Influence of Walnut (Juglans regia) Intake on Acute Adverse Effects Induced by High Saturated Fat Meals in Obese and Diabetic Women

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RESEARCH PROJECT

Influence of Walnut (Juglans regia) Intake on Acute Adverse Effects Induced by High Saturated Fat Meals in Obese and Diabetic Women

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Initial Considerations

The consumption of high-fat meals, particularly those rich in saturated fats, is increasing in Brazil and contributing to the rise of chronic non-communicable diseases, including obesity and diabetes. The known chronic effects of these diets arise from acute adverse effects, such as increased inflammation and oxidative stress, making it crucial to investigate the potential of certain foods to counteract the harmful effects of an unhealthy diet. In this context, chronic nut consumption is associated with reduced cardiovascular outcomes. Although some evidence also suggests benefits in the short term, studies of short duration are very limited. However, the mechanisms involved in the beneficial effects of nut intake remain to be elucidated. In our study, we aim to investigate whether circulating microRNA profiles and metabolomic profiles can contribute to pathways related to the beneficial effects of nut intake. These pathways are identified through bioinformatics analysis and may inform the mechanisms of action of nuts, as well as aid in obesity prevention and treatment. Additionally, bioinformatics analysis and pathway identification may contribute to developing diagnostic and prognostic algorithms in artificial intelligence.

This study employs advanced methodologies and may contribute to the treatment of obesity and type II diabetes, conditions affecting more than 20% of the Brazilian population. The clinical trial will be conducted at the Botucatu Medical School, and biological material will be collected for further analysis at Monash University, Australia. Both institutions have a strong research tradition and laboratory facilities to support the project. Professor Marcos Minicucci, the project coordinator, has expertise in nutrition and studies on acute supplementation of bioactive compounds. He is an Associate Professor in the Department of Clinical Medicine at Botucatu

Medical School – Unesp, with 191 full articles published in journals and 29 book chapters. He has an H-index of 33 with over 4,209 citations (Google Scholar), has received six regular grants from FAPESP and three from CNPq, holds a level 1D research productivity scholarship, served as Head of the Clinical Medicine Department from 2017 to 2021, and is currently Editor-in-Chief of the journal Nutrire of the Brazilian Society of Food and Nutrition. Professor Bárbara R. Cardoso, the international collaborator on this project, is an internationally recognized researcher in nutrition biochemistry and holds a Senior Lecturer position in the Department of Nutrition, Dietetics, and Food at Monash University, ranked among the top 50 universities worldwide. She is recognized as one of the most significant researchers in the field of nuts, ranking in the top 1% globally (http://expertscape.com). She has expertise in clinical trials, epidemiological studies, genomics, and proteomics, particularly related to nuts. She has published 48 full journal articles and 15 book chapters, with an H-index of 25 and over 2,400 citations (Google Scholar). She has received funding from the Australian National Health and Medical Research Council (NHMRC) and philanthropic entities.

The combination of these expertises will produce a unique, globally relevant work with the potential to inform international guidelines on healthy eating and diabetes treatment. Furthermore, the proposed partnership will allow for a doctoral student to be supervised by Professor Marcos with co-supervision by Professor Bárbara, access to advanced technologies, and postgraduate student mobility, strengthening the exchange between Monash University and Unesp. This will expand opportunities for the training and development of Brazilian researchers and promote academic and scientific excellence.

Summary

Obesity is a multifactorial disease in which excess body fat accumulation increases the risk of other chronic diseases such as type 2 diabetes. The primary cause of obesity is the energy imbalance between calories consumed and expended. A balanced diet is essential for weight control and maintaining a healthy body weight. In this context, the consumption of high saturated fat meals (HSFM) plays a detrimental role, contributing to the development of obesity, type 2 diabetes, cancer, cardiovascular diseases, and exacerbating chronic inflammation. Additionally, it may induce metabolic endotoxemia and modulate circulating microRNA profiles. Thus, due to the harmful health impacts of HSFM, studies on metabolomics and circulating microRNA levels could enhance understanding of the mechanisms by which walnuts (Juglans regia) prevent and treat diseases.

The objective of this study is to evaluate whether acute walnut supplementation attenuates the inflammatory response, oxidative stress, glycemic response, and regulates the metabolomic and circulating microRNA responses induced by HSFM in women with obesity and type 2 diabetes. The study will be randomized and cross-over, including obese and non-insulin-dependent diabetic women over 18 years of age without other comorbidities. In the first session, participants will arrive at the clinic after a 10-hour fast and will be offered HSFM with or without 30g of walnuts. In the second session, those who did not receive walnuts will now receive them with HSFM, and vice versa. Blood samples will be collected, and serum insulin, glucose, inflammatory biomarkers, and oxidative stress will be assessed. TNF-alpha, IL-6, IL-1β, IL-1ra, IL-8, and IL-10 will be measured by ELISA, and MDA by HPLC. Additionally, metabolomic and microRNA

analyses will be conducted. Data analysis will be performed using JAMOVI v 2.3.21, with a significance level of 5%.

Introduction

Obesity is a multifactorial disease in which excess body fat accumulation leads to negative health effects [1]. It is considered a global public health problem, mainly due to its association with an increased risk of developing other non-communicable chronic diseases (NCDs) [2]. According to the World Obesity Atlas 2023 report, 38% of the global population is currently overweight or obese. Additionally, it is projected that 78% of adults in the U.S. will be overweight/obese [1,3]. In Brazil, it is estimated that one-quarter of the population is obese, making this a significant risk factor for pathological aging [4].

The primary cause of obesity is the energy imbalance between calories consumed and expended, and its high prevalence is associated with physical inactivity and sedentary behavior [5]. A balanced diet, with the consumption of whole and minimally processed foods, is known to be essential for weight control. However, in the current scenario, there has been a significant increase in the consumption of ultra-processed foods and a reduction in the intake of whole and minimally processed foods, resulting in a substantial impact on the prevalence of obesity [6, 7].

Obesity is one of the most relevant risk factors for type 2 diabetes, a disease that is increasingly prevalent worldwide and is considered one of the health emergencies of the 21st century. Diabetes impacts morbidity and mortality and is also a major risk factor for stroke, renal dysfunction, cardiovascular diseases, vision loss, and neuropathy [8]. Non-pharmacological strategies for controlling diabetes typically include nutritional

intervention, regular physical activity, smoking cessation, and maintaining a healthy body weight [10, 11].

Acute intake of macronutrients can induce adverse responses even in healthy individuals [9–19], which can lead to chronic effects over time. In this context, the consumption of high saturated fat meals (HSFM) plays an important role, as chronic consumption of an HSFM diet is known to contribute to the development of obesity, type 2 diabetes, cancer, and cardiovascular diseases, in addition to exacerbating chronic inflammation induced by insulin resistance and excess weight [9]. Furthermore, studies show that an HSFM can also induce metabolic endotoxemia and modulate circulating microRNA profiles [20]. MicroRNAs are non-coding RNA molecules approximately 22 nucleotides in length that regulate gene expression at the post-transcriptional level by degrading mRNA or inhibiting the translation of target genes [21]. Thus, microRNAs may contribute to the adverse effects of an HSFM.

Given the detrimental health impacts of HSFM, studies have been conducted with acute supplementation of foods with anti-inflammatory and antioxidant properties, such as orange juice and green tea [17-22], in order to minimize the negative effects of an unhealthy diet. A study conducted by our research group showed that acute supplementation of green tea inhibited the expression of 62 microRNAs induced by HSFM intake [22]. Bioinformatics analysis indicated that these microRNAs regulated genes associated with TGF-beta, CARM1, RSK, and BMP pathways [22]. The identification of these miRNAs and their molecular pathways can contribute to understanding the benefits of dietary compounds for health and disease.

Regular walnut (Juglans regia) consumption is associated with better cardiovascular health [23] and insulin response, possibly due to the regulation of insulin response by reducing the activity of carbohydrate-digesting enzymes [24]. However, the

literature remains limited and inconclusive regarding the effects of walnuts on the inflammatory and antioxidant response [25]. Additionally, there is only a limited number of studies investigating the acute effects of walnuts, with indications of positive response. In this regard, postprandial attenuation of insulin resistance, improved endothelial function, oxidative stress, and satiety have been demonstrated in some but not all studies [26-29]. Interestingly, Torabian et al. [27], in an intervention study, observed that acute walnut consumption increased plasma concentrations of polyphenols, as well as enhancing total antioxidant capacity and reducing plasma lipid peroxidation.

In addition to the limited evidence regarding the acute effects of walnuts, their effects alongside HSFM on metabolomic and microRNA profiles have not yet been studied. Metabolomics was initially defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to physiopathological stimuli or genetic modification. Thus, it directly reflects the phenotype of a particular biological system at the molecular metabolic level, also allowing for the detection of changes in previously unknown, uncharacterized, or rarely reported metabolites [29]. In this way, metabolomics can provide important insights into the mechanisms of walnuts in the prevention and treatment of diseases. The same applies to the study of microRNAs, which can aid in identifying the intracellular pathways related to the beneficial effects of walnut consumption.

Therefore, this study will generate new evidence regarding the acute effects of walnuts in attenuating the adverse effects of HSFM. Furthermore, mitigating the adverse effects of HSFM could yield significant benefits, particularly for patients with insulin resistance, such as the obese and diabetics.

Objective

To evaluate whether acute supplementation with walnuts (Juglans regia) attenuates inflammatory markers, oxidative stress, metabolomic profiles, and circulating microRNAs induced by high saturated fat meals (HSFM) in obese and diabetic women.

Methods

The project has been approved by the Ethics Committee of the Faculty of Medicine of Botucatu. The project is currently being registered in Clinical Trials. This study will be randomized and crossover, including women with obesity (body mass index [BMI] ≥ 30) and non-insulin-dependent diabetes aged over 18, recruited during outpatient consultations at the Faculty of Medicine of Botucatu. The exclusion criteria will include: use of dietary supplements, use of corticosteroids in the last 3 months, cancer, heart failure, kidney diseases, liver diseases, lung diseases, neurological diseases, allergy to any component of HSFM or walnuts, and habitual walnut consumption (≤3 times per week) in the last month.

The sample size was estimated based on a previous study that showed a 60% reduction in IL-6 levels after meals with HSFM associated with orange juice compared to the control group (without orange juice) [12]. Considering this reduction, a type I error of 5%, and a power of 90%, this study will include 15 individuals.

Patients who sign the Informed Consent Form (ICF) will be evaluated at two time points, separated by a minimum washout of one week. In the first meeting, after fasting for 10 hours, HSFM will be offered with or without 30g of walnuts. In the second meeting, the condition will be switched. This dosage of walnuts refers to the daily recommended intake of nuts proposed by different dietary guidelines [30, 31]. Simple randomization will be performed by a nurse who will not participate in the research. HSFM will be offered in the outpatient clinic of the hospital, and patients will be required to consume

the entire meal and all the walnuts. During the study protocol, patients will be instructed to avoid walnut consumption for 2 weeks before the first study time point.

Blood samples will be collected at time points 0 (immediately before the meal), 15 minutes, 30 minutes, 60 minutes, and 150 minutes after the interventions. From time points 0 to 60 minutes, serum insulin and glucose will be measured, while at time points 0 and 150 minutes, serum biomarkers of inflammation, oxidative stress, circulating microRNAs, and metabolomic profiles will be assessed. Additionally, we will evaluate satiety using a visual analog scale (VAS) at all time points after the meal. Figure 1 illustrates the study design.

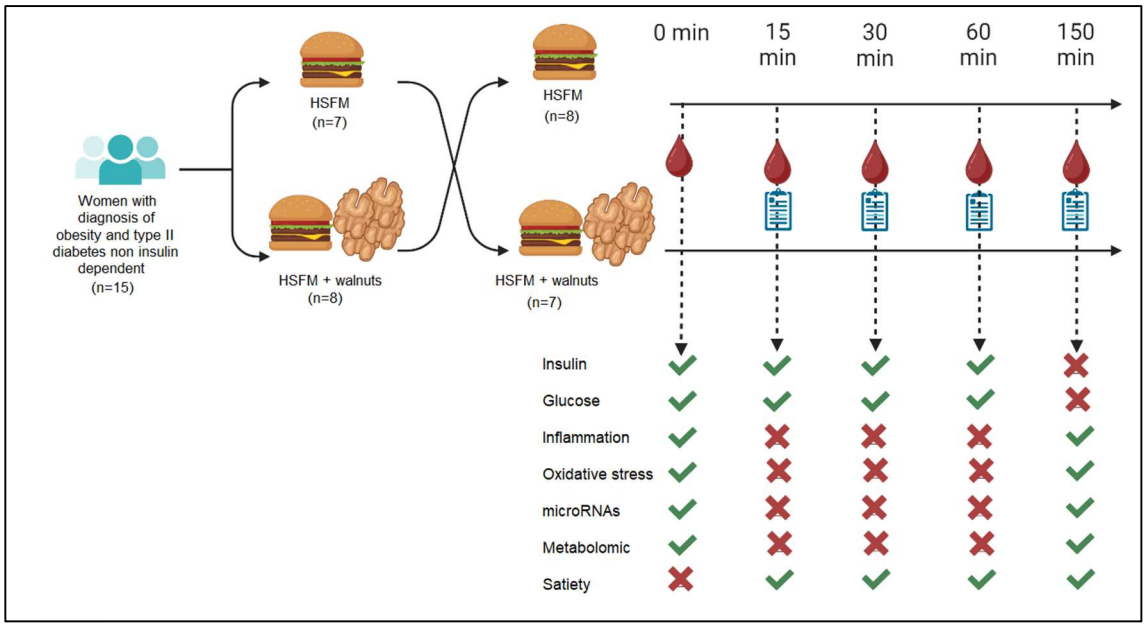

Figure 1. Randomized crossover study. HSFM, high saturated fat meal.

Biochemical, Inflammation, and Oxidative Stress Analysis

The cytokines TNF-alpha, IL-6, IL-1β, IL-1ra, IL-8, and IL-10 will be measured using ELISA following the manufacturer's instructions for the kits. Serum concentrations of malondialdehyde (MDA) will be measured by high-performance liquid chromatography (HPLC) based on the reaction with thiobarbituric acid [32]. Insulin will

be measured using a chemiluminescent immunoassay, and glucose will be assessed using the dry chemistry method.

Metabolomic Analysis

Analyses will be conducted using liquid chromatography on a Thermo Dionex Ultimate 3000 system coupled to a high-resolution mass spectrometer, Thermo QExactive Plus, with electrospray ionization operating in both positive and negative modes. Separation will be achieved using a Waters® Acquity BEH C18 column (100 x 2.1 mm x 1.7 µm particle diameter) maintained at 40 °C with a flow rate of 0.350 mL/min for the mobile phase and a sample injection of 5 µL. The mobile phases (MP) will be: (MP A) ultrapure water with 0.1% formic acid and 5 mM ammonium formate, and (MP B) methanol with 0.1% formic acid. The chromatographic separation will be performed using a gradient elution mode: 0-1.0 min 5% B; 1.0-3.0 min 35% B; 3.0-13.0 min 95% B; 13.0-17.0 min 95% B; 17.0-20.0 min 5% B. General source conditions will include: sheath gas and auxiliary gas: 50 and 15 arbitrary units, respectively; spray voltage: ± 3600 V; S-lens voltage: 50 V; capillary temperature: 320 °C; source temperature: 400 °C. Data will be acquired in full ion scan mode over the range of m/z 100-1000 using a resolution of 35,000 FWHM (Full Width at Half Maximum), combined with data-dependent acquisition experiments (DDA) using a resolution of 17,500 FWHM, AGC 1e5, IT 50 ms; NCE 15-35%, and an isolation window of 1.2 Da.

The processing of the raw files obtained from high-resolution liquid chromatography-mass spectrometry (LC-MS) analyses will be conducted using the MS-DIAL software (RIKEN, version 4.9) for the extraction of detected signals, spectral deconvolution, and peak alignment [33]. Parameters for processing will include: MS1 tolerance of 0.005 Da and MS2 tolerance of 0.05 Da; minimum peak height of 1.0E5;

mass width of 0.05 Da; sigma value for deconvolution window of 0.5; and alignment tolerance of 0.3 min and 0.005 Da. A sample blank (extraction solvent) will be used to subtract interfering signals.

For the putative annotation of key metabolites, experimental MS/MS spectra will be compared to data from a spectral library from the laboratory that combined the public library Mass Bank of North America and the commercial NIST MSMS 2020, considering a similarity level of over 80% between spectra. An error of less than 8 ppm for similarity between experimental and theoretical m/z values will also be considered. Detected ions will be exported to Excel (Microsoft Office 2016) for generating a data matrix table. Additionally, molecular formulas will be determined using MS-FINDER (RIKEN, version 3.44) [34]. Analytical standards will be used for compound identification based on comparisons of m/z of precursor ions, fragmentation spectrum, and retention time. Analyses will be performed at the Monash Proteomics & Metabolomics Platform (MPMP).

Circulating MicroRNA Analysis

Following RNA extraction, we will utilize the human miRNA array 4.0 Affymetrix GeneChip® (ThermoFisher Scientific), according to the manufacturer's instructions. This array contains 2,578 sets of probes for mature human miRNAs and 2,025 sets of probes for human pre-miRNAs (stem-loop). Probe sets (a total of 1,996) for determining the presence of human snoRNA and scaRNA are also included in this array. GeneChips will be scanned using the Affymetrix GeneChip G3000 7G scanner with standard settings to capture signal intensities for the miRNAs. Raw intensity data will be imported into the Affymetrix Expression Console software (v1.4.1.46) for signal preprocessing, including background correction using the robust multi-array average

(RMA) algorithm, median polish summarization of probe signals at the probe set level, and quantile normalization across multiple arrays. A detection call for the miRNA signal strength will be performed using the Affymetrix "Detection Above Background" (DABG) algorithm, which generates a p-value for each signal above the background probability. The method will follow the protocol recommended by the manufacturer. Analyses will be conducted at Monash in Hudson Genomics.

Statistical Analysis

Data will be presented as mean and standard deviation when normally distributed, median and 25-75% percentiles when non-normally distributed, or percentage. For comparisons between the two groups (with and without walnuts), we will use the Student's t-test when the distribution is normal, the Mann-Whitney test when the distribution is non-normal, and the Chi-Square test for categorical variable comparisons. Additionally, generalized estimating equations (GEE) will be used to evaluate repeated measures. Data analyses will be performed using JAMOVI v 2.3.21. The significance level will be set at 5%.

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