoxygenase, was observed after consuming HSP+ compared to HSP. Generally, CYP epoxygenase enzymes produce oxylipins with anti-inflammatory and vasodilatory effects (29). Interestingly, all of the other epoxy oxylipins produced by the action of CYP epoxygenase (CYP e) including 17,18-epoxy-eicosatetraenoic acid (EPA-derived) 15,16 and 9,10-EpODE (ALA-derived), 5,6-epoxy-eicosatrienoic acid (AA-derived) and 9,10 and 12,13-epoxy-octadecenoic acid (LA-derived) showed similar trends after consuming three treatments however, those did not achieve statistical significance. It might have been necessary to have a larger sample size to detect a significant difference. Epoxy-eicosatrienoic acids (EpETrEs) possess strong vasodilatory properties, regulate renal hemodynamics and blood pressure, and exhibit anti-inflammatory characteristics (30). It is worth noting that in vitro results

**Table 6.3.** Treatment Effects on Plasma n-3 and n-6 Oxylipins in Participants after 6 Weeks Consumption of Casein, HSP and HSP$^+$

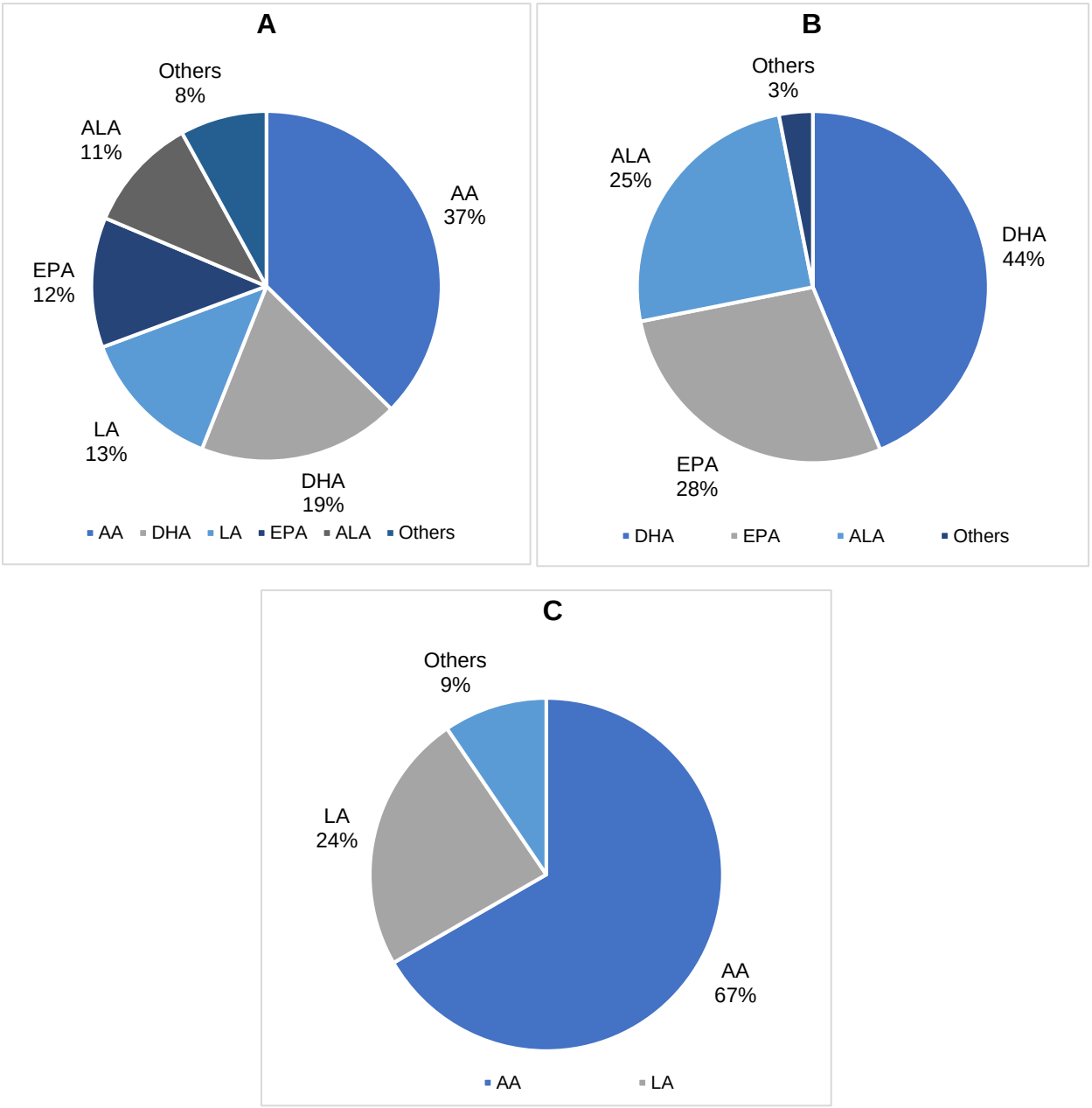
| | Casein | HSP | HSP$^+$ | *p* |
|---|---|---|---|---|
| **n-6 PUFA** ( ng/mL) | 233.17 ± 25.27 | 234.47 ± 25.27 | 218.11 ± 25.27 | 0.87 |
| **AA-derived** (ng/mL) | 38.77 ± 6.01 | 43.78 ± 6.01 | 37.33 ± 6.01 | 0.53 |
| **11,12 DiHETrE** | 0.17 ± 0.01 | 0.16 ± 0.01 | 0.18 ± 0.01 | 0.18 |
| **11-HETE** | 0.82 ± 0.11 | 0.76 ± 0.11 | 0.75 ± 0.11 | 0.86 |
| **12epi LTB4** | 0.004 ± 0.008 | 0.021 ± 0.008 | 0.008 ± 0.008 | 0.25 |
| **12-HETE** | 4.39 ± 1.84 | 7.80 ± 1.84 | 7.74 ± 1.84 | 0.21 |
| **12-HHTrE** | 3.11 ± 0.90 | 4.05 ± 0.90 | 2.97 ± 0.90 | 0.50 |
| **12-oxoETE** | 7.73 ± 3.50 | 7.08 ± 3.50 | 5.30 ± 3.50 | 0.31 |
| **14,15 DiHETrE** | 0.22 ± 0.01 | 0.21 ± 0.01 | 0.22 ± 0.01 | 0.46 |
| **15d PGJ2** | 0.23 ± 0.04 | 0.18 ± 0.04 | 0.21 ± 0.04 | 0.57 |
| **15-HETE** | 1.40 ± 0.17 | 1.44 ± 0.17 | 1.47 ± 0.17 | 0.94 |
| **15-oxoETE** | 0.39 ± 0.04 | 0.36 ± 0.04 | 0.35 ± 0.04 | 0.81 |
| **15R-LXA4** | 0.29 ± 0.03 | 0.28 ± 0.03 | 0.22 ± 0.03 | 0.20 |
| **16-HETE** | -0.03 ± 0.02 | -0.06 ± 0.02 | -0.07 ± 0.02 | 0.51 |
| **17-HETE** | 0.016 ± 0.003 | 0.019 ± 0.003 | 0.012 ± 0.003 | 0.43 |
| **18-HETE** | 0.053 ± 0.006 | 0.050 ± 0.006 | 0.059 ± 0.006 | 0.55 |
| **20-cooh-AA** | 4.42 ± 0.34[b] | 3.53 ± 0.34 | 3.45 ± 0.34[b] | 0.03 |
| **20-HETE** | 0.04 ± 0.05 | 0.14 ± 0.05 | 0.06 ± 0.05 | 0.34 |
| **5,6 DiHETrE** | 0.18 ± 0.01 | 0.18 ± 0.01 | 0.19± 0.01 | 0.59 |
| **5,6 EpETrE** | 0.49 ± 0.08 | 0.56 ± 0.08 | 0.68 ± 0.08 | 0.17 |
| **5-HETE** | 1.65 ± 0.2 | 1.58 ± 0.2 | 1.74 ± 0.2 | 0.82 |
| **5-oxoETE** | 0.83 ± 0.09 | 0.79 ± 0.09 | 0.75 ± 0.09 | 0.79 |
| **6t LTB4** | 0.08 ± 0.04 | 0.12 ± 0.04 | 0.11 ± 0.04 | 0.70 |
| **6t, 12epi LTB4** | 0.067 ± 0.04 | 0.058 ± 0.04 | 0.089 ± 0.04 | 0.83 |
| **8,9 DiHETrE** | 0.18 ± 0.01 | 0.16 ± 0.01 | 0.18 ± 0.01 | 0.68 |
| **8-HETE** | 1.22 ± 0.17 | 1.08 ± 0.17 | 1.14 ± 0.17 | 0.83 |
| **9-HETE** | 0.74 ± 0.11 | 0.60 ± 0.11 | 0.64 ± 0.11 | 0.64 |
| **HXB3** | 0.002 ± 0.007 | 0.007 ± 0.007 | -0.010 ±0.007 | 0.13 |
| **LTB4** | 0.005 ± 0.008 | 0.023 ± 0.008 | 0.006 ± 0.008 | 0.15 |
| **PGE2** | 0.12 ± 0.02 | 0.14 ± 0.02 | 0.15 ± 0.02 | 0.58 |
| **tetranor 12-HETE** | 0.123 ± 0.009 | 0.114 ± 0.009 | 0.133 ± 0.009 | 0.22 |
| **TXB2** | 9.43 ± 2.18 | 12.01 ± 2.18 | 8.14 ± 2.18 | 0.29 |
| **LA-Derived** (ng/mL) | 190.54 ± 24.27 | 187.31 ± 24.27 | 177.08 ± 24.27 | 0.91 |
| **12,13 diHOME** | 1.97 ± 0.21 | 1.57 ± 0.21 | 1.85 ± 0.21 | 0.26 |
| **12,13 EpOME** | 8.59 ± 0.97 | 7.87 ± 0.97 | 10.21 ± 0.97 | 0.17 |
| **13-HODE** | 34.75 ± 3.44 | 28.32 ± 3.44 | 31.59 ± 3.44 | 0.35 |

| | | | | |
|---|---|---|---|---|
| **13-oxoODE** | 10.89 ± 1.52 | 8.10 ± 1.52 | 8.27 ± 1.52 | 0.28 |
| **9,10 diHOME** | 1.54 ± 0.20 | 1.22 ± 0.20 | 1.29 ± 0.20 | 0.46 |
| **9,10 EpOME** | 4.40 ± 3.10 | 2.76 ± 3.10 | 9.26 ± 3.10 | 0.31 |
| **9,10,13 triHOME** | 31.86 ± 4.05 | 29.16 ± 4.05 | 32.31 ± 4.05 | 0.80 |
| **9,12,13 triHOME** | 15.13 ± 2.03 | 15.02 ± 2.03 | 15.96 ± 2.03 | 0.91 |
| **9-HODE** | 39.55 ± 18.36 | 60.70 ± 18.36 | 32.36 ± 18.36 | 0.52 |
| **9-oxoODE** | 41.78 ± 3.7[a] | 32.55 ± 3.7[a] | 33.93 ± 3.7 | 0.03 |
| **GLA-derived** (ng/mL) | | | | |
| **13-HOTrE-y** | 0.39 ± 0.03 | 0.33 ± 0.03 | 0.40 ± 0.03 | 0.32 |
| **DGLA-derived** (ng/mL) | | | | |
| **15-HETrE** | 0.35 ± 0.03 | 0.33 ± 0.03 | 0.32 ± 0.03 | 0.74 |
| **8-HETrE** | 0.23 ± 0.02 | 0.22 ± 0.02 | 0.22 ± 0.02 | 0.85 |
| **n-3 (ng/mL)** | 41.27 ± 4.74 | 39.17 ± 4.74 | 49.93 ± 4.74 | 0.20 |
| **ALA-derived** (ng/mL) | 20.03 ± 2.47 | 16.89 ± 2.47 | 20.90 ± 2.47 | 0.40 |
| **12,13 diHODE** | 0.060± 0.02 | 0.123 ± 0.02 | 0.129 ± 0.02 | 0.14 |
| **12,13 EpODE** | 0.45 ± 0.06 | 0.40 ± 0.06[c] | 0.57 ± 0.06[c] | 0.02 |
| **13-HOTrE** | 2.69 ± 0.32 | 2.15 ± 0.32 | 2.40 ± 0.32 | 0.43 |
| **15,16 EpODE*** | 7.67 ± 1.39 | 6.80 ± 1.39 | 9.94 ± 1.39 | 0.23 |
| **9 oxoOTrE** | 2.84 ± 0.39 | 2.20 ± 0.39 | 1.99 ± 0.39 | 0.20 |
| **9,10 diHODE** | 1.72 ± 0.22 | 1.27 ± 0.22 | 1.64 ± 0.22 | 0.25 |
| **9,10 EpODE** | 1.59 ± 0.21 | 1.45 ± 0.21 | 1.66 ± 0.21 | 0.72 |
| **9-HOTrE** | 2.96± 0.35 | 2.44 ± 0.35 | 2.53 ± 0.35 | 0.44 |
| **EPA-derived** (ng/mL) | 4.97 ± 0.71 | 5.73 ± 0.71 | 6.53 ± 0.71 | 0.14 |
| **11-HEPE** | 0.15 ± 0.04 | 0.18 ± 0.04 | 0.22 ± 0.04 | 0.50 |
| **12-HEPE** | 0.27 ± 0.19[b] | 0.63 ± 0.19 | 0.82 ± 0.19[b] | 0.04 |
| **14,15 diHETE** | 0.79 ± 0.09 | 0.80 ± 0.09 | 0.84 ± 0.09 | 0.86 |
| **15-HEPE** | 0.27 ± 0.06 | 0.29 ± 0.06 | 0.39 ± 0.06 | 0.34 |
| **17,18 diHETE** | 2.73 ± 0.31 | 3.03 ± 0.31 | 3.22 ± 0.31 | 0.36 |
| **17,18 EpETE** | 0.07 ± 0.02 | 0.10 ± 0.02 | 0.13 ± 0.02 | 0.27 |
| **18-HEPE** | 0.36 ± 0.07 | 0.40 ± 0.07 | 0.49 ± 0.07 | 0.31 |
| **5-HEPE** | 0.19 ± 0.04 | 0.15 ± 0.04 | 0.23 ± 0.04 | 0.41 |
| **8-HEPE** | 0.09 ± 0.03 | 0.10 ± 0.03 | 0.15 ± 0.03 | 0.39 |
| **DHA-derived** (ng/mL) | 14.77 ± 2.56 | 15.21 ± 2.56 | 20.77 ± 2.56 | 0.18 |
| **10-HDoHE** | 0.50 ± 0.08 | 0.42 ± 0.08 | 0.59 ± 0.08 | 0.32 |
| **11-HDoHE** | 0.49 ± 0.08 | 0.46 ± 0.08[c] | 0.69 ± 0.08[c] | 0.05 |
| **13-HDoHE** | 0.93 ± 0.13 | 0.73 ± 0.13 | 0.98 ± .13 | 0.38 |
| **14-HDoHE** | 1.78 ± 1.02 | 2.93 ± 1.02 | 4.34 ± 1.02 | 0.21 |
| **16,17 DiHDoPE** | 0.056 ± 0.005[b] | 0.059 ± 0.005 | 0.069 ± 0.005[b] | 0.04 |

| | | | | |
|---|---|---|---|---|
| **16,17 EpDoPE** | 0.99 ± 0.18[b] | 1.15 ± 0.18 | 1.52 ± 0.18[b] | 0.05 |
| **16-HDoHE** | 0.72 ± 0.11 | 0.63 ± 0.11 | 0.81 ± 0.11 | 0.54 |
| **17-HDoHE** | 2.56 ± 0.45 | 2.46 ± 0.45 | 3.28 ± 0.45 | 0.34 |
| **19,20 DiHDoPE** | 0.52 ± 0.05 | 0.50 ± 0.05 | 0.6 ± 0.05 | 0.27 |
| **19,20 EpDoPE** | 0.27 ± 0.04 | 0.23 ± 0.04[c] | 0.36 ± 0.04[c] | 0.02 |
| **20-HDoHE** | 1.73 ± 0.22 | 1.68 ± 0.22[c] | 2.31 ± 0.22[c] | 0.04 |
| **4-HDoHE** | 2.64 ± 0.36 | 2.67 ± 0.36 | 3.44 ± 0.36 | 0.21 |
| **7-HDoHE** | 0.64 ± 0.11 | 0.51 ± 0.11 | 0.71 ± 0.11 | 0.46 |
| **8-HDoHE** | 0.87 ± 0.14 | 0.74 ± 0.14 | 1.02 ± 0.14 | 0.36 |

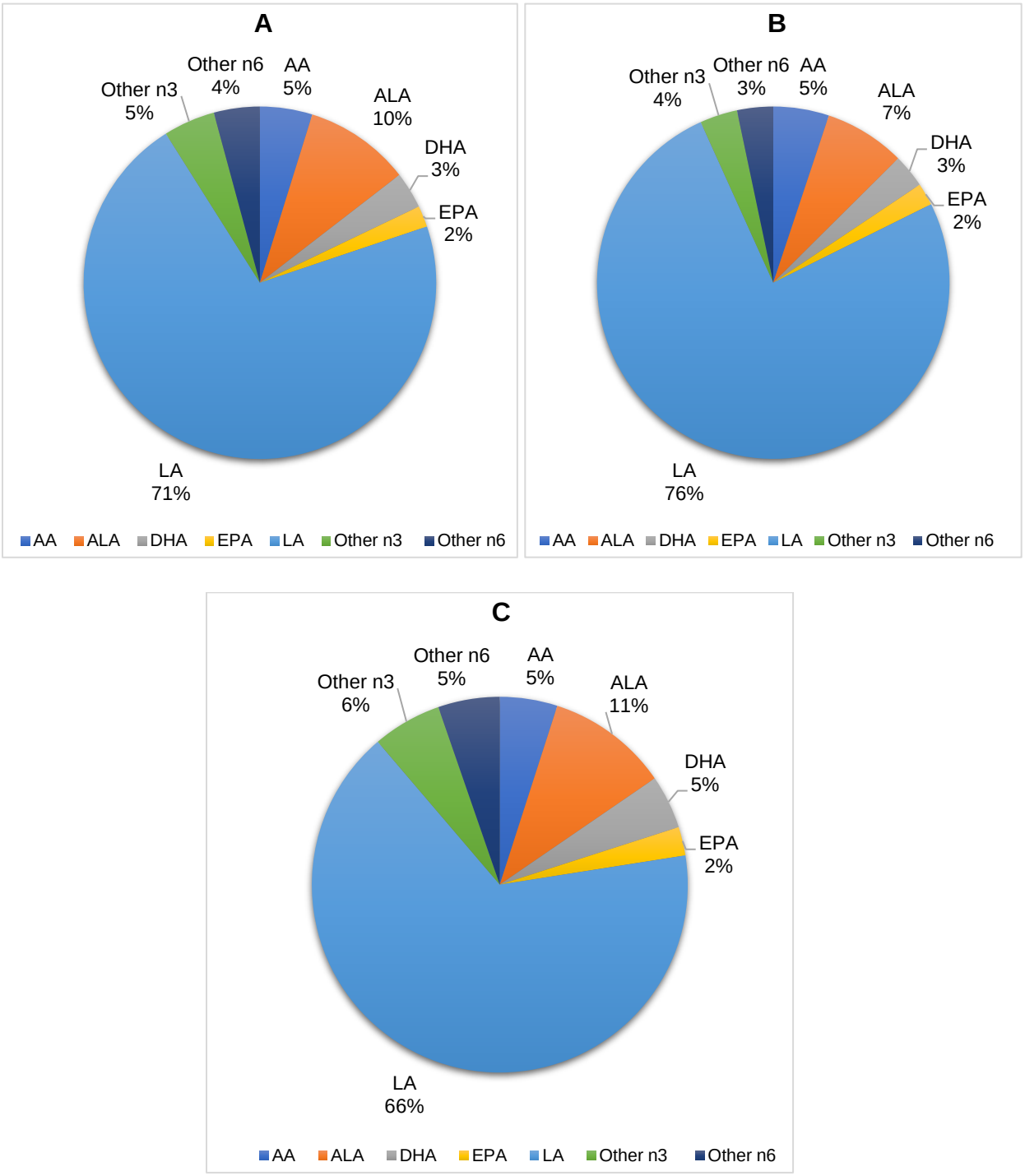
20-cooh-AA, 20-carboxy Arachidonic acid; AA, arachidonic acid; d, deoxy; ALA, α-linolenic acid; d, deoxy; DGLA, dihomo-γ- linolenic acid; DHA, docosa hexaenoic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxyoctadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-docosapentaenoic acid; EpETE, epoxy-eicosatetraenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; GLA, γ-linoleic acid; HDoHE, hydroxy-DHA; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; HXB3, hepoxilin B3; LA, linoleic acid; LT, leukotriene; LXA4, Lipoxin A4; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; oxoOTrE, oxo-octadecatrienoic acid; PG, prostaglandin; PUFA, poly unsaturated fatty acid; TriHOME, trihydroxy-octadecenoic acid; Tx, thromboxane. Results are presented as Least Squares Means ± standard error of the mean (SEM) for oxylipin concentrations. [a] represents significant difference between casein and HSP, [b] represents significant difference between casein and HSP+ and [c] represents significant difference between HSP and HSP+.* denotes oxylipin was not quantified due to lack of available standard.

**Figure 6.1.** The proportion of the plasma oxylipins scanned and quantified in participants provided treatments in total (A) and based on n-3 (B) and n-6 (C) Derived FA Precursors.



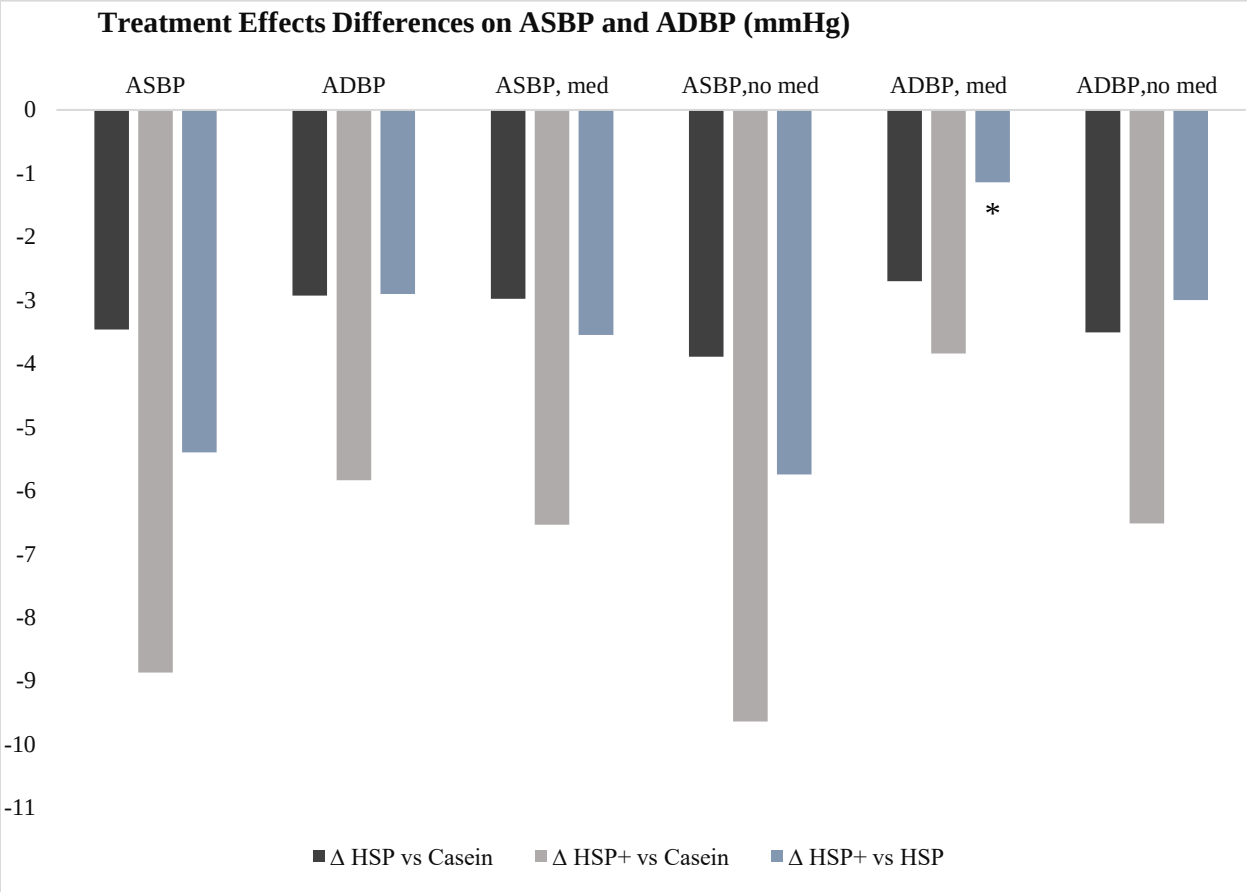

AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid.

**Figure 6.2.** Proportion based on mean oxylipin concentrations after treatment A, B, and C consumption



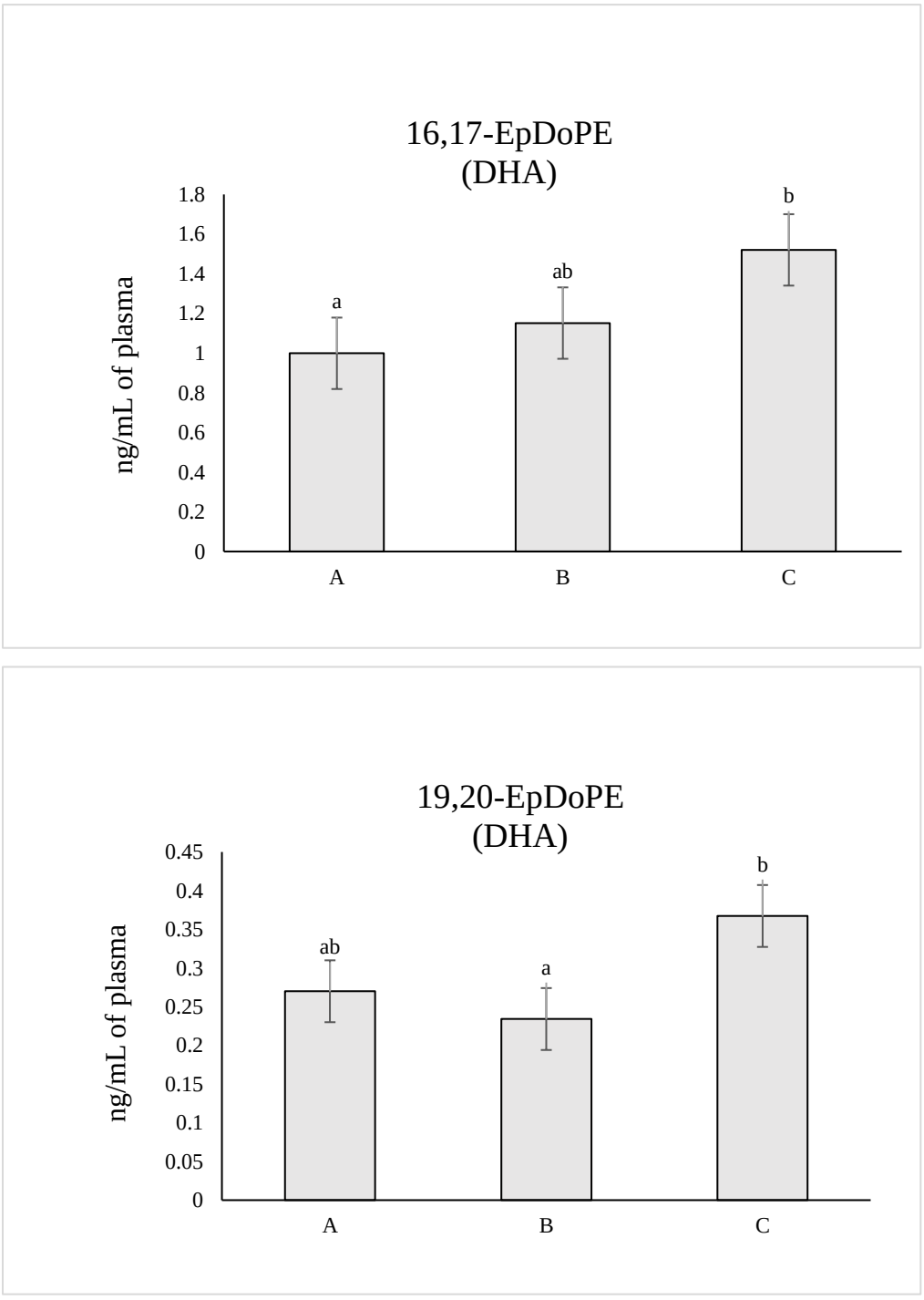

Concentration of oxylipins derived from each FA after consumption of treatment A (casein), B (hemp seed protein or HSP) and C (HSP plus HSP hydrolysate or $HSP^{+}$) are presented in percentages. AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosa hexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid.

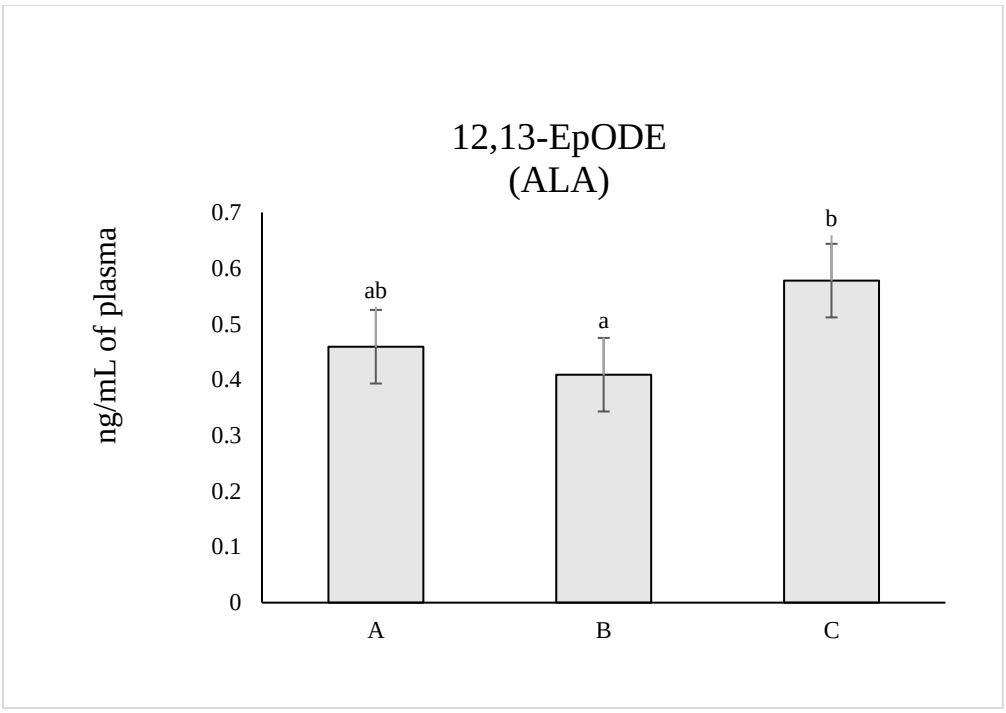
**Figure 6.3.** Changes in mean ASBP and ADBP after consumption of treatments casein, HSP and HSP$^{+}$

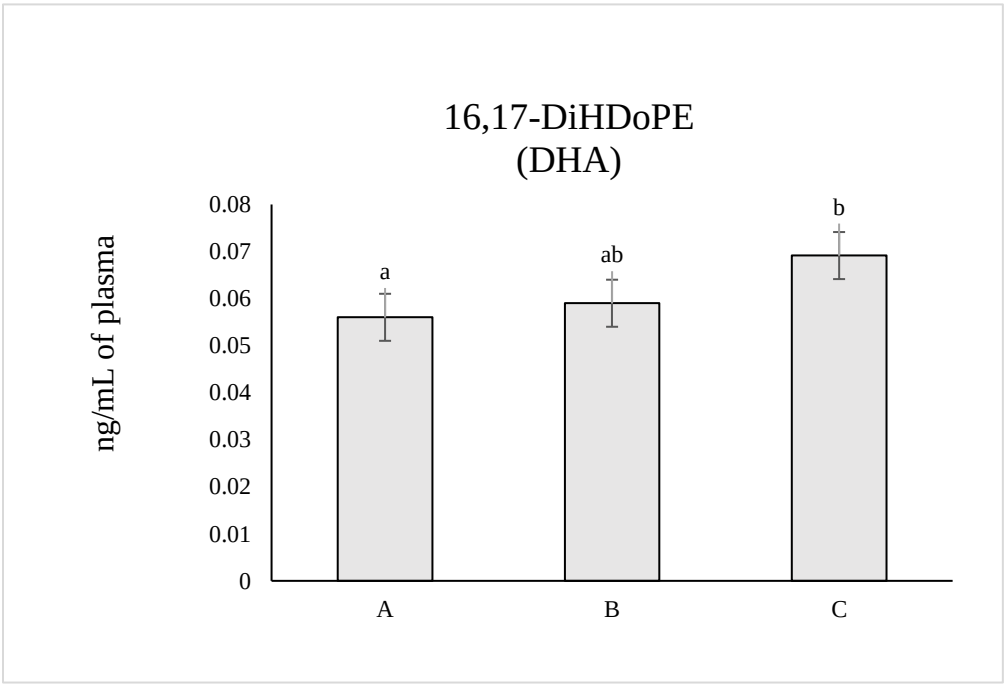


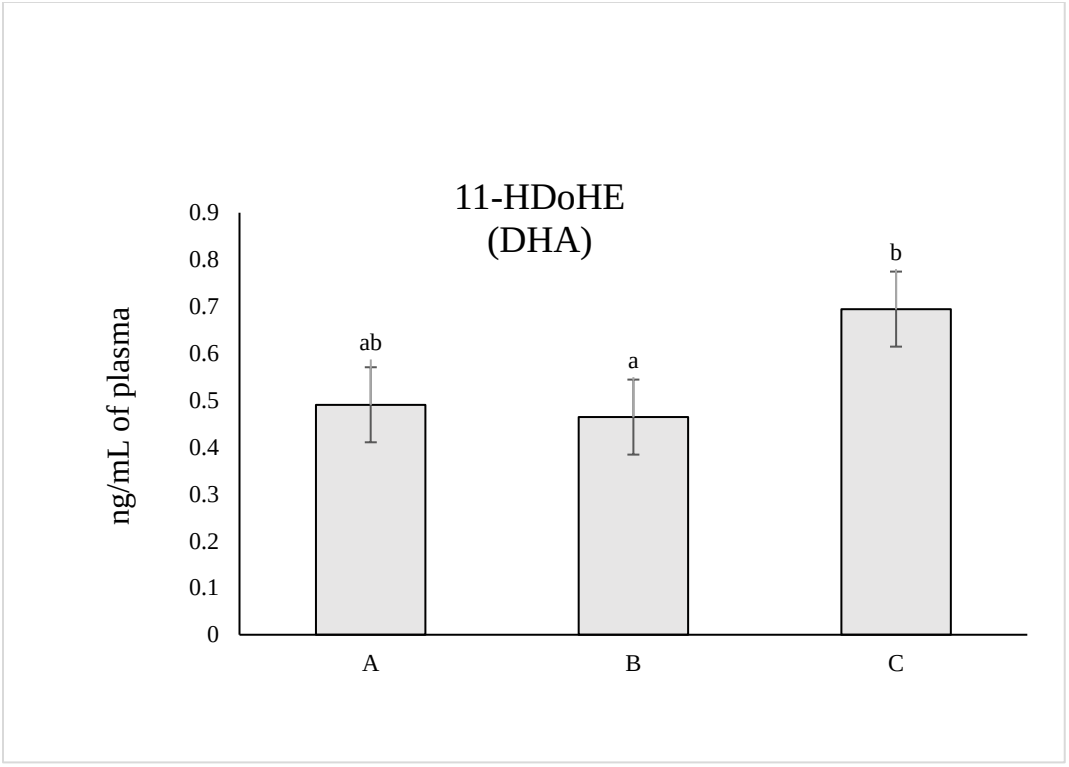

ADBP, ambulatory diastolic blood pressure; ASBP, ambulatory systolic blood pressure; HSP, hemp seed protein; HSP+, HSP plus HSP derived bioactive peptides; mmHg, millimetre of mercury; med, participants on blood pressure medication; no med, participants on no blood pressure medication. * this comparison was not statistically different.

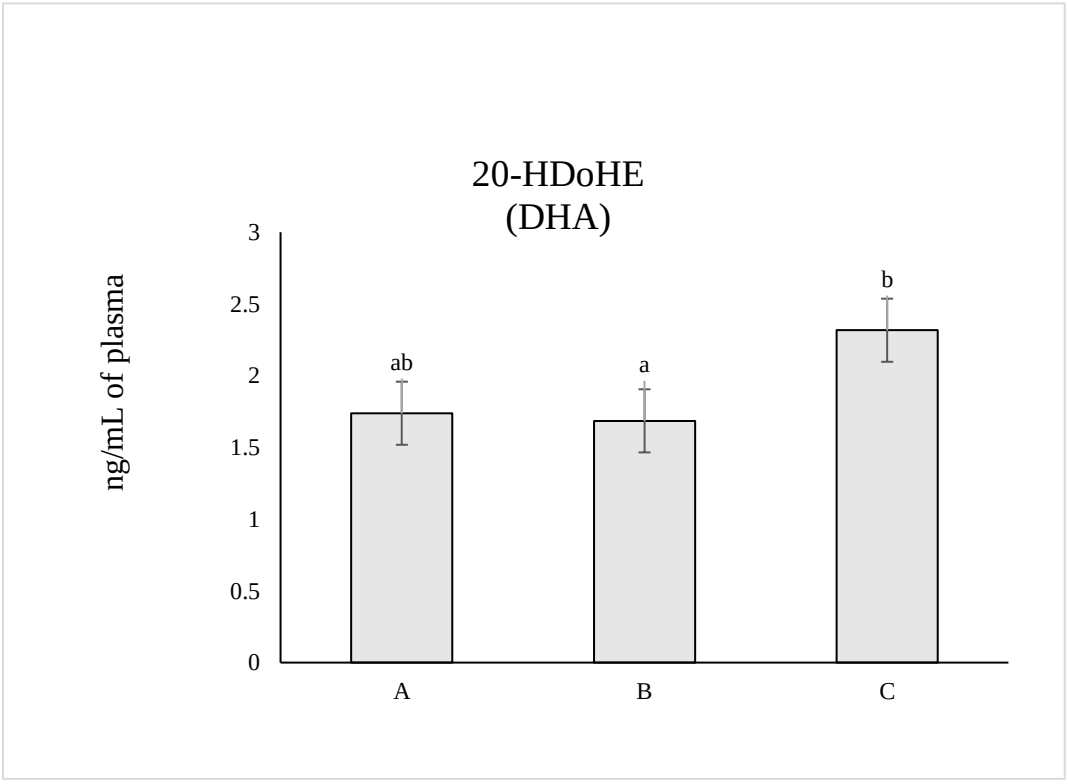
**Figure 6.4.** Mean concentrations of oxylipins after consumption of treatment A, B and C

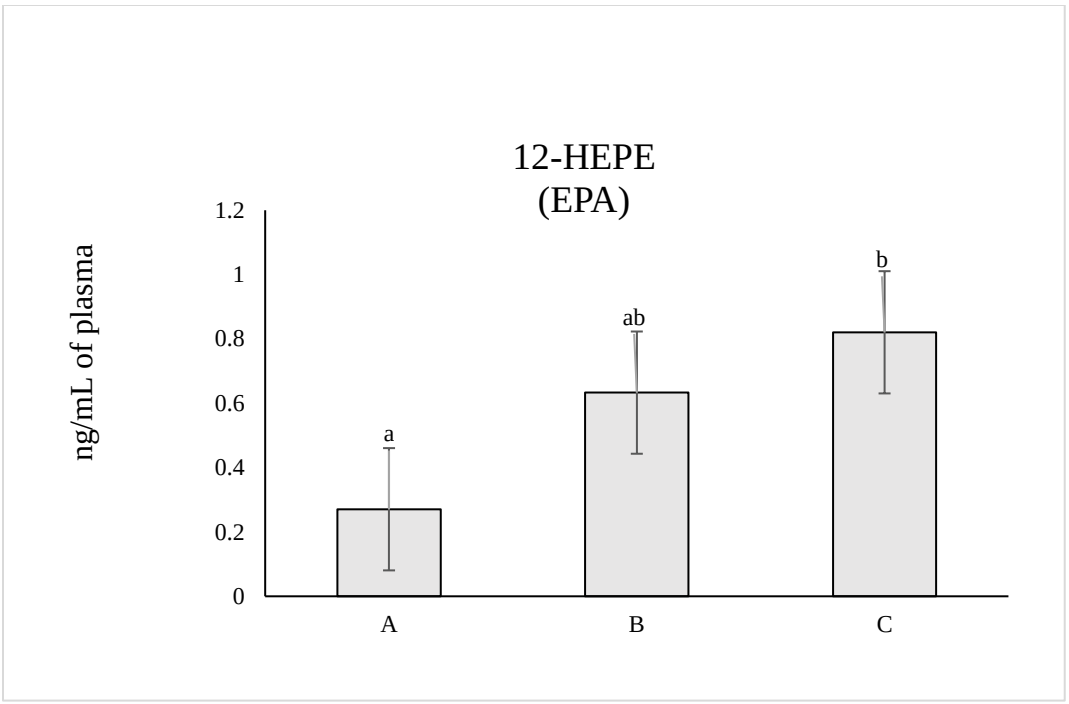


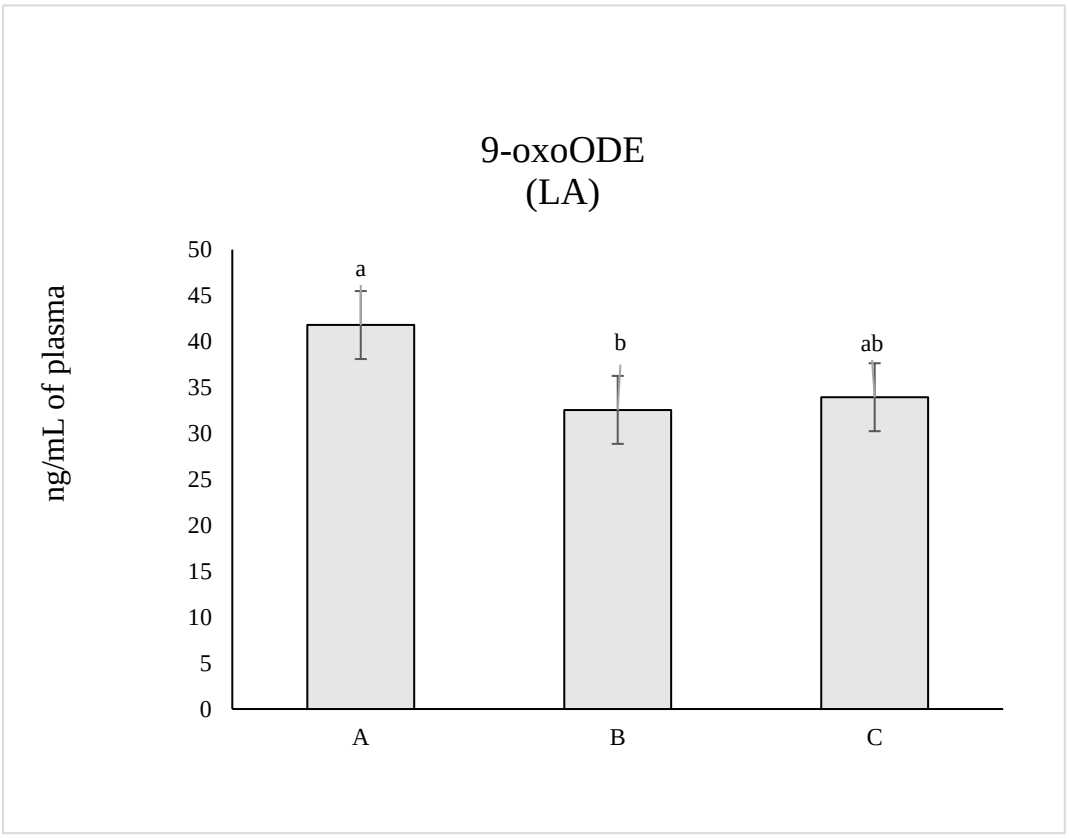

12,13-EpODE
(ALA)
ng/mL of plasma
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ab
a
b
A
B
C

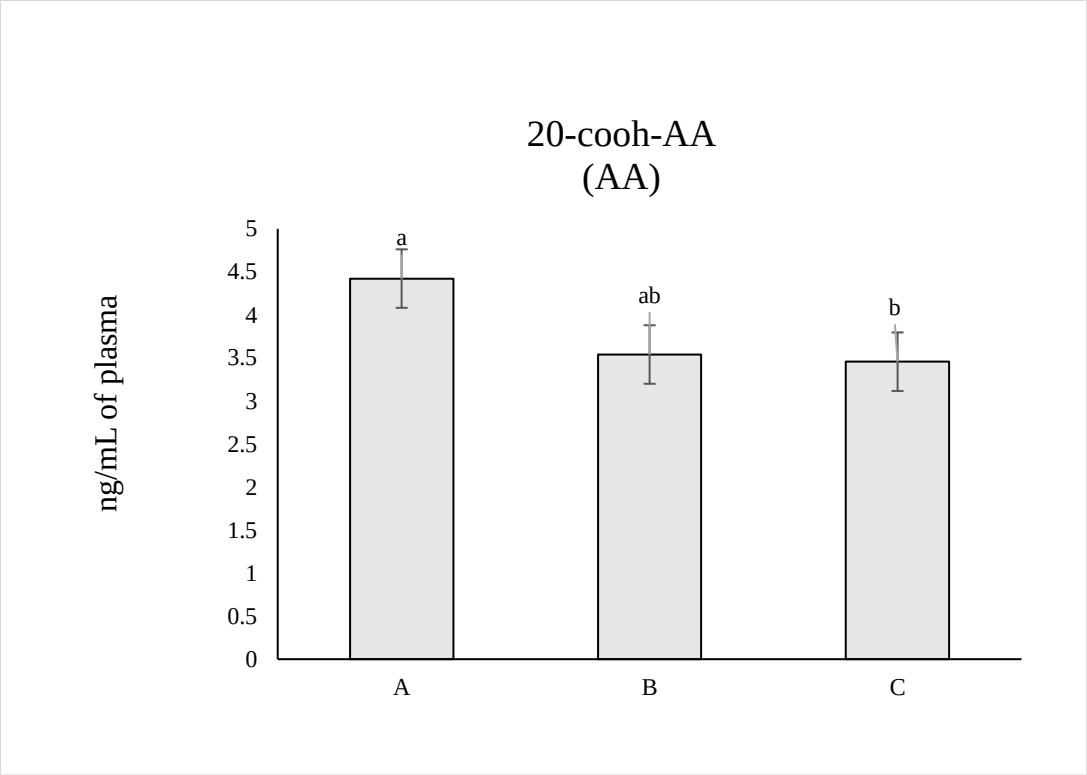

16,17-DiHDoPE
(DHA)
ng/mL of plasma
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
ab
b
A
B
C


11-HDoHE
(DHA)
ng/mL of plasma
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ab
a
b
A
B
C


20-HDoHE
(DHA)
ng/mL of plasma
3
2.5
2
1.5
1
0.5
0
ab
a
b
A
B
C


12-HEPE
(EPA)
ng/mL of plasma
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
b
A
B
C


9-oxoODE
(LA)
ng/mL of plasma
50
45
40
35
30
25
20
15
10
5
0
a
b
ab
A
B
C




Least Squares Mean Concentration of Oxylipins after Consumption of treatment A (casein), B (hemp seed protein or HSP) and C (HSP plus HSP hydrolysate or HSP+). Bars with superscript letters “a” or “b” are significantly different and “ab” represents no significant difference. 20-cooh-AA, 20-carboxy Arachidonic acid; AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosa hexaenoic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-docosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; HDoHE, hydroxy-DHA; HEPE, hydroxy-eicosapentaenoic acid; LA, linoleic acid; oxoODE, oxo-octadecadienoic acid.

have revealed that DHA and EPA epoxides exhibit a significantly higher potency (10- to 100-fold) in inducing vascular relaxation compared to their AA counterparts (31).

Likewise, we observed a similar trend for 16,17-DiHDoPE, which had the highest concentration after consuming HSP+ compared to casein. The observed finding was not surprising as 16,17-DiHDoPE is produced from 16,17-EpDoPE through the action of soluble epoxide hydrolase (sEH), which converts the epoxide to its diol form. The plasma level of sEH was not measured in the current study, so it is unknown whether it was increased or decreased. However, in a randomized clinical trial involving flax seed consumption in hypertensive individuals, it was observed that flax seeds decreased the level of sEH and some of its dihydroxy products. Nonetheless, 16,17-DiHDoPE did not show a reduction as a result of the flax seed intervention (22).

Both 11 and 20-HDoHE, derived from DHA, showed significantly higher concentrations in the HSP+ group. 11-HDoHE is generated by LOX while 20-HDoHE is produced by CYP ω-hydroxylase. Regarding EPA-derived oxylipins, the only one that differed between the treatment groups was 12-HEPE, produced by LOX. The highest level of 12-HEPE was obtained after the consumption of HSP+. This finding is consistent with a study on mice that showed an increase in 12-HEPE levels after a high-protein diet (35% energy) intake for 13 weeks (33). 12-HEPE is associated with the accumulation of anti-inflammatory molecules (34–36) and has shown a strong inverse relationship with systolic blood pressure and arterial stiffness in clinical trials (37). Higher amounts of 12-HEPE and its correlation with cardioprotective factors are consistent with the well-known anti-inflammatory effects of n-3 PUFAs.

On the other hand, consuming casein resulted in the highest level of 20-cooh-AA in plasma samples among the three treatment groups. 20-cooh-AA is formed through the metabolic breakdown of 20-HETE by alcohol dehydrogenases (38). It was shown that 20-HETE has the potential to induce hypertension by enhancing the vascular response to vasoconstrictors however, 20-cooh-AA is recognized as a moderately potent vasodilator (39). Our data showed that dietary proteins from different sources was not able to alter most of n-6 derived oxylipins which is consistent with a previous research comparing high protein with low protein diet in rats (40). Physiological effects of AA oxylipins in the kidney play a vital role in the maintenance of renal function, the renin-angiotensin system and fluid-electrolyte balance (41). Moreover, another an

*in vitro* study showed that diets containing 40% casein compared to 6% casein for 8 weeks enhanced production of vasoactive COX derived AA oxylipins (42).

Additionally, our findings demonstrated that the level of 9-oxoODE, was significantly higher following the consumption of casein. 9-oxoODE is an oxylipin that can be produced both through enzymatic and non-enzymatic oxidation of LA. While LOX is involved in the enzymatic production of 9-oxoODE, the non-enzymatic pathway involves the autoxidation of linoleic acid under oxidative stress conditions, such as exposure to free radicals or ROS (43,44). Previous research on individuals with liver disease also found elevated levels of 9-oxoODE in plasma samples of patients with nonalcoholic steatohepatitis (NASH) compared to those with simple steatosis or normal liver biopsy (45). Considering the role of free radicals in the production of oxidized fatty acids such as 9-oxoODE and oxidative processes involved in the development of chronic diseases such as hypertension, the importance of oxylipins like 9-oxoODE in the occurrence and progression of hypertension should be emphasized.

**Conclusion**

The primary outcome of our study was the significant increase in oxylipins involved in maintaining vascular function in hypertensive individuals who consumed hemp seed protein along with its bioactive peptides for 6 weeks, compared to the control group receiving casein. Notably, DHA-derived oxylipins exhibited the most prominent differences among all the metabolites when comparing the three treatment groups. Collectively, these findings suggest that oxylipins could play a crucial role as a group of metabolites that can be modified by the consumption of bioactive peptides, potentially leading to the modulation of high blood pressure and mitigating the risk of hypertension. Targeting specific DHA oxylipins, particularly epoxides, holds promise for effectively preventing or treating high blood pressure.

**Limitations**

The solid phase extraction-method used, although being one of the most efficient, has its limitations as every extraction method exhibits a preference for certain types of oxylipins (46,47). Another limitation was the relatively small sample size, which stemmed from two factors: firstly, the limited number of participants who fulfilled all the inclusion and exclusion criteria, and secondly, the constraints of time and resources that hindered our ability to recruit a larger number. Also, we did not analyze the dietary consumption of FAs and their potential

modulatory effects on plasma free oxylipin profile. Additionally, our study did not include a treatment group that solely consumed bioactive peptides without the inclusion of protein isolate. Future investigations should consider comparing protein isolate with bioactive peptides derived from protein hydrolysate to elucidate the specific role of bioactive peptides in modulating of oxylipin profile.

## AUTHORS' CONTRIBUTIONS

The authors' contributions were as follows—REA, HMA, DSM, and RCM: conceptualized the research and designed the research program; MS: prepared University of Manitoba Ethics and Health Canada documents and REA: submitted the documents for approvals; MS: was responsible for the screening, data collection, and clinical trial coordination; TW: trained MS and MS: performed sample extraction and TW: analyzed the samples in HPLC/MS/MS; MS: performed the statistical analyses and wrote the first draft of the manuscript; REA: was the Principal Investigator and administrative lead for the study; all authors: critically reviewed the manuscript, and approved the final manuscript. None of the authors reported a conflict of interest related to the trial.

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# CHAPTER VII

# GENERAL DISCUSSION AND CONCLUSIONS

Hypertension is the most prevalent health condition in developed countries and afflicts 20% to 50% of the adult population in the world. High blood pressure leads to various organs damage such as brain, eyes, and kidney dysfunction, and in particular, it can cause serious heart issues such as heart failure and stroke. All blood pressure medications have potential side effects ranging from mild to more severe complications affecting hypertensive patients. Therefore, researchers are actively seeking for natural solutions like functional foods and nutraceuticals to lower blood pressure with a lot fewer side effects compared to pharmaceutical drugs. In the recent years, researchers have discovered numerous bioactive characteristics in peptides obtained from food-sources of proteins.

The involvement of bioactive peptides in cellular interactions and their potency in modulating pathways through mediators have generated considerable interest in the last couple of decades. This significant interest has prompted the exploration of further potentials inherent in these peptides and this area of research continues to be extensively investigated. The research has revealed that bioactive peptides possess a wide range of potentials including anti-inflammatory, antioxidant, antihypertensive, antimicrobial, immunomodulating, antiproliferative and antidiabetic properties. Hence, the food industries are rapidly engaged in the development of different food-derived bioactive peptides as prominent source of functional foods with beneficial impacts on human health.

To date, different types of bioactive peptides have been produced and examined from numerous plant sources of protein such as soybean, pea, wheat, barley, and hemp seed. However, the majority of research conducted have been limited to *in vitro* experiments or animal studies with relatively few investigations focusing on the impacts of these novel peptides in human subjects. In particular, to our knowledge, the peptides derived from hemp seed protein have never been tested in a clinical trial involving human participants. In order to establish hemp seed bioactive peptides as therapeutics and nutraceutical agents, it is crucial to conduct human trials and validate the findings obtained regarding their beneficial health effects.

This thesis as the first clinical trial involving hemp seed bioactive peptides consumption has novel findings listed below.

1. The main outcome investigated in this thesis was the effect of hemp seed bioactive peptides on 24-hrBP. By utilizing GIT enzymes and stimulating GIT model of digestion, we were able to generate bioactive peptides from hemp seed protein that exhibited reducing effects on both 24-hrSBP and 24-hrDBP. Our investigation confirmed our first hypothesis that both treatments with hemp protein effectively lowered 24-hrBP in comparison to casein protein. Furthermore, HSP+ exhibited more pronounced BP-reducing effects compared to HSP, thereby confirming our second hypothesis. In addition, the produced peptides increased NO level, which supports their potential role in BP regulation.

2. This thesis also assessed the capabilities of HSP and hemp protein peptides in inhibition of ACE and renin activities as the crucial enzymes involved in BP control with vasoconstrictive activities. We hypothesized that the consumption of HSP treatments, as opposed to casein, would lead to the downregulation of plasma enzymes associated with hypertension. Subsequently, our results confirmed our third hypothesis, as the consumption of HSP treatments was able to mitigate ACE and renin activities, in comparison to casein which demonstrated their potential role in modulating the mechanisms responsible for BP management.

3. In order to deepen our understanding of how hemp peptides contribute to the mechanisms responsible for BP control, we evaluated their effects on oxidative and antioxidant enzymes. We hypothesized that the intake of HSP treatment would alter antioxidant or oxidative biomarkers linked to BP regulation in comparison to casein. Our findings indicate that HSP treatments reduce the levels of ROS/RNS and PTPs in plasma compared to casein that confirmed our fourth hypothesis. Additionally, they elevate CAT levels compared to casein. Notably, while HSP+ increases SOD levels compared to casein, HSP alone does not exhibit the same effect. Hemp seed peptides demonstrated the strongest antioxidant activities among the three treatment groups. This finding proposes a possible relationship between their potency with respect to BP reduction and antioxidant properties.

4. Additionally, this work evaluated the oxylipin profile of study participants to examine any possible alterations in eicosanoid levels. We discovered that the hemp seed derived peptides primarily modulated epoxy oxylipins, that are recognized for their role in vasorelaxation and

antioxidant effects. This result further supports our previous findings indicating the potential pathways through which these peptides may exert their health-promoting functions.

The outcomes obtained in this research will have significant advantages on multiple aspects. Firstly, hemp seed protein offers a wide range of health benefits that are beneficial to the consumers who seek to incorporate healthier food choices in their diet and those who seek alternatives for pharmaceutical drugs. Additionally, the benefits of hemp seed protein are not limited to the health-boosting properties. Hemp seed industry is rapidly growing especially in Canada as it ranks among the top countries in hemp crop production. Incorporating hemp products with potential health benefits into diet will contribute to the acceleration of the hemp industry. Growth of the hemp industry will impact Canada's economy in various sectors as it expands the agriculture, manufacturing, research and development, marketing, and retail. Hemp seed as a sustainable source of protein has gained significant attention especially in the past two decades. Introducing novel perspectives that extend beyond the basic benefits of hemp seed protein and the bioactive peptides derived from it will facilitate international trade and export opportunities for Canada as one of the pioneers in the hemp industry. Furthermore, the findings of this study have also introduced new perspectives on the health advantageous of hemp seed protein. This research has opened the door for further research in humans to unravel the hemp seed protein potentials in the functional foods and nutraceutical industries as it contains novel bioactive peptides involved in several physiological pathways within the human body.

**NOVELTY**

The novelty of our research lies in being the first effort to evaluate hemp seed-derived bioactive peptides in a clinical trial involving hypertensive individuals. This study seeks to identify effective interventions for managing blood pressure. The results obtained in our study highlighted the lowering effect of these peptides on both SBP and DBP, indicating their potential as antihypertensive agents. Additionally, we detected these peptides may have influenced BP through their inhibitory activities against renin and ACE, essential enzymes involved in the BP regulation. Defining the mechanisms by which these peptides exhibit their hypotensive properties would assist us for developing targeted therapies for hypertension. Furthermore, they exhibited potential antioxidant properties by modulating oxidative stress-related enzymes that contribute to their additional value as potential health-promoting agents. Generally, our study

contributes to the existing body of knowledge obtained from *in vitro* and animal research by offering additional evidence and insights into the therapeutic capabilities of hemp seed bioactive peptides.

**LIMITATIONS**

1. The primary limitation of this study was the absence of existing literature investigating the effects hemp seed bioactive peptides in individuals with high blood pressure to corroborate our findings. Besides, no clinical trial had previously evaluated hemp seed derived bioactive peptides and we were the first group embarked generating them in the laboratory setting and testing in human subjects.

2. We did not collect diet and physical activity information from the patients due to the additional commitment required from the participants. Although, we advised them to maintain their regular diet and physical activity, any variances could have affected the study outcomes.

3. We were not able to supervise the participants every day regarding their treatment consumption given the long period of the study. Moreover, we assessed the compliance according to the participant's report rather than testing a blood biomarker representing the treatment consumption.

4. We determined the oxylipin profile only in individuals not on any antihypertensive medications due to time and financial constraints. The limited number of samples may have impacted our ability to achieve statistically significant results.

Overall, as the pioneering effort, we obtained promising results that can contribute to the future research endeavors for adopting a more comprehensive approach and considering all relevant aspects that may influence the study findings.

## FUTURE RESEARCH

The findings of this research, being the first clinical trial on hemp seed bioactive peptides, emphasizes the necessity of further exploration of this subject in order to first validate the results obtained and second to optimize the utilization of hemp seed peptides for hypertensive individuals.

A few key aspects that need to be addressed in the future investigations are as follows.

1) Designing dose-response trials to determine minimum yet effective and the safe upper-level doses that offer the maximum health benefits.
2) Establishing a separate group with bioactive peptides as a treatment solely and not in combination with the protein isolate.
3) Assessing the potential relationship between human genomics and its effect on response to different treatments including bioactive peptides derived from hemp protein.

It is necessary to expand our knowledge regarding the potentials offered by hemp derived bioactive peptides and the mechanisms through which these peptides perform their roles in managing high blood pressure. Enhancing our understanding of hemp seed derived bioactive peptides will assist further investigation of their potential implications in preventing and treating chronic diseases.