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Acute Impact of Dark Chocolate on High-Intensity Interval Training-Induced Oxidative Stress in Healthy Female Adults

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Nina Todorovic BSc

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Abstract

Moderate levels of reactive oxygen species (ROS) are essential for maintaining redox homeostasis in the human body. However, an excessive production of ROS can lead to oxidative stress, a condition associated with the pathogenesis of various lifestyle diseases. The generation of ROS can be triggered by a variety of endogenous and exogenous factors, including strenuous physical activity. In a healthy organism, the balance of ROS is typically maintained by antioxidants. Dark chocolate is notable as a source, among other things, of secondary plant compounds such as polyphenols and flavonoids, which exhibit antioxidant effects. This Master's thesis aims to evaluate the acute effects of pre-exercise dark chocolate consumption on oxidative stress induced by intense physical activity in female participants in the context of a high-intensity interval training (HIIT) strength training circuit. Twenty-two healthy women aged 18 to 40 years took part in a randomized and controlled intervention study conducted by the Faculty of Life Sciences at the University of Vienna within the Department of Nutritional Sciences. In addition to dark chocolate, participants consumed five other plant-based foods and water as a control measure in a randomized and alternating sequence before engaging in a HIIT strength training circuit over a total of seven intervention days. On each intervention day, four venous blood samples were collected—pre-meal, pre-HIIT, immediately post-HIIT, and 15 minutes after HIIT—and ROS levels were measured as oxidative stress markers using electron paramagnetic resonance (EPR) spectroscopy. Immediately post-HIIT, a slight increase in ROS was observed following dark chocolate consumption compared to the water control; however, this increase was not statistically significant ($p > 0.05$). In conclusion, the findings suggest that acute consumption of dark chocolate before HIIT does not mitigate exercise-induced oxidative stress in healthy female adults.

Zusammenfassung

Moderate Mengen an reaktiven Sauerstoffspezies (ROS) sind entscheidend für die Aufrechterhaltung der Redox-Homöostase im menschlichen Körper. Jedoch kann eine übermäßige Produktion von ROS oxidativen Stress verursachen. Oxidativer Stress steht in Zusammenhang mit der Pathogenese diverser Zivilisationskrankheiten. Die Entstehung von ROS kann durch eine Vielzahl von endogenen und exogenen Faktoren ausgelöst werden, einschließlich anstrengender körperlicher Aktivität. In einem gesunden Organismus wird das Gleichgewicht von ROS typischerweise durch Antioxidantien aufrechterhalten. Bitterschokolade zeichnet sich als Quelle unter anderem für sekundäre Pflanzenstoffe wie Polyphenole und Flavonoide aus, welche eine antioxidative Wirkung aufweisen. Diese Masterarbeit zielt darauf ab, die akuten Auswirkungen des vor dem Training erfolgenden Verzehrs von Bitterschokolade auf durch intensive körperliche Aktivität im Rahmen eines hochintensiven-Intervalltrainings (HIIT) Krafttrainingszirkeltraining bei weiblichen Studienteilnehmerinnen verursachten oxidativen Stress zu bewerten. Zweiundzwanzig gesunde Frauen im Alter von 18 bis 40 Jahren nahmen an einer randomisierten und kontrollierten Interventionsstudie teil, die von der Fakultät für Lebenswissenschaften an der Universität Wien im Department für Ernährungswissenschaften durchgeführt wurde. Neben Bitterschokolade konsumierten die Teilnehmerinnen fünf andere pflanzliche Lebensmittel und Wasser als Kontrollmaßnahme in einer randomisierten und abwechselnden Reihenfolge, bevor sie an einem HIIT Krafttrainingszirkeltraining über insgesamt sieben Interventionstage teilnahmen. An jedem Interventionstag wurden vier venöse Blutproben entnommen - vor der Mahlzeit, vor dem HIIT, unmittelbar nach dem HIIT und 15 Minuten nach dem HIIT - und ROS-Spiegel wurden als Marker für oxidativen Stress mittels Elektronenspinresonanz-(EPR)-Spektroskopie gemessen. Unmittelbar nach dem HIIT wurde eine leichte Zunahme von ROS nach dem Verzehr von Bitterschokolade im Vergleich zur Kontrolle mit Wasser beobachtet; jedoch war diese Zunahme nicht statistisch signifikant ($p > 0,05$). Zusammenfassend legen die Ergebnisse nahe, dass der akute Verzehr von Bitterschokolade vor HIIT den durch Bewegung induzierten oxidativen Stress bei gesunden erwachsenen Frauen nicht mildert.

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List of Acronyms and Abbreviations

°C	Degrees Celsius
•OH	Hydroxyl radical
5-RM	Five-repetition maximum
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
As	Arsenic
AVA	Avenanthramides
BIA	Bioelectrical Impedance Analysis
BMI	Body Mass Index
CAT	Catalase
Cd	Cadmium
CMH	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine
CS	Citrated synthase
Cu	Copper
Cu^{+}	Cuprous ion
DNA	Deoxyribonucleic acid
ecSOD	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
EKG	Electrocardiogram
EPR	Electron paramagnetic resonance
Fe	Iron
Fe^{2+}	Ferrous ion
GPX	Glutathione peroxidase
GSH	Glutathione
H_2O	Water
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HF	Heart rate
Hg	Mercury
HIIT	High-intensity interval training
HOCl	Hypochlorous acid
hs-CRP	High-sensitivity C-reactive protein
IL	Interleukin

LDL	Low density lipoprotein
LKB1	Liver kinase B1
Mn	Manganese
MnSOD	Mn superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO•	Nitric oxide
NOS	Nitric oxide synthase
O_2	Molecular oxygen
$O_2•^-$	Superoxide radical
$ONOO^-$	Peroxynitrite
PAL	Physical activity level
Pb	Lead
PC	Protein carbonylation
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
RCT	Randomized controlled trial
ROS	Reactive oxygen species
Se	Selenium
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
UA	Uric acid
UV	Ultraviolet lights
VO_2 max	Maximal oxygen uptake
Zn	Zinc

1 Scientific Background

1.1 Dark Chocolate

1.1.1 History and Production

The history of chocolate began in South America over five thousand years ago, when the Maya people started cultivating the cocoa plant. During that period, chocolate was consumed in the form of a cocoa drink prepared with hot water along with cinnamon and pepper, which were frequently used as flavors. Cocoa beans would later become a form of currency in Mesoamerica, holding great value. Cocoa was first introduced to Europeans at the beginning of the 16th century when Christopher Columbus came across a canoe that contained cocoa beans – an unfamiliar commodity at the time. Dubbed “brown gold”, cocoa became popular in Europe when it was brought to King Charles of Spain in 1528. It was not until 1753 that a Swedish scientist called Carl Linnaeus named the cocoa plant *Theobroma cacao*. The name was derived from the Latin Theobroma – ‘food of the Gods’ and the Aztec word xocolatl – xococ (bitter) and atl (water) (Montagna et al., 2019).

Despite Europeans' fascination with this new delicacy, Europe was an inadequate environment for cocoa to grow in. Cocoa plants thrive in relatively high temperatures and high relative humidity, with tropical regions having the most favorable conditions for their development. More specifically, a flourishing cocoa tree can be found in the lower level of an evergreen rainforest (Montagna et al., 2019). The tree can reach up to 12 meters in height, bearing its fruit after about 5 years. The fruit, called pod or cabosside, can carry 20 to 40 cocoa beans. Usually, a cocoa tree will yield 20 to 50 cabossides per year. The production of cocoa is a complex process that involves several stages. To produce 1 kilogram of cocoa about 10 cabossides are needed. Once the beans are extracted from the fruit, they are first fermented and dried in the sun, and later packed for local consumption or manufactured into cocoa and chocolate. In order to gain their characteristic brown color in the process of making cocoa or chocolate, the beans are carefully roasted and after being pulled from their shell, they are finally ground into a fine grain or powder (Verna, 2013).

Cocoa liquor, referred to as “percent cacao” on food packaging, is a paste produced by roasting the beans at higher temperatures. The paste contains both non-fat cocoa solids and

cocoa butter. Extracting some of the cocoa butter from the liquor results in dry residual cocoa powder. In order to produce solid chocolate, cocoa liquor is mixed with cocoa butter and sugar. The darkness of chocolate exhibits varying degrees depending on the proportion of cocoa liquor in the final product. Bittersweet chocolate, better known as dark chocolate, must contain a minimum of 35% by weight of cocoa liquor. Milk chocolate, on the other hand, is made by adding condensed or powdered milk to the chocolate mixture. It typically contains 10%-12% cocoa liquor. Finally, combined with sweeteners and dairy ingredients, white chocolate contains only cocoa butter, 20% by weight (Katz et al., 2011).

1.1.2 Bioactive Components

Dark chocolate, specifically its major ingredient cocoa liquor, is an important foodstuff because of its many bioactive components (Katz et al., 2011). Besides being responsible for the organoleptic properties of cocoa and cocoa-derived products, bioactive components are secondary metabolites that protect the cocoa tree from UV lights, parasites, and pathogens. They are abundant in dark chocolate as it is rich in total phenolic compounds, catechin, epicatechin, caffeine, and flavonoids. These compounds may negatively influence dark chocolate's appeal compared to its sweeter counterpart milk chocolate as they give it an intense bitter taste, astringency, and cocoa flavour (Samanta et al., 2022).

Polyphenols, the main components found in cocoa beans, can be classified as phenolic acids, stilbenes, flavonol and flavan-3-ols, and anthocyanins. As the total polyphenol content in cocoa varies depending on the cultivators of cocoa beans, so does the quantity of polyphenols in different cocoa-derived foods depending on the manufacturer. Having high amounts of polyphenols for protection against different herbivores and pathogens, cocoa beans in their natural raw form are practically inedible. A fresh, unrefined cocoa bean usually contains 5-6% polyphenol causing it to have extreme bitterness and astringency (Samanta et al., 2022). Throughout the years, cocoa polyphenols have been proven to possess many health benefits. Epidemiological studies illustrate the important role that cocoa polyphenols play in the prevention of cardiovascular diseases, inflammatory processes, and metabolic disorders, while *in vitro* and animal studies demonstrate their potential anti-carcinogenic effects (Andújar et al., 2012). Polyphenols effectively inhibit lipid peroxidation and also decrease the oxidation of LDL cholesterol. Besides modulating the glycemic response, platelet functioning, and inflammation, they can moderate systolic and diastolic blood

pressure. Cocoa polyphenols reduce neutrophil infiltration and the expression of different transcription factors, thus, delaying intestinal inflammation. As a result, the production of pro-inflammatory cytokines is decreased. Additionally, polyphenols hold anti-carcinogenic, anti-proliferative, and anti-mutagenic, as well as chemoprotective effects (Samanta et al., 2022).

Flavonoids are a widely distributed group of naturally occurring polyphenolic compounds present in fruits, vegetables, and plant-derived beverages, with the flavan nucleus characterizing their chemical structure (Babu & Liu, 2009). Typically, the flavonoid content in dry cocoa ranges from 12% to 18%, a higher percentage in comparison to that found in apples, onions, and wine. Flavonoids can be classified into multiple subcategories for example flavonols, flavones, anthocyanin, flavanone, flavan 3-ols, and anthocyanins. High concentrations of flavonols contribute to the characteristic bitterness of dark chocolate. Furthermore, flavonol enhances the skin's healing capacity and protection against UV light, while also improving dermal blood flow (Samanta et al., 2022). Possessing antibacterial, antiviral, and anti-inflammatory effects, flavonoids serve as dietary supplements playing a role in disease prevention and health promotion. Epidemiological, clinical, and animal studies have demonstrated the potential protective effects of flavonoids against various non-communicable diseases such as cardiovascular disease, cancer, and other diseases related to oxidative stress (Babu & Liu, 2009). Cocoa flavonoids exhibit higher effectiveness in brain function, protecting vulnerable neurons and improving their capabilities. They prevent neuronal degeneration by interacting with signaling proteins crucial for pro-survival pathways. In that way, flavonoids provide protection against Alzheimer’s disease and Parkinson’s disease (Samanta et al., 2022).

Alongside polyphenols, cocoa also contains methylxanthine compounds, primarily theobromine making up about 2% to 3% of its weight (Katz et al., 2011). Caffeine is present in smaller amounts, while theophylline is found only in traces. As purine alkaloids and secondary metabolites, they give cocoa and its derived products a bitter taste and astringency (Jean-Marie et al., 2021).

Theobromine has been shown to have relatively high bioavailability and possess a number of physiological benefits. It increases serum HDL cholesterol and has an influence on heart and lung muscle activity. Theobromine stimulates heart muscle and induces relaxation of smooth

muscles in the bronchial tubes of the lungs, thus, playing an important role in the transmission of intracellular signals. Since theobromine exhibits antioxidant activity, it is utilized in the treatment of depressive disorders. Its antioxidant role is crucial for scavenging free radicals produced in the skin due to UV light exposure. In this way, theobromine prevents those free radicals from having adverse effects on signaling pathways in the skin (Samanta et al., 2022).

1.2 Oxidative Stress and the Antioxidant System

1.2.1 Reactive Oxygen Species and Oxidative Stress

Oxidative stress is a physiological condition that arises when there is an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues and the body's ability to detoxify these reactive products or repair the damage that they cause (Pizzino et al., 2017).

ROS is a term used to describe various substances that contain reactive oxygen, including free radicals such as hydroxyl (•OH) and superoxide (O_2•$^-$), as well as non-radical species like hydrogen peroxide (H_2O_2). Besides oxygen, certain reactive species may also carry elements like nitrogen, chlorine, and sulphur (Demirci-Çekiç et al., 2022). They occur naturally as by-products of the human metabolism. Free radicals contain one or more unpaired electrons in their outer atomic or molecular orbital and are therefore highly reactive compounds that readily react with different organic substrates to enhance their stability (Zeng et al., 2021).

ROS are generated due to molecular oxygen's gradual uptake of electrons from numerous substrates during the aerobic process (Demirci-Çekiç et al., 2022). The production of ROS is dependent on enzymatic and non-enzymatic reactions. The enzymatic reactions capable of producing ROS are an inherent part of the respiratory chain, prostaglandin synthesis, cytochrome P450 system, and phagocytosis. NADPH oxidase, xanthine oxidase, and peroxidases all generate O_2•$^-$, which later takes part in multiple reactions that subsequently produce H_2O_2, •OH, peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), etc. Amino acid oxidase and xanthine oxidase are some of the many oxidase enzymes that produce the non-radical H_2O_2. Furthermore, the reaction between H_2O_2 and O_2•$^-$ catalyzed by either Fe^{2+} or Cu^+, also known as the Fenton reaction, produces the most reactive free radical species *in vivo* - •OH. Another free radical that fulfills several important physiological functions is nitric oxide (NO•). Composed of one nitrogen atom and one oxygen atom, it is generated through the oxidation of arginine to citrulline by nitric oxide synthase (NOS). Non-enzymatic ROS production can occur in several different ways. For example, it can take place during mitochondrial respiration or be triggered by cells' exposure to ionizing radiation. It can also result from the reaction of oxygen with certain organic compounds (Pizzino et al., 2017).

Both endogenous and exogenous sources can instigate the production of free radicals (Figure 1). Some of the endogenous sources are health problems such as inflammation, ischemia, infection, mental stress, and cancer, as well as biological processes like immune cell activation and aging. Interestingly, endogenous free radical production can also be set off by excessive exercise. Exogenous sources that trigger the generation of free radicals are environmental pollutants, cigarette smoke, alcohol, certain drugs (like cyclosporine, tacrolimus, gentamycin, bleomycin), chemical solvents, heavy metals (such as Cd, Hg, Fe, Pb, and As), radiation, and even cooking (smoked meat, oxidized oil and fat). Once these external substances enter the body, they are degraded or metabolized, leading to the generation of free radicals as by-products of these reactions (Pizzino et al., 2017).

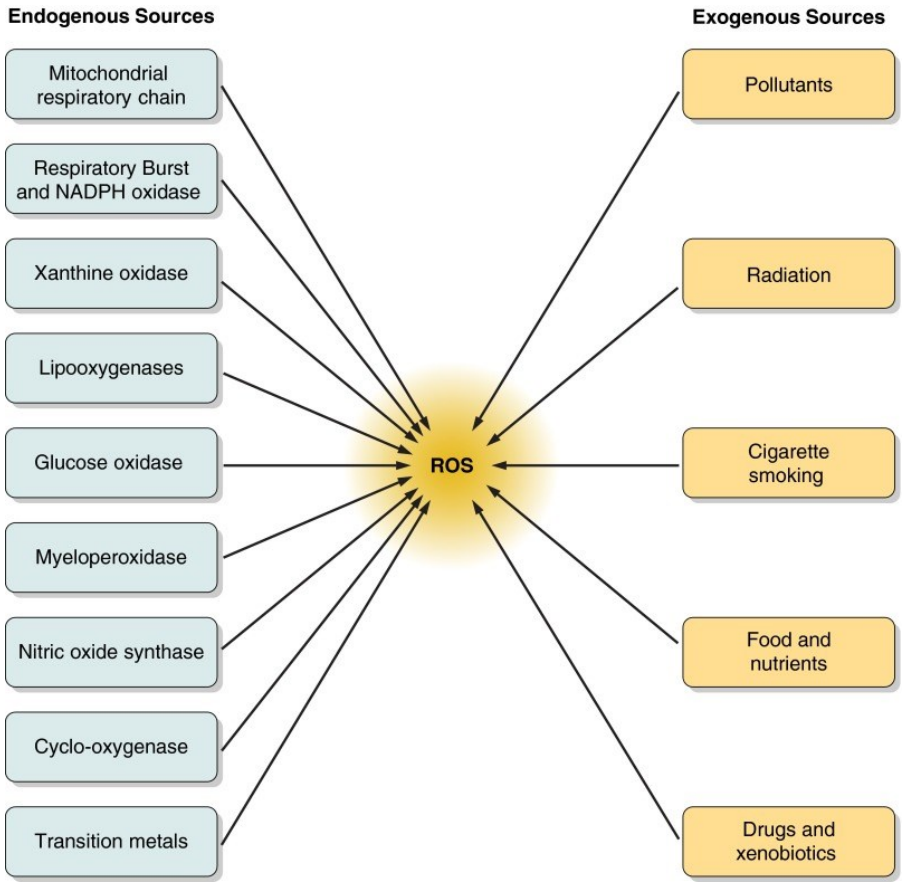

Figure 1: Endogenous and exogenous factors influencing ROS production (Adapted from Bhattacharyya et al., 2014)

1.2.2 Antioxidants

The human body exhibits a complex antioxidant system to protect its cells and organ systems against free radicals. The detrimental effects of free radicals can be neutralized by

endogenous enzymatic and non-enzymatic, as well as exogenous or dietary antioxidants (Figure 2). The antioxidants synthesized in our body such as glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), uric acid (UA), and ubiquinone, alongside the antioxidants acquired through dietary intakes like vitamins E, C, and A, and flavonoids mitigate the negative impacts of free radicals through numerous mechanisms. These mechanisms encompass electron donation, catalytic removal, binding radicals, and gene expression regulation. Antioxidants work together to form a mutually supportive defense system against ROS to sustain the balance between oxidants and antioxidants (Janciauskiene, 2020).

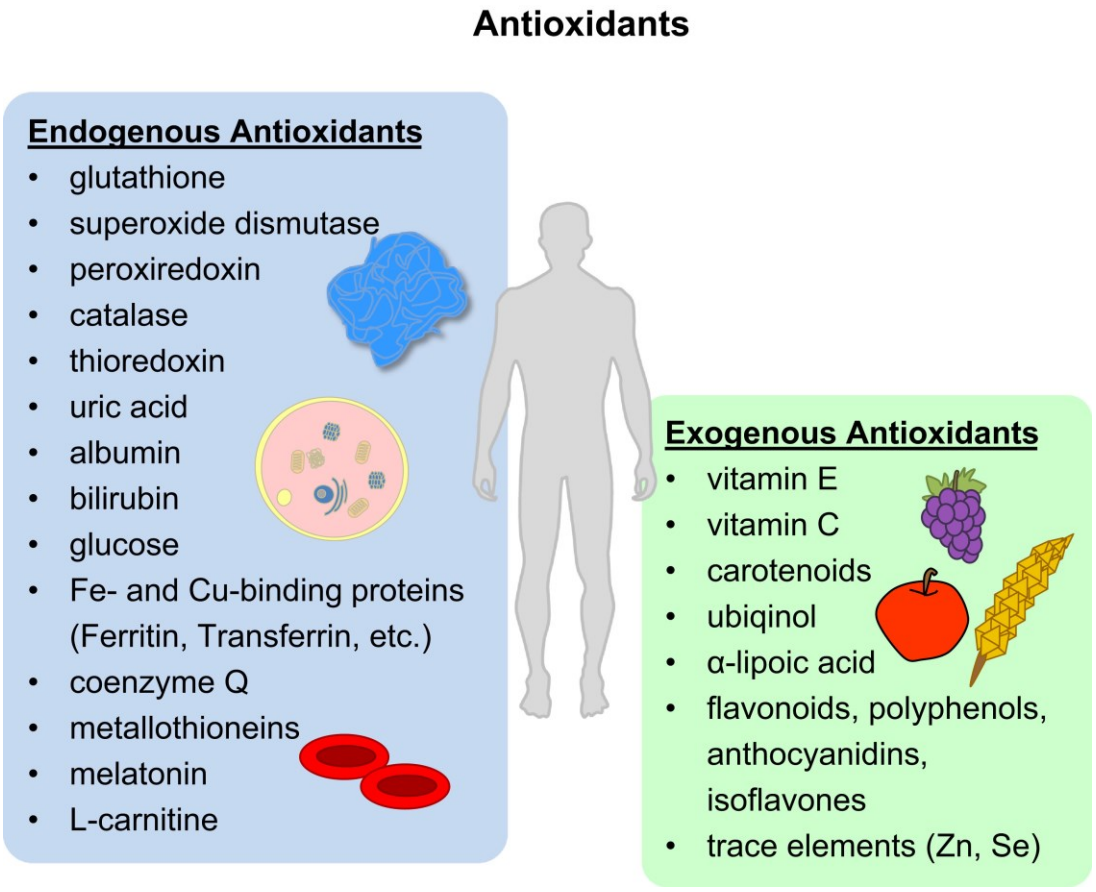

Figure 2: Overview of endogenous and exogenous antioxidants (Adapted from Höhn et al., 2017)

In a healthy organism, ROS can typically be balanced by antioxidants. The role of the human immune system is to keep humans protected from various diseases and ensure their survival. ROS are crucial here, as they actively participate in eliminating infecting pathogens. However, continuing the activation of the immune system once the threat is removed also has harmful

consequences. ROS are important for redox signaling in the body, and as previously mentioned, they play a role in adapting to conditions caused by ischemia and intense exercise. The human antioxidant defense system is responsible for eliminating excessive amounts of ROS without impeding their beneficial role. This process is crucial for maintaining a healthy life. In order to uphold the cells' natural balance, also known as “redox homeostasis”, the quantity of ROS must be regulated by the cellular antioxidant system. The components of the cellular antioxidant system can be classified as enzymatic and non-enzymatic species. The enzymatic species primarily consist of SOD, CAT, and GPX, while non-enzymatic species include GSH as the main antioxidant. SOD catalyzes the dismutation reaction of $O_2\bullet^-$ resulting in O_2 and H_2O_2. Subsequently, CAT and GPX break down H_2O_2 into H_2O and O_2 (Demirci-Çekiç et al., 2022).

Aside from endogenous antioxidants, varieties of different antioxidants originating from dietary sources have protective effects on human health. Vitamins such as vitamin E, vitamin C, and pro-vitamin A carotenoids possess antioxidant activity and are present in fruits and vegetables. They are situated on the cell membrane, intracellularly, or extracellularly where they react with free radicals either eliminating or inhibiting them. Vitamin E is the primary scavenger for peroxyl radicals, while carotenoids suppress singlet oxygen, hindering lipid peroxidation. Vitamin C functions as a ROS scavenger rescuing vitamin E, which is anchored in the cell membrane and inhibits lipid peroxidation (Janciauskiene, 2020).

Certain minerals are considered antioxidants because of their essential role in the activity of antioxidant enzymes. The minerals in question are selenium (Se), copper (Cu), zinc (Zn), and manganese (Mn). The effective functioning of GPX relies on Se, enabling them to act as scavengers of H_2O_2 in subcellular compartments (Janciauskiene, 2020). Research has demonstrated the significance of sufficient Se concentrations as lower levels of serum Se were observed in an elderly Swedish population compared to other parts of Europe, with those in the lowest Se quartile exhibiting increased mortality (Alehagen et al., 2016). Although Se supplementation benefits human health, excessive amounts of it can also cause adverse effects. Se has an extremely narrow range between deficiency and toxicity. The non-linear dose-response relationship between Se status and health is U-shaped and quite complex. Individuals with insufficient baseline Se levels may benefit from supplementation; however, those with sufficient or elevated Se levels may face detrimental effects. Due to

variations in baseline Se levels across different populations, safe methods, appropriate dosages, and the suitable baseline Se range for supplementation require further research (Sun et al., 2023). Zn serves as a cofactor for enzymes of the antioxidant system. It hinders the prooxidant enzyme – NADPH oxidase, and elicits the synthesis of metallothionein, which is crucial for •OH reduction. Mn is an inherent part of Mn superoxide dismutase (MnSOD) – the primary ROS scavenger during mitochondrial oxidative stress. Besides playing an important role in the synthesis and activation of various enzymes, Mn partakes in the regulation of glucose and lipid metabolism. However, the antioxidant abilities of Mn face competition from cellular iron when binding to MnSOD. This means that iron might counteract the positive effects of Mn antioxidants. Furthermore, Mn is capable of obstructing the development of diseases caused by bacteria. Since several bacterial proteins rely on Mn for their activation, the host's ability to sequester Mn has the potential to interfere with bacterial pathogenesis (Janciauskiene, 2020).

Other valuable dietary antioxidants are flavonoids. As previously stated, they possess numerous health benefits and play a significant role in various pharmacological activities. Predominantly found in plants, these polyphenolic compounds have been intensively studied because of their antioxidant properties. Flavonoids have functional hydroxyl groups that are capable of scavenging free radicals and/or binding to metal ions. The conformational disposition of these functional groups is a determining factor for the effectiveness of their antioxidant activity. Key factors influencing the mechanisms of antioxidant activity such as metal chelation and ROS scavenging include the configuration, substitution, and total number of hydroxyl groups. Flavonoids play a role in suppressing the production of ROS, hindering enzymes, or chelating trace elements that contribute to the generation of free radicals. Furthermore, they act as ROS scavengers and improve antioxidant defense. A flavonoid compound that is worth mentioning in the context of dietary antioxidants is genistein. This soy isoflavone holds several pharmacological activities and can efficiently scavenge ROS, demonstrating its useful antioxidant properties. Genistein can enhance a cell's antioxidant defenses, thereby inhibiting the apoptotic process by altering various genes and proteins (Pizzino et al., 2017).

Nonetheless, if there is an overproduction of ROS, the cellular antioxidant system is unable to neutralize them successfully, which can cause a disruption of balance in favor of oxidants resulting in oxidative stress (Demirci-Çekiç et al., 2022).

Prolonged exposure to elevated levels of ROS can initiate oxidative damage. When the antioxidant system fails to effectively scavenge ROS, cellular molecules such as deoxyribonucleic acid (DNA), proteins, and lipids are susceptible to damage and modification by these high concentrations of ROS. This can ultimately lead to serious health issues. Previous studies have demonstrated that oxidative stress contributes to the onset of a variety of different chronic diseases (Zeng et al., 2021). Some of the relevant diseases linked to oxidative stress are cancer, atherosclerosis, diabetes, and neurological diseases such as Alzheimer's disease and Parkinson's disease, as well as chronic inflammation. For instance, in comparison to healthy cells, cancer cells contain increased levels of ROS. Furthermore, some research states that protein carbonyls, end products of oxidative protein damage, are observable in the brains of Parkinson's disease patients. Worth mentioning too is that there might be a possible relationship between oxidative stress and Down syndrome. Specific neurodegenerative genes caused by oxidative stress are present on chromosome 21, which is characteristic of Down syndrome (Demirci-Çekiç et al., 2022).

Cellular senescence and the aging process have also been linked to oxidative stress. The nervous, endocrine, and immune systems are all impacted by age-related oxidative stress. This can initiate a harmful cycle where chronic oxidative stress and inflammation mutually assist each other, increasing age-related morbidity and mortality levels (Janciauskiene, 2020).

2 Study Protocol

2.1 Study Design

The research project titled “Acute Impact of different Plant-Based Foods on Exercise-Induced Oxidative Stress and Inflammation” is an initiative of the Faculty of Life Sciences at the University of Vienna, led by Markus Gassner, MSc, within the Department of Nutritional Sciences under the guidance of Univ.-Prof. Dr. Daniel König, Division of Nutrition, Exercise and Health.

This research project aims to investigate the acute impact of plant-based foods on inflammation markers and oxidative stress two hours after consumption and following intense physical activity within the context of a human randomized and controlled intervention study. The selection of foods was primarily based on the “Dietary Inflammatory Index” developed by Tabung et al. (2016). Twenty-two healthy female participants, aged 18 to 40 years, were each assigned to consume a specific food after overnight fasting in the morning. Subsequently, they were instructed to rest for a period of two hours before engaging in a 30-minute High-Intensity Interval Training (HIIT) strength training circuit. Four blood samples were collected from the arm vein on each intervention day – in the morning (fasted state), two hours after food consumption, immediately after training, and 15 minutes after training. This protocol was performed seven times at intervals of at least one week until all foods assigned to the participants were consumed. As a control measure, each participant performed the strength training circuit once without prior food consumption, only drinking water before.

This study design is based on a pilot study with a similar study model by Zeng et al. (2020), which demonstrated a significant reduction in oxygen radicals immediately after a HIIT strength training session following the consumption of oatmeal.

This research project was approved by the Ethics Committee of the University of Vienna; reference ID 00743, on 01.12.2021.

2.2 Methods

2.2.1 Participants

The recruitment process for this study was initiated in April 2023, utilizing various social media platforms to draw attention to the research. A study poster containing all relevant information for potential participants, such as study objectives, eligibility criteria, and contact information, was shared across multiple social media groups.

Healthy women within the age range of 18 to 40 years could apply for participation in this research project. To meet the inclusion criteria, applicants needed to exhibit a Body Mass Index (BMI) between 18.5 and 25 kg/m², a minimum body fat percentage of 20%, engage in regular physical activity for no more than 120 minutes per week, and express a willingness to both consume the specified foods in the study and actively participate in strength training circuit sessions. Applicants were excluded if they had any medical conditions that contraindicated involvement in strength training interventions, such as metabolic, pulmonary, cardiovascular diseases, or known autoimmune disorders affecting the immune system. Additionally, individuals with pre-existing injuries or injuries sustained before the study that hindered participation in the training intervention were not considered eligible. Subjects who did not tolerate the HIIT training in the morning after overnight fasting also had to be excluded. Further exclusion criteria included the use of medications that could influence oxidative stress and/or inflammation, any known incompatibility with the foods used in the study, and a history of engaging in regular strength training more than once per week in the six months leading up to the intervention. In the event of an acute infection occurring within one week before a scheduled intervention day, the study day needed to be postponed.

The randomized controlled trial (RCT) by Zeng et al. (2020) was used to estimate case numbers for this research project. The study included 34 participants, of which 17 consumed oatmeal and milk before a HIIT-strength training session, and 17 exclusively performed HIIT-strength training, resulting in significantly lower ROS levels immediately after physical activity following the consumption of oatmeal (Zeng et al., 2020). This was used for the calculation of case numbers. A G-Power analysis, including a safety margin (n=4), indicated that 24 participants per intervention day were required.

All individuals who applied for this study received a Participant Information and Consent Form containing detailed explanations of the intervention procedures and potential risks associated with study participation. Prior to inclusion, informed consent was obtained from each participant.

2.2.2 Screening Process

To assess their health status and suitability for the study, potential participants underwent a two-day screening process before the intervention began. For both screening days, participants were instructed to refrain from engaging in sports, consuming alcohol, and taking supplements and medications the day before. During the first screening session, a consultation and examination were carried out by a physician of the University of Vienna to assess potential risks. Additionally, participants were presented with a Participant Information and Consent Form for signature, and provided with clarifications if necessary. Subsequently, participants were directed to an examination room for an assessment of their body measurements. This included measuring height, weight, and BMI, as well as obtaining body fat percentage and phase angle through Bioelectrical Impedance Analysis (BIA). They then reclined on an examination table for the evaluation of blood pressure, followed by an Electrocardiogram (EKG) measurement. After these assessments, participants engaged in a 30-minute test training session with sports scientific supervision. On the second day of the screening process, initial blood samples were collected for analysis. For this reason, participants were instructed to fast for 12 hours prior to the appointment. In instances where data was incomplete from the previous screening day, the BIA, EKG, or blood pressure measurements were repeated. Subsequently, participants performed a five-repetition maximum (5-RM) test to assess their training weights for circuit training, lasting approximately 30-45 minutes. This marked the conclusion of the screening process, leaving the follow-up appointment to be scheduled once the initial blood test results became available, typically within one to two weeks.

2.2.3 Intervention Protocol

The intervention period spanned from April 2023 to February 2024 at the Nutrition and Training Laboratory (NuTraLab) of the Department of Nutritional Sciences, University of Vienna, located at Zieglergasse 6, 1070 Vienna.

In this study, participants underwent a total of seven intervention days, which consisted of six sessions involving both strength training and food consumption and one session exclusively focused on strength training without food consumption. Six designated plant-based foods and water as a control measure were consumed in a randomized and alternating sequence. The selected foods included broccoli, dark chocolate, blueberries, flaxseed oil, whole-grain bread, and white bread. To ensure sufficient recovery, a wash-out period of at least seven days was applied between two intervention days.

Intervention appointments were scheduled in the mornings based on participants availability, each lasting approximately 3.5 hours. Throughout the intervention period, participants were required to maintain their usual dietary and exercise routines – maximum two hours of exercise per week – but to abstain from additional resistance exercise. Additionally, they were instructed to refrain from the consumption of any dietary supplements from seven days before their initial appointment until the completion of all seven intervention days.

Participants were requested to keep track of their menstrual cycle throughout the intervention, with this information being recorded for subsequent evaluation. Furthermore, participants were reminded to ensure that their last occurrence of a cold, vaccination, or blood donation had taken place at least seven days before each scheduled study appointment. In the event of feeling unwell, they were advised to reschedule their appointment.

Twenty-four hours before each intervention morning, participants were instructed to abstain from physical exercise, alcohol consumption, and the intake of supplements and medications. Hormonal contraceptives were permitted. Additionally, participants were required to observe a 12-hour fasting period. On the morning of every blood sampling, they were advised to drink at least 0.5 liters of water.

To avoid physical exertion on the morning of the intervention, participants were instructed not to commute to the study center by bicycle. Instead, they were advised to use other means of transportation such as walking, public transportation, or car. Upon arrival, participants were directed to the laboratory and asked to recline on an examination table for a 30-minute resting period. During this time, participants underwent a 24-hour dietary recall

interview. After the 30-minute rest and one minute before the first blood sample was collected, participants were asked to rate their stress level and BORG Scale of Perceived Exertion. This process was repeated for every blood draw. Once the first blood sample was acquired, participants were provided with their designated food of the day and instructed to rest for a duration of two hours. Following the rest period, the second blood sample was obtained just before participants commenced a 30-minute HIIT-strength training circuit, which was supervised by a trained instructor. Further details on the HIIT circuit will be discussed in the upcoming chapter. Immediately after the training circuit was completed, the third blood draw took place, followed by a 15-minute rest. Subsequently, the fourth and final blood sample was collected, concluding the intervention day.

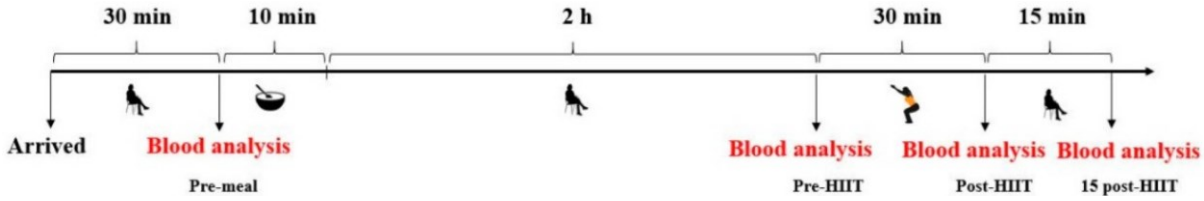

Figure 3: Study design overview. HIIT: high-intensity interval training (Adapted from Zeng et al., 2020)

2.2.4 High-Intensity Interval Training (HIIT)

HIIT consists of alternating short periods of high-intensity exercise with recovery intervals or light physical activity (Zeng et al., 2020). The circuit training used for this study was modeled after the HIIT-strength training by Bizheh & Jaafari (2011), which was able to acutely increase the inflammatory marker hs-CRP. Preceding the seven intervention days, participants underwent a test training session under the guidance of a trainer who provided detailed instructions for each exercise. The 5-RM test carried out during the screening process, established the maximum weight for each participant. For the intervention, illustrated in Figure 4, participants performed three sets of five exercises, including rowing, chest press, leg curl, lat pulldown, and leg press. After completing a warm-up and a trial round, participants initiated the first set of exercises. Each exercise involved 40 seconds of exertion with a goal of 20 repetitions, accompanied by a 10-second rest period between exercises, and a 60-second break between sets. Following each set of exercises, participants were asked to assess their perceived exertion levels using the BORG scale. The obtained values were

documented by the trainer. A training protocol, specifying a detailed sequence of exercises and predetermined weights for each participant, was followed throughout the study period.

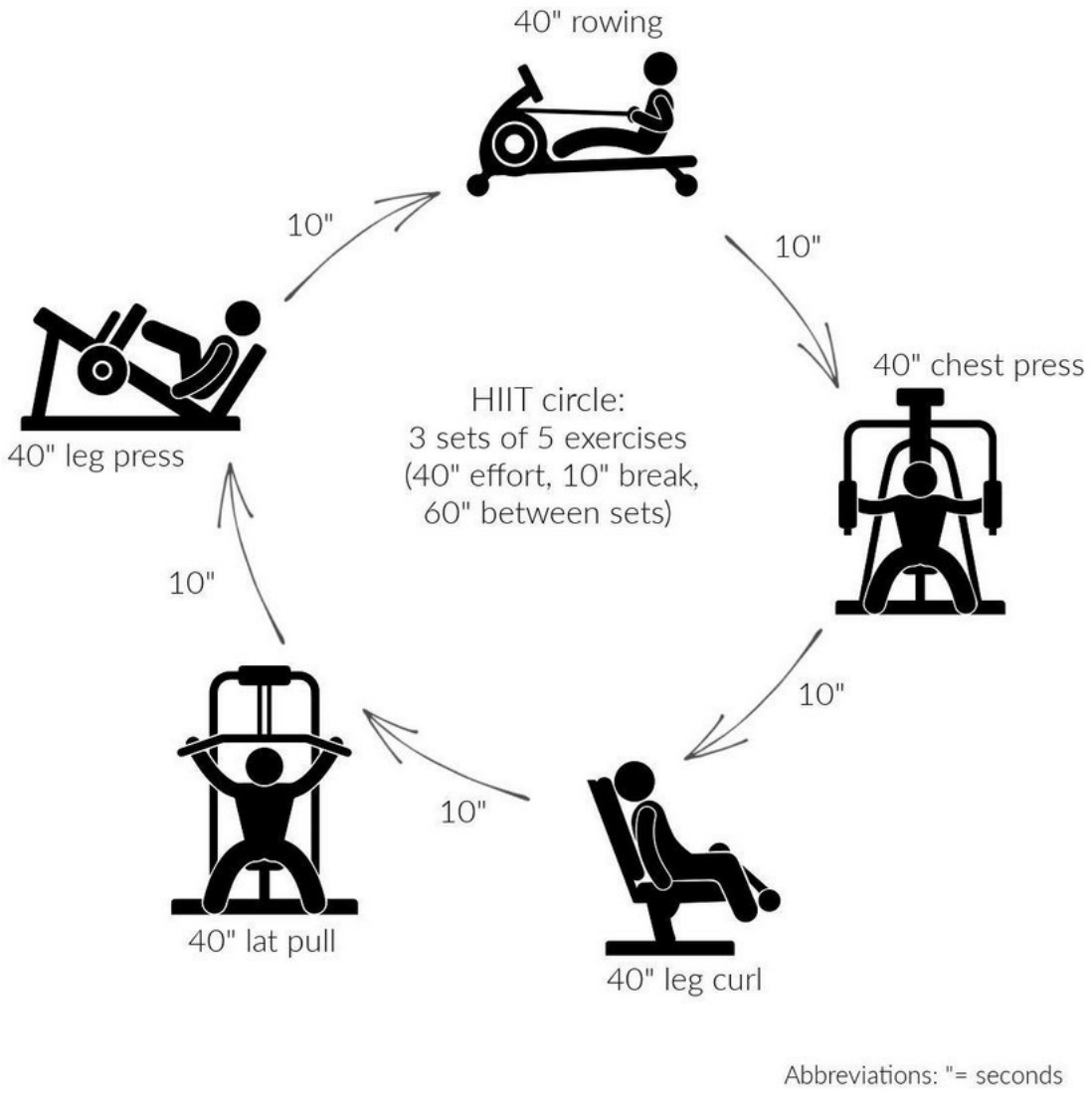

Figure 4: HIIT strength training circuit (Adapted from Gassner, M. 2023)

2.2.5 Dark Chocolate Intervention

Over the course of the seven intervention days, participants were provided with the plant-based foods in a randomized and alternating sequence. As one of the intervention foods, each participant was assigned to consume 40 g of dark chocolate. The precise weight of the dark chocolate was measured using a gram scale. This consumption took place immediately after the first blood collection, followed by a two-hour rest period. Subsequently, participants underwent the HIIT strength training intervention. The dark chocolate used in

this study contained a cacao content of 70% and was purchased from a nearby supermarket ('Fairglobe' brand, manufactured by LIDL-Stiftung & Co. KG in Germany).

2.2.6 Blood Collection

All blood samples collected in this study were obtained from the arm vein by a trained medical professional. On each intervention day, a total of four blood samples, amounting to 20 mL, were collected. Ethylenediaminetetraacetic acid (EDTA) was used as an anticoagulant for the plasma samples. One EDTA tube was filled for each blood draw, with an additional serum tube filled exclusively during the first collection. All blood draws were conducted with participants in a lying position. From the EDTA tube, containing 4 mL of blood, 20 µL of whole blood was extracted for the measurement of ROS. Two portions of 50 µL of whole blood were transferred to two separate 0.5 mL Eppendorf tubes and immediately frozen at -80°C. Subsequently, the sealed EDTA tube underwent centrifugation for 15 minutes to separate plasma from cellular components. A 1.5 mL Eppendorf tube was filled with 300 µL of Trichloroacetic acid (TCA), to which 300 µL of centrifuged plasma was added. After vortexing for 20 seconds, until a homogenously milky consistency was achieved, the Eppendorf tube was placed in a microcentrifuge for 10 minutes at 4°C. From the centrifuged TCA-plasma mixture, two portions of 150 µL from the supernatant were extracted and placed in two 0.5 mL Eppendorf tubes, labeled with TCA, and frozen at -80°C. The remaining centrifuged plasma was divided into 0.5 mL Eppendorf tubes and stored at the same temperature.

2.2.7 ROS Measurement

The ROS measurement protocol was adapted from Zeng et al. (2020). A portion of whole blood from each of the four blood samples collected during the intervention days was utilized for the measurement of ROS. The electron paramagnetic resonance (EPR) technique (Bruker, Germany) was employed to assess the total ROS formation rate under *in vivo* conditions at 37°C, using a temperature and gas controller BIO-III (TGC-BIO, Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany). For each sample test, 20 µL of whole blood from the arm vein of each participant was sampled using a standard Eppendorf pipette (Eppendorf, Research Plus, Hamburg, Germany). This blood sample was then mixed with 20 µL of the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 400 µmol/L) diluted in Krebs-HEPES buffer. The interaction of released ROS in the venous blood with CMH

in both intracellular and extracellular spaces forms the stable radical CM•. Following this, the 40 µL mixture was transferred into a capillary tube (Hirschmann, Eberstadt, Germany), and the ROS formation rate was measured using the following EPR settings: center field: g = 2.011, sweep width: 60 G, sweep time: 5.24 s, frequency: 9.76 GHz, power: 20 mW, gain: 1×10^3, modulation amplitude: 1.0 G, and number of scans: 10. The EPR signal was calibrated using a standard 10 µM solution (Zeng et al., 2020).

3 Results

This section presents the results of the 22 participants who successfully completed the trial, with no reported adverse events during the intervention period.

3.1 Participant Characteristics

The group consisted of 22 healthy women aged 18 to 40, with a mean BMI of 21.6 kg/m^2. Further participant characteristics are presented in Table 1.

Table 1: Participants Descriptive and Anthropometric Characteristics (*n* = 22)

Measurements	Mean	Standard Deviation	Minimum	Maximum	Median
Body height (m)	1.67	0.05	1.57	1.78	1.67
Body weight (kg)	60.99	4.55	52.40	71.15	60.65
BMI (kg/m²)	21.63	1.47	19.12	24.07	21.58
Phase angle (°)	4.93	0.47	4.30	6.10	4.90
Body fat (%)	27.86	4.28	20.20	34.80	28.30
Waist circumference (m)	0.71	0.06	0.61	0.86	0.70
Muscle mass (kg)	20.11	2.57	15.40	25.20	19.70
Blood pressure systolic (mmHg)	119.89	9.56	98.00	135.00	120.00
Blood pressure diastolic (mmHg)	80.63	10.11	60.00	100.00	80.00
PAL	1.66	0.09	1.60	1.80	1.60

BMI: Body Mass Index; PAL: Physical Activity Level

3.2 ROS Production

The results were analyzed using SPSS Statistics software version 27.0 (IBM, Armonk, NY, USA), incorporating Repeated Measures ANOVA for statistical examination.

Table 2: Descriptive Statistics

Measurements	Time Point	Intervention	Mean	Standard Deviation	N
ROS (µmol/min)	Baseline	DC	2.20	0.67	22
		Water	2.22	0.70	22
		Total	2.21	0.67	44
	PreHIIT	DC	1.92	0.54	22
		Water	1.78	0.50	22
		Total	1.85	0.52	44
	PostHIIT	DC	2.46	0.61	22
		Water	2.37	0.72	22
		Total	2.41	0.66	44
	15PostHIIT	DC	2.00	0.41	22
		Water	2.03	0.55	22
		Total	2.02	0.48	44

DC: Dark Chocolate; HIIT: High-Intensity Interval Training; ROS: Reactive Oxygen Species

In Table 2, it is evident that the mean ROS value at the post-HIIT time point is higher following the consumption of dark chocolate compared to the water control. This indicates that dark chocolate did not alleviate exercise-induced oxidative stress, instead, it appeared to elevate it. However, it should also be borne in mind that at the pre-HIIT time point, the ROS values were already somewhat higher when participants consumed dark chocolate compared to when they consumed water. Moreover, the lower standard deviation (SD=0.61) observed after dark chocolate consumption at the post-HIIT time point, indicates reduced variability in ROS levels among participants compared to water consumption (SD=0.72). These findings suggest potentially more consistent individual responses to the dark chocolate intervention relative to water.

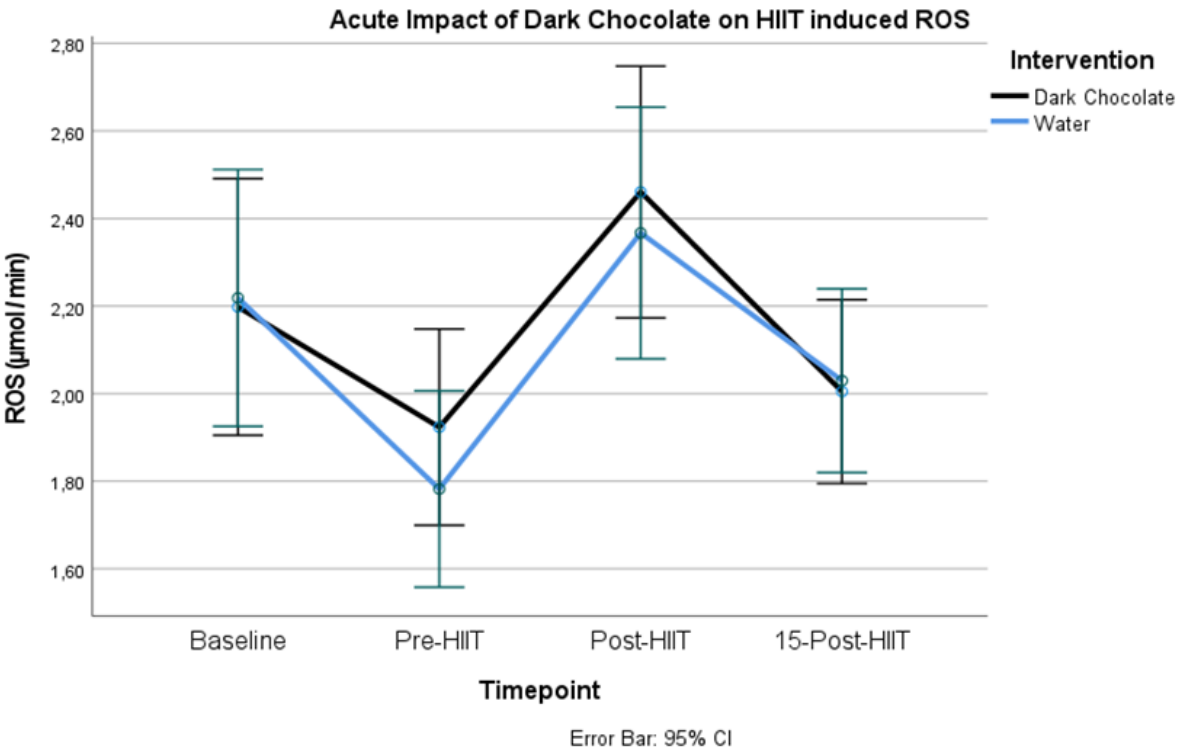

Figure 5: Acute Impact of Dark Chocolate on HIIT-induced ROS Formation

Figure 5 serves as a visual representation of the descriptive statistics presented in Table 2, illustrating the dynamics of ROS levels in response to the dark chocolate and water control interventions. Each line represents the mean ROS levels over time for the respective intervention, with individual data points (dots) indicating mean ROS levels at each of the four time points (Baseline, Pre-HIIT, Post-HIIT, and 15-Post-HIIT). Error bars, representing the 95% confidence intervals (CI), accompany each data point and demonstrate the variability around the mean ROS levels.

Table 3: Descriptive Statistics: PreHIIT vs. PostHIIT change in ROS

Intervention	Mean	Standard Deviation	N
DC	0.54	0.27	22
Water	0.58	0.36	22
Total	0.56	0.32	44

DC: Dark Chocolate

The descriptive statistics in Table 3 present the changes in ROS levels from pre-HIIT to post-HIIT measurements among participants who consumed dark chocolate, water, and the total

sample. Specifically, the mean change in ROS levels for the dark chocolate intervention is 0.54 µmol/min, compared to 0.58 µmol/min for the water control.

Table 4: Parameter Estimations: PreHIIT vs. PostHIIT change in ROS

Parameter	Regression Coefficient B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Constant Term	0.59	<.001	0.45	0.72
Intervention = DC	-0.05	0.63	-0.24	0.15
Intervention = Water	0[a]			

a. This parameter is set to zero as it is redundant.

DC: Dark Chocolate

In Table 4, a significance value of 0.63 can be observed, indicating that the change in ROS levels from pre-HIIT to post-HIIT measurements associated with the dark chocolate intervention is not statistically significant ($p > 0.05$). Therefore, the consumption of dark chocolate resulted in a non-significant (since $0.63 > 0.05$) decrease in ROS elevation by 0.05 µmol/min from pre-HIIT to post-HIIT.

Table 5: Descriptive Statistics: PreHIIT vs. 15PostHIIT change in ROS

Intervention	Mean	Standard Deviation	N
DC	0.08	0.30	22
Water	0.25	0.20	22
Total	0.16	0.26	44

DC: Dark Chocolate

Similarly to Table 3, the descriptive statistics in Table 5 demonstrate the changes in ROS levels from pre-HIIT to 15-post-HIIT measurements for both interventions. A mean change in ROS levels of 0.08 µmol/min is evident for the dark chocolate intervention, compared to a mean change in ROS levels of 0.25 µmol/min for the water control.

Table 6: Parameter Estimations: PreHIIT vs. 15PostHIIT change in ROS

Parameter	Regression Coefficient B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Constant Term	0.25	<.001	0.14	0.36
Intervention = DC	-0.17	0.04	-0.32	-0.01
Intervention = Water	0[a]			

a. This parameter is set to zero as it is redundant.

DC: Dark Chocolate

Table 6 shows a statistically significant ($p < 0.05$) change in ROS levels from pre-HIIT to 15-post-HIIT measurements for the dark chocolate intervention.

3.3 HIIT Performance

In addition to the primary focus of this master's thesis, this section will present supplementary findings pertaining to the participants' HIIT performance.

Table 7: HIIT Performance

Intervention		Upper Body Performance (kg)	Lower Body Performance (kg)	Total Daily Performance (kg)
DC	N	22	22	22
	Mean	2 888.53	9 376.98	12 265.51
	Standard Deviation	546.14	2 130.90	2 524.90
Water	N	22	22	22
	Mean	2 740.39	9 374.39	12 114.77
	Standard Deviation	524.82	1 969.92	2 286.15
Total	N	44	44	44
	Mean	2 814.46	9 375.68	12 190.14
	Standard Deviation	534.60	2 027.99	2 381.54

DC: Dark Chocolate

Table 7 presents performance metrics across interventions for upper body, lower body, and total daily performance. For upper body performance, the mean value for the dark chocolate intervention (2 888.53 kg) is higher than that for the water control (2 740.39 kg). Similarly,

for lower body performance, the mean value for the dark chocolate intervention (9 376.98 kg) is slightly higher than that for the water control (9 374.39 kg).

Table 8: Heart Rate (HF) & BORG Scale

Intervention		HF_PreHIIT	HF_PostHIIT	BORG_PreHIIT	BORG_PostHIIT
DC	N	22	22	22	22
	Mean	85.81	156.45	6.14	16.68
	Standard Deviation	9.62	13.70	0.35	1.42
Water	N	22	22	22	22
	Mean	83.78	153.95	6.05	16.77
	Standard Deviation	12.41	17.35	0.21	1.52
Total	N	44	44	44	44
	Mean	84.80	155.20	6.09	16.73
	Standard Deviation	11.02	15.50	0.29	1.45

DC: Dark Chocolate; HF: Heart Rate; HIIT: High-Intensity Interval Training

Table 8 presents measurements of heart rate variability (HF) and perceived exertion levels (BORG scale) before and after HIIT sessions for both dark chocolate and water interventions. For HF measurements, the mean value at pre-HIIT is 85.81 for dark chocolate and 83.78 for water, indicating slightly higher heart rate variability before HIIT following dark chocolate consumption. At post-HIIT, the mean value for HF is 156.45 for dark chocolate and 153.95 for water, once again suggesting a slightly higher post-exercise heart rate variability after dark chocolate consumption. Similarly, for the BORG scale, the mean value at pre-HIIT is 6.14 for dark chocolate and 6.05 for water, with slightly higher perceived exertion levels before HIIT following dark chocolate consumption. At post-HIIT, the mean value is 16.68 for dark chocolate and 16.77 for water, indicating slightly lower perceived exertion levels after exercise following dark chocolate consumption.

Table 9: Parameter Estimations: Upper Body Performance (kg)

Parameter	Regression Coefficient B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Constant Term	2 740.39	< .001	2 509.95	2 970.83
Intervention = DC	148.15	0.36	-177.75	474.04
Intervention = Water	0[a]			

a. This parameter is set to zero as it is redundant. Dependent Variable: Upper Body Performance (kg)

DC: Dark Chocolate

In the parameter estimations Table 9 for upper body performance, a regression coefficient (B) of 148.15 can be observed for the dark chocolate intervention. This indicates that, on average, after consuming dark chocolate 148.15 kg more weight was moved in the upper body compared to water consumption. However, the significance level for this coefficient is 0.36, indicating that the effect of dark chocolate on upper body performance is not statistically significant ($p > 0.05$).

Table 10: Parameter Estimations: Lower Body Performance (kg)

Parameter	Regression Coefficient B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Constant Term	9 374.39	< .001	8 491.50	10 257.27
Intervention = DC	2.59	1.00	-1 245.99	1 251.18
Intervention = Water	0[a]			

a. This parameter is set to zero as it is redundant. Dependent Variable: Lower Body Performance (kg)

DC: Dark Chocolate

Furthermore, in Table 10 for lower body performance, it can be seen that, on average, 2.59 kg more weight was moved in the lower body after the consumption of dark chocolate compared to the water control. However, once again, a significance level of 1.00 is evident, suggesting that there is no statistically significant effect of dark chocolate on lower body performance ($p > 0.05$).

Table 11: Parameter Estimations: Total Daily Performance (kg)

Parameter	Regression Coefficient B	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
Constant Term	12 114.77	< .001	11 078.51	13 151.04
Intervention = DC	150.74	0.84	-1 314.77	1 616.24
Intervention = Water	0[a]			

a. This parameter is set to zero as it is redundant. Dependent Variable: Total Daily Performance (kg)

DC: Dark Chocolate

Finally, in Table 11 for total daily performance, it is apparent that, on average, there was an increase of 150.74 kg in total weight moved following the dark chocolate intervention compared to the water control. However, with a significance level of 0.84, it is evident that the effect of dark chocolate on total daily performance is not statistically significant ($p > 0.05$).

4 Discussion

The primary outcome of this study, as presented in this master's thesis, reveals that consuming dark chocolate before intense physical exercise does not mitigate exercise-induced ROS formation. Moreover, ROS levels show a slight increase after dark chocolate consumption following intense exercise compared to the water control.

The potential reason for this finding could be explained by the dual nature of polyphenols. Under specific conditions, such as elevated concentrations, high pH levels, and the presence of redox-active transition metals, phenolic compounds can exhibit pro-oxidant properties. This occurs through the generation of a labile aroxil radical or the formation of a labile redox complex with a metal cation. Consequently, this aroxil radical can react with oxygen to produce $O_2^{\bullet -}$, and may additionally create ternary compounds involving DNA, Cu, and flavonoids (León-González et al., 2015). Given that the exact polyphenol content of the dark chocolate utilized in the study of this master's thesis is still yet to be determined, the extent to which polyphenols contributed to this effect remains uncertain.

Furthermore, it is important to note that dark chocolate, used as the intervention, contains calories from various macronutrients, unlike water, which is calorie-free. This disparity in caloric content between the intervention and control may have influenced the observed outcomes.

The mild elevation in ROS levels post-exercise observed in this study following the consumption of 40 g dark chocolate may also be interpreted as beneficial. It is noteworthy that a moderate increase in ROS production during exercise serves a pivotal role in the regulation of signaling pathways that are necessary to promote beneficial physiological adaptations within active skeletal muscles. These adaptations include mitochondrial biogenesis, the synthesis of antioxidant enzymes, and stress protein expression. However, excessively high levels of ROS production can lead to detrimental effects, causing damage to macromolecular structures such as proteins, lipids, and DNA (Powers et al., 2020).

4.1 Previous Literature

The comprehensive narrative review of human studies conducted by Zeng et al. (2021) outlined a limited number of studies that have demonstrated the beneficial antioxidant properties of dark chocolate.

Four studies explored the potential beneficial impact of dark chocolate on humans. Davison et al. (2012) and Allgrove et al. (2011) focused on the influence of dark chocolate consumption on oxidative stress induced by prolonged exercise. Taub et al. (2016) investigated the effects of dark chocolate on exercise capacity in sedentary subjects, while Wiswedel et al. (2004) specifically examined the impact of a flavanol-rich cocoa drink on oxidative stress.

In the studies mentioned, F2-isoprostanes, derived from lipoperoxidation due to oxidative damage, were predominantly used as oxidative markers. Simultaneously, protein carbonylation (PC) served as an indicator of protein damage (Zeng et al., 2021).

To investigate the impact of acute dark chocolate consumption, rich in polyphenols, on plasma antioxidant capacity, oxidative stress markers, and immunoendocrine responses during prolonged exercise, Davison et al. (2012) examined the association between elevated plasma epicatechin levels and F2-isoprostanes. Fourteen healthy men participated in a randomized-counterbalanced design, cycling for 2.5 hours at approximately 60% maximal oxygen uptake (VO_2 max), two hours after consuming 100 g of dark chocolate, an isomacronutrient control bar, or neither. Blood samples were assessed before and immediately after exercise. Dark chocolate exhibited a pre-exercise enhancement in antioxidant status and, compared to the isomacronutrient control bar, showed a trending reduction in plasma free F2-isoprostane levels one hour post-exercise. Furthermore, F2-isoprostane levels increased post-exercise in the isomacronutrient control bar and baseline trials but not in the dark chocolate trials. In addition, minimal effects on immunoendocrine responses were observed with dark chocolate. These findings demonstrate that acute dark chocolate consumption may influence antioxidant status and oxidative stress responses during prolonged exercise (Davison et al., 2012).

Similarly, this next study aimed to identify the acute antioxidant effects after pre-exercise consumption of a flavanol-rich cocoa drink (Wiswedel et al., 2004). To assess whether flavan-

3-ols found in cocoa and chocolate beverages can mitigate an increase in plasma oxidative stress markers, Wiswedel et al. (2004) compared the impact of a low flavanol and a high-flavanol cocoa drink on the plasma concentrations of F2-isoprostanes in healthy male subjects, both at rest and during physical exercise through cycling for 29 minutes. The study involved 20 non-smoking male volunteers, aged 20 to 40 years, primarily students between 20 and 25 years old, with limited endurance training experience. Employing a comparative randomized double-blind crossover design, the participants ingested either a high-flavanol cocoa drink (HFCD; 187 mg flavan-3-ols/100 ml) or a low-flavanol cocoa drink (LFCD; 14 mg/100 ml). In a subset of 10 individuals, the treatments were combined with strenuous physical exercise. The low-flavanol cocoa drink led to a slight increase in mean plasma F2-isoprostane concentrations two and four hours after ingestion, potentially attributed to postprandial oxidative stress. In contrast, this increase was not observed with the high-flavanol cocoa drink. Notably, the disparity in F2-isoprostane levels two and four hours after consumption of the high-flavanol cocoa drink versus the low-flavanol cocoa drink became statistically significant when combined with physical exercise. The study's findings suggest that dietary flavanols, exemplified by cocoa drinks, have the potential to reduce plasma levels of F2-isoprostanes, serving as indicators of *in vivo* lipid peroxidation (Wiswedel et al., 2004).

In contrast, Allgrove et al. (2011) explored the impact of regular intake of dark chocolate, high in polyphenol content, on plasma metabolites, hormones, and markers of oxidative stress following prolonged exhaustive exercise. Twenty physically active men participated in a cycling regimen, maintaining 60% VO_2 max for 1.5 hours, with intensity peaking at 90% VO_2 max for a 30-second interval every 10 minutes, culminating in a ride to exhaustion at 90% VO_2 max. In the two weeks preceding exercise, participants followed a randomized, counterbalanced, crossover design, consuming either 40 g of dark chocolate or an isocarbohydrate-fat control cocoa liquor-free chocolate twice daily and once two hours before exercise. Venous blood samples were collected at distinct time points: immediately before exercise, post-exercise (fixed duration), post-exhaustion, and after a one-hour recovery period. This study discovered a significant decrease in F2-isoprostanes at exhaustion and after one hour of recovery with dark chocolate. Additionally, oxidized LDLs exhibited a significant reduction with dark chocolate both before and after exercise, as well

as at the point of exhaustion. However, changes in circulating interleukin (IL)-6, IL-10, and IL-1ra, which served as inflammation markers, remained unaffected by the treatment. Time to exhaustion at 90% VO_2 max did not exhibit a significant difference between trials, and no noticeable effects on exercise performance were observed with dark chocolate. Collectively, these findings suggest that regular dark chocolate consumption is linked to a reduction in oxidative stress markers following exercise (Allgrove et al., 2011).

In a different approach, Taub et al. (2016) delved into the mechanisms responsible for the prolonged antioxidant effects of dark chocolate, employing human muscle samples as the main focus. This study aimed to evaluate the impact of (-)-Epi-rich dark chocolate on exercise capacity in middle-aged, sedentary individuals. The investigation focused on examining the underlying mechanisms, specifically targeting upstream metabolic control systems, mitochondrial density, function, and oxidative stress endpoints. The study involved twenty sedentary subjects aged 40 to 75, predominantly around 50 years old, randomized into placebo or dark chocolate groups. Participants consumed 20 g of the assigned products daily for three months, totaling approximately 100 calories per day. Assessments before and after treatment included bicycle ergometry for VO_2 max and work capacity, skeletal muscle biopsy from *quadriceps femoris* to evaluate changes in mitochondrial density, function, and oxidative stress, and blood sampling for metabolic endpoints. Results indicated that the dark chocolate group exhibited increased VO_2 max and maximum work capacity, unlike the placebo group. Furthermore, the dark chocolate group showed elevated HDL levels and reduced triglycerides. In the skeletal muscle of the dark chocolate group, significant increases were observed in protein levels for liver kinase B1 (LKB1), adenosine monophosphate (AMP)-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), along with their active forms (phosphorylated AMPK and LKB1), and citrated synthase (CS) activity. However, there were no changes in mitochondrial density. Post-exercise, the dark chocolate group displayed significant reductions in PC and increased GSH levels in skeletal muscle. The improvements in maximum work achieved and VO_2 max observed in the dark chocolate group can be attributed to the activation of upstream control systems and the enhancement of skeletal muscle mitochondria efficiency (Taub et al., 2016).

However, contrary to these findings, the study of this master's thesis did not observe a decrease in oxidative stress markers, in this case ROS production, following exercise. To investigate potential explanations for this finding, specific elements within the study design that might have contributed to this outcome will be analyzed in the following section.

4.2 Comparison of Study Designs

To begin, it is evident that the studies from prior literature, including the study examined in this thesis - Gassner (2023), featured diverse study designs. From participant selection to the type and quantity of the dark chocolate intervention, as well as the nature of the training interventions, these studies exhibited heterogeneous designs. This diversity makes it challenging to pinpoint a specific aspect of the study that could have contributed to the discrepancies in our findings. Therefore, it is important to examine and compare various elements of the designs (Table 12).

Table 12: Effects of Dark Chocolate on Exercise-Induced Oxidative Stress: Study Comparison

Study	Type of Dark Chocolate	Amount Consumed	Nutritional Protocol	Type and Intensity of Exercise
Gassner (2023)	DC 70% cacao	40 g	DC vs. water, 2 h before exercise	Body weight HIIT, 30 min
Davison et al. (2012)	DC (39.1 mg catechin, 96.8 mg epicatechin)	100 g	DC vs. cocoa-liquor-free control bar vs. neither, 2 h before exercise	Cycling, 2.5 h
Wiswedel et al. (2004)	HFCD (187 mg flavan-3-ols/100 ml)	100 ml	HFCD vs. LFCD, 2 h before exercise	Cycling, 29 min
Allgrove et al. (2011)	DC 70% cocoa	40 g	DC vs. isocarbohydrate-fat control cocoa-liquor-free chocolate, twice/d, 2 weeks	Cycling for 90 min followed by 25 min exhaustion time trial
Taub et al. (2016)	HFDC (175.2 mg cocoa flavanols)	20 g	HFDC vs. LFDC, once/d, 3 months	Ramped exercise on stationary bicycle, ~10 min

Study	Detection Method			
	ROS Production	**Oxidative Stress Marker**	**Inflammation Marker**	**Antioxidant Activity**
Gassner (2023)	ROS	N/A	N/A	N/A
Davison et al. (2012)	N/A	F2-isoprostane	Circulating leucocyte, IL-6	N/A
Wiswedel et al. (2004)	N/A	F2-isoprostane	N/A	N/A
Allgrove et al. (2011)	N/A	F2-isoprostane	Circulating leucocyte, IL-6, IL-10, IL-1Ra	N/A
Taub et al. (2016)	N/A	PC	N/A	GSH/GSSH

DC: Dark Chocolate; GSH/GSSH: Glutathione/Oxidized Glutathione; HFCD: High-Flavanol Cocoa Drink; HFDC: High-Flavanol Dark Chocolate; HIIT: High Intensity Interval Training; IL-6: Interleukin-6; IL-10: Interleukin-10; IL-1Ra: Interleukin-1 receptor antagonist; LFCD: Low-Flavanol Cocoa Drink; LFDC: Low-Flavanol Dark Chocolate; PC: Protein Carbonyls; ROS: Reactive Oxygen Species

(Adapted from Zeng et al., 2021)

Analyzing the studies that demonstrated a reduction in oxidative stress markers, we can first compare the type of dark chocolate used as an intervention. It is worth noting that a limiting factor in comparing Gassner's study (2023) with others is that the exact polyphenol content of the dark chocolate used is currently unknown and will be determined at a later stage. Gassner (2023) employed 40 g of 70% cacao dark chocolate, a choice similar to that of Allgrove et al. (2011), who also utilized 40 g of 70% chocolate. On the other hand, Davison et al. (2012) opted for 100 g of dark chocolate with a composition of 39.1 mg catechin, 96.8 mg epicatechin, 58.4 mg dimer B2, 7.3 mg dimer B5, 34.7 mg trimer C, and 10.5 mg tetramer D. In contrast, Wiswedel et al. (2004) utilized 100 mL of a high-flavanol cocoa drink containing 187 mg flavan-3-ols per 100 mL. Finally, Taub et al. (2016) selected a high-flavanol dark chocolate with a total of 175.2 mg cocoa flavanols amounting to 20 g, including 30.6 mg monomers (-)-epicatechin (26 mg) and (+)-catechin (4.6 mg), 128 mg Theobromine, and 13 mg Caffeine.

What makes caffeine intriguing is its dual role as both a free radical scavenger and an agent with pro-oxidant potential. It is noteworthy that caffeine demonstrates antioxidant effects at low concentrations, whereas at higher concentrations, it may enhance cytotoxicity, thereby promoting the generation of ROS (Ko et al., 2020). One possibility to consider is that the dark chocolate utilized in Gassner's study (2023) might have contained a high concentration of caffeine, potentially contributing to the observed increase in ROS levels post-exercise.

The frequency and duration of the dietary intervention are crucial to consider. While Gassner (2023) implemented acute consumption, it is noteworthy that both Davison et al. (2012) and Wiswedel et al. (2004) also adopted acute consumption before exercise. However, Gassner (2023) stands out as the only study employing a smaller acute amount. In contrast, the other studies implementing acute consumption utilized larger quantities of the intervention food: Davison et al. (2012) employed 100 g of dark chocolate, and Wiswedel et al. (2004) used 100 mL of the cocoa drink.

Gassner (2023) administered 40 g of dark chocolate once, two hours before exercise. This approach of a smaller, single acute dosage might have contributed to the observed lack of a decrease in ROS production post-exercise. In contrast, Allgrove et al. (2011) also utilized 40 g of dark chocolate, but the intake was spread twice daily over two weeks preceding exercise.

The extended duration of consumption suggests the potential for a cumulative effect, in contrast to the acute consumption in Gassner (2023). A similar pattern is evident in the study by Taub et al. (2016), where participants consumed a smaller amount (20 g of dark chocolate) once daily for three months. In this study, prolonged consumption resulted in decreased PC and increased GSH levels (Taub et al., 2016).

Generally, due to their complicated structures and high molecular weights, dietary polyphenols have low bioavailability and limited absorption in the small intestine. Approximately 90% of dietary polyphenols remain intact upon reaching the large intestine, where they undergo biotransformation by the gut microbiota, converting into bioactive, low-molecular-weight phenolic metabolites (Wang et al., 2022). Subsequently, these metabolites are absorbed into the portal vein, where they can be transported to peripheral tissues attached to albumin, or excreted via the kidneys in urine. However, the absorption and bioavailability of polyphenols depend on various factors, including age, sex, BMI, physical activity level (PAL), smoking status, food processing, storage conditions, chemical structure of the polyphenols, and the presence of specific gut microbiota (Clarke et al., 2020). Given the unknown polyphenol content and chemical structures of the dark chocolate used in Gassner's study (2023), as well as the undetermined gut microbial composition of the participants, drawing conclusions about factors affecting their absorption rate would be difficult.

Additionally, considering the variability in the half-life of urinary polyphenol metabolites, ranging from one to 24 hours, with each metabolite exhibiting differing times to reach maximum concentration (Clarke et al., 2020), it is plausible that the dark chocolate employed in Gassner's study (2023) may have contained polyphenols with longer times to maximum concentration. Consequently, these polyphenols might not have reached sufficient concentrations in the bloodstream within the two-hour window between consumption and the commencement of the HIIT circuit, potentially leading to inadequate effects in counteracting exercise-induced ROS production.

Regular moderate training is generally associated with beneficial effects on oxidative stress and overall health. In contrast, acute exercise results in increased oxidative stress (Pingitore et al., 2015). For this reason, all four previously mentioned studies, along with Gassner (2023), implemented acute exercise to induce oxidative stress. However, only Gassner (2023)

incorporated resistance training, specifically a HIIT strength training circuit, while the four studies from previous literature employed endurance training, cycling to be precise, albeit with varying durations and intensities.

Although acute bouts of both aerobic and anaerobic exercises, such as resistance and endurance training, have been reported to cause a state of oxidative stress, the extent of oxidation depends on factors like exercise mode, intensity, and duration (Bloomer, 2008). Considering this, it is possible that the HIIT strength training in Gassner (2023) induced a more pronounced oxidative response compared to the endurance training in the other studies. Moreover, Gassner (2023) stands out as the only study that applied a smaller acute amount of dietary intervention, which might have been insufficient to counteract the increase in oxidative stress post-exercise.

Interestingly, successful reduction of post-HIIT strength training oxidation markers has previously been reported with dietary interventions. As Zeng et al. (2020) discovered, acute pre-HIIT consumption of oatmeal reduces ROS production after exercise in women. However, it is important to consider the composition of oatmeal in this context, particularly the presence of avenanthramides (AVA), which are not a common component in cocoa-based products. AVAs, a group of di-phenolic compounds found exclusively in oats, have previously demonstrated strong antioxidant and anti-inflammatory effects in human studies (Zhang et al., 2020). This distinction suggests that the efficacy of mitigating ROS production post-HIIT in Zeng et al.'s (2020) study could be attributed to the presence of AVAs in oatmeal, or conversely, the lack of AVAs in dark chocolate may have contributed to the unsuccessful mitigation of ROS production in the study of Gassner (2023).

Presumably, the acute consumption of 70% dark chocolate in the quantity of 40 g might not have exerted a strong enough antioxidant effect to counteract ROS production induced by HIIT strength training. Adjusting the intensity and duration of the training intervention is also valuable to consider for future research. Applying the same acute amount of 40 g 70% dark chocolate in combination with a HIIT circuit of different duration or intensity would be interesting to explore in subsequent studies. More research, particularly with different adjusted quantities of dark chocolate in combination with resistance training, is needed to draw definitive conclusions about the antioxidant properties of dark chocolate in response to this type of anaerobic exercise.

4.3 Limitations

It is important to mention that this study has some limitations. Firstly, the participant selection was restricted to young female individuals. Previous research has highlighted gender-related differences in oxidative stress, indicating that, under normal physiological conditions, females generally tend to be less susceptible to oxidative stress compared to males. This phenomenon may be attributed to the antioxidant properties of estrogen (Kander et al., 2017). Specifically 17beta-estradiol has been shown to reduce angiotensin II-induced free radical production in vascular smooth muscle cells as well as upregulate the expression and enzyme activity of MnSOD and extracellular superoxide dismutase (ecSOD) (Strehlow et al., 2003). For this reason, including only young and premenopausal female participants may compromise the validity of the results regarding the true antioxidant effects of dark chocolate. However, at a later stage of the overall research project, data that is specifically relevant to women, such as hormone status and menstrual cycle, will also be statistically taken into account to increase the validity of the results. Given the observed differences in oxidative stress between genders and the influence of other factors on ROS production such as age and training status, the applicability of the study's findings to other populations may be limited.

Another limitation of this study is the potential influence of other dietary antioxidants on participants' responses to oxidative stress post-exercise. The participants were not provided with a specific dietary protocol or instructions regarding food restrictions but were rather encouraged to maintain their regular dietary habits throughout the intervention period. While participants were questioned about their 24-hour dietary recall on the morning of each intervention day, inquiries about their food intake over an extended period were not conducted. Consequently, the types of foods they might have consumed in the days preceding the intervention or habitually over a more extended period could have impacted their oxidative stress response. These dietary elements may have included fruits and vegetables rich in antioxidants such as vitamin E, vitamin C, and pro-vitamin A, as well as foods containing oxidized oils, fats, and smoked meats that can trigger the generation of free radicals. This variability in dietary factors could potentially compromise the validity of the study results. To address this issue, future studies could consider implementing a

standardized dietary protocol for participants during the intervention to ensure consistent food intake conditions and establish a uniform baseline for all individuals.

Finally, dark chocolate, used as the intervention, contains calories from various macronutrients, whereas water, serving as the control, has no caloric value. This difference in caloric content between the intervention and control could have potentially influenced the observed results. Furthermore, the exact polyphenol composition of the dark chocolate utilized in this study is not available at the time of writing this master's thesis and will be determined at a later stage of the research project. Therefore, this limitation restricts drawing conclusions about the effects of specific components of the dark chocolate that may have contributed to the findings regarding exercise-induced ROS production in this study.

5 Conclusion

This Master's thesis aimed to investigate the acute effects of pre-exercise dark chocolate consumption on exercise-induced oxidative stress in healthy female adults. Twenty-two participants underwent a randomized controlled intervention study, where they consumed 40 g of dark chocolate before engaging in a HIIT strength training circuit. As acute intense exercise is known to increase oxidative stress, the HIIT circuit was selected to induce ROS production, which was measured using electron paramagnetic resonance (EPR) spectroscopy. Data analysis was conducted using SPSS 27.0, revealing that despite the antioxidant properties of dark chocolate, its acute consumption before HIIT did not mitigate exercise-induced ROS formation compared to a water control. In fact, it appeared to elevate ROS levels, however this elevation was not statistically significant. Several factors may have contributed to these findings, including the type and quantity of dark chocolate used, the frequency and duration of the dietary intervention, as well as the type and intensity of the training regimen. Considering that a moderate increase in ROS production during exercise plays an important role in promoting positive physiological adaptations within active skeletal muscles, these findings may also be seen as beneficial. Nevertheless, certain limitations in this study, such as the limited diversity among participants and the lack of a standardized dietary protocol during the intervention, may have influenced the results. Overall, the findings in this thesis suggest that acute consumption of 40 g of dark chocolate may not effectively mitigate exercise-induced oxidative stress in healthy women. However, further investigations, utilizing different quantities of dark chocolate in combination with HIIT, are warranted to draw definitive conclusions regarding its antioxidant properties in response to this type of exercise. In conclusion, understanding the interplay between dietary antioxidants and ROS production during exercise is essential for optimizing strategies to promote redox homeostasis and overall health. Therefore, while dark chocolate remains a promising dietary source of antioxidants, its acute effects on exercise-induced oxidative stress require more research for better understanding and practical application.

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