University of Nevada, Reno

**Use of Saliva Biomarkers to Monitor Efficacy of Vitamin C**

**in Exercise-Induced Oxidative Stress**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Nutrition

by

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MASTER OF SCIENCE

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**Abstract**

Saliva is an appealing biospecimen and can be easily obtained for health research. A popular discussion in health research is oxidative stress. Systemic biomarkers that represent oxidative stress and antioxidant status such as malondialdehyde and vitamin C can be found in saliva. It is unclear, however, if saliva is an accurate biospecimen as is blood and plasma for systemically monitoring such biomarkers. Exercise can induce oxidative stress, resulting in a trend of antioxidant supplementation to combat its assumed detriments. Vitamin C is a popular antioxidant supplement in the realm of sports and exercise but its role in the area is unconfirmed. One potential avenue for evaluating exercise-induced oxidative stress is through assessment of biomarkers like vitamin C, thiobarbituric acid reactive substances (TBARS), and malondialdehyde (MDA) in saliva.

Salivary vitamin C was examined before and after a vitamin C supplementation period of four weeks. Oxidative stress was induced via resistance exercise. Free malondialdehyde and TBARS were used as the salivary biomarkers to assess oxidative stress before and after exercise and vitamin C supplementation. Peak muscular force was measured before and after the supplementation period to examine any exercise performance enhancement. Salivary vitamin C increased after the supplementation period along with post-resistance exercise. Free malondialdehyde in saliva increased after resistance exercise and was reduced after the supplementation period; similar results have been experienced with systemic biomarker examination. An increase in peak muscular force was experienced after the supplementation period. Saliva could potentially be used to assess vitamin C status and exercise-induced oxidative stress. Further, vitamin C may be a potentially beneficial supplement for resistance exercise and warrants further research.

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# 1    Introduction

Few studies have used saliva as the biospecimen to assess exercise-induced oxidative stress. Fewer studies have experimented with supplementing vitamin C for anaerobic-exercise performance enhancement. The following experiments were conducted to examine vitamin C and oxidative stress biomarkers in saliva. Resistance exercise was used to induce oxidative stress; salivary malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) were used to examine the possibility of oxidative stress. Vitamin C was supplemented in human participants and exercise performance was examined via peak muscular force.

Free radicals and their potentially destructive nature were recognized in a laboratory as early as 1900 [1]. A free radical can be defined as a compound that has one or more unpaired electrons in its orbital and is capable of finite existence [2, 3]. Free radicals have extremely high chemical reactivity, which can in turn cause damage to cells. Moses Gomberg and other researchers investigating carbons and hydrocarbons acknowledged that oxygen derivatives were causing reactions within their experiments [1]. "Oxidation" was termed as the environment's oxygen as it bound with chemical constituents. Since this century-old occurrence, studies have continued to examine oxidation, its properties, and how it can affect a multitude of health-related outcomes. The adverse effects of oxidation and free radicals were hypothesized by the mid-1900s and further examined by Gerschman et al. [4], who compared and identified the dangers of oxidation and radiation. Their findings led to future research more closely examining free radicals and consequences associated with their presence. It should be noted that the generation of free radicals is not always negative.  A number of reactions essential to life, such

as the generation of phagocytic cells to kill invading pathogens, along with those related to intercellular and intracellular signaling, are important beneficial effects [2].

Free radial species include reactive oxygen species (ROS), reactive nitrogen species (RNS), and a variety of compounds that can become reactive from surrounding free radicals; such compounds can include carbons, carbonyls, lipids, proteins, sulfur, halogen, nitrogen, and nucleotides [5-13]. It can be argued that with time, individual organisms are exposed to more and more oxidizing elements resulting in more free radicals. These oxidizing elements are found in everyday life. Theoretically speaking, this would cause an accumulation of free radical damage needing to be repaired in order to avoid the correlated dangers of such instances. Antioxidants can reduce free radicals to less-harmful compounds at the expense of such antioxidants, which in turn become oxidized. One factor recognized as causing increased oxidation is exercise, known as exercise-induced oxidative stress. Davies was one of the first who witnessed exercise-induced oxidative stress as exhaustive exercise increased free radical production [14]. This has led to an ongoing area of research.

Biomarkers of oxidative stress have been a major source of debate related to monitoring oxidative stress and the possible resulting damage. Biomarkers of oxidative stress have been measured in plasma, whole blood, urine, and in respired gases [2]. One possible biospecimen that is still requiring investigation is saliva, as it has the potential to be utilized for measuring a variety of biomarkers in relation to antioxidants and oxidative stress. Saliva is potentially a novel biospecimen for a number of reasons.

Because indices for oxidative stress increase during and possibly from exercise, dietary supplements are often used to counter such negative effects as soreness and fatigue.

Furthermore, dietary supplements are often used to enhance exercise performance. Vitamin C is

an antioxidant that has been used as a dietary supplement to mitigate exercise-induced

oxidative stress, the negative results of unchecked free radicals, and the possible performance

enhancement that might come of the vitamin's consumption.

## 2    Literature Review

The following literature review has been submitted to the journal of *Antioxidants.*

### 2.1    <u>Saliva</u>

2.1.1    Basics of Saliva

Saliva is a fluid found in the oral cavity that has potential to identify compounds under

different diagnostic conditions in health, disease, and oxidative stress. It is comprised mostly of

water but also contains a variety of organic and inorganic components such as proteins,

enzymes, electrolytes, immunoglobulins, hormones, and micronutrients such as vitamin C [15-

20]. These components are essentially blood-based and permeate to the saliva due to different

capillaries, acinar cells, and ductal cells [21]. Saliva is secreted into the oral cavity via major and

minor glands with stimulation by the medulla [22]. The three major glands are the parotid gland,

the sublingual gland, and the submandibular gland; the minor glands are the labial gland, the

buccal gland, the lingual gland, and the palatal gland [23, 24]. Each individual gland produces

different amounts and flow rates of saliva with different compositions of its components at

different times of the day. Such amounts and flow rates can also depend on if it is stimulated or

unstimulated [20]. Some main functions of salivary components include the breakdown of local

bacteria, digestion of carbohydrates and lipids, food lubrication, taste, and oral health [25-27].

An average individual can produce between 1 to 1.5 liters of saliva per day [28] making it a very opportune biospecimen in multiple sampling. Furthermore, saliva requires little to no safety-training for extraction and is a noninvasive alternative to blood and serum sample procedures. While it has been seen that some systemic biomarkers in health correlate well between saliva and blood in diagnosis (i.e. HIV [29], hepatitis [30], oral cancer [31], periodontal disease [32, 33], and obesity [34]), saliva is not always the best biospecimen in that it does not always fully represent the systemic concentration such as with amylase, some proteomes, and phosphate [35].

2.1.2    Biomarkers of Oxidative Stress in Saliva

Technologies have advanced over the last century as well as the accuracy to identify salivary components. With such advancement, biomarkers, such as; malondialdehyde (MDA), 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), thiobarbituric acid-reacting substances (TBARS), total antioxidant capacity (TAC), and protein carbonyls, have been identified in the saliva. These biomarkers have been used in research studies focusing on oxidative stress and its association with health and disease. Malondialdehyde has been used as a biomarker in recurrent aphthous ulceration (RAU) research with both serum and saliva used as a biospecimen [36, 37]. Diabetes mellitus has also been studied with malondialdehyde as a biomarker of oxidative stress in both serum and saliva despite lacking statistical significance in the control and experimental groups' levels [38]. Therefore, saliva has been and can be used successfully in monitoring oxidative stress.

Saliva has been utilized to monitor oxidative stress via resistance exercise in the past [39]. These results suggest that resistance training can induce oxidative stress. TBARS was not

significantly increased in the saliva after resistance training but was so in the plasma. The bench

press, cable pull down, overhead press, leg extension, leg flexion, and leg press were the

exercises used. Three sets of 10 repetitions for each exercise were performed by the

participants with the weight determined by the maximal effort sets. It is possible the change in

salivary TBARS depends on the intensity of the resistance-exercise bout.

## 2.2     **Oxidative Stress**

### 2.2.1    Basic Concepts of Oxidative Stress

Reduction and oxidation reactions, or redox reactions, involve the process of electron

transport between molecular orbitals. When a compound is reduced, it gains an electron in its

orbital for stabilization; alternatively, when a compound is oxidized, it loses an electron from its

orbital. Oxidization usually leads to an unpaired electron, turning it into a free radical or a

compound known as a reactive species. Redox reactions are coupled in systems such as

biological organisms. Normally, these are balanced, and otherwise healthy, unless some sort of

"stress" should cause an imbalance between reduction and oxidation. The imbalance is usually

skewed towards oxidation and is defined as oxidative stress; that is, a buildup of reactive

species/free radicals (such as reactive oxygen species or reactive nitrogen species) which are not

being reduced at an equal pace by antioxidants and leading to potential damage [40].

It should be recognized that oxygen is one of the more toxic chemicals in oxidative

stress when not reduced. Oxygen is, however, a necessity for a wide range of organisms, being

the final acceptor in electron transportation during ATP synthesis. Because of oxygen's

molecular structure, it is very capable of free radical formation and contributes highly to

reactive oxygen species as a whole. This is interesting, given not only its biological necessity, but

also given it is the most abundant chemical in the Earth's atmosphere at 21% [41, 42]. Exposure to high levels of oxygen or to anything that can lead to toxic levels of oxygen that cannot be reduced is known to be detrimental to organisms such as plants, animals, and bacteria [43-45]. To combat oxygen toxicity and its role in free radical formation, such organisms require antioxidant defenses from both endogenous and exogenous sources. Humans are especially susceptible to high levels of oxygen exposure in the lungs because of their physiological function [46], but other tissues are capable of high levels as well. Oxidative stress and its relative damage can therefore be present systemically in humans.

The simplest free radical is atomic hydrogen. Free radicals can be highly reactive to other components and compounds in that they search for electrons to pair with their unpaired electrons. Such electrons often come from nucleotides, lipids, proteins, and/or other molecules throughout the surrounding system that are paramagnetic. This process, in turn, oxidizes these substances, potentially resulting in an array of problems such as mutation and dysfunction. The superoxide radical is one of the more common free radicals; it can either oxidize substances or create other free radicals [2]. Other known free radicals are hydroperoxyl, hydroxyl, peroxyl, alkoxyl, carbonate, carbon dioxide, $O_2$ and singlet oxygen [47]. Some compounds are considered non-radicals but have still been associated with oxidative stress, such as hydrogen peroxide, peroxynitrite, peroxynitrous acid, nitrosoperoxycarbonate, hypochlorous acid, hypobromous acid, ozone, and singlet oxygen. Nitrogen, chlorine, bromine, and sulfur have also been shown to produce their own versions of reactive species [48]. Free radicals can be formed under a variety of different circumstances including cell/tissue injury [49] and exercise.

2.2.2    Health, Disease, and Disorders Associated with Oxidative Stress

Based on the existing research on free radicals and oxidative stress and their potential

association with negative outcomes, it is easy to misinterpret the available information and

conclude that these are unhealthy and (at times) detrimental compounds. Possible cell death

can occur via oxidative stress and free radical exposure. The list of clinical conditions associated

with oxidative stress and free radicals is exhaustive; however, whether they are causative for

these conditions or simply byproducts is not yet established. Some examples of research

involving health conditions and their associations with free radicals are described below:

Porphyria is a condition in humans where heme's biosynthesis is abnormal in such a way

that leads to skin and tissue damage in severe cases, and often, a buildup of free

radicals [50, 51]. Atherosclerosis and cardiovascular disease are two of the leading

causes of death worldwide [52]. Inflammatory and oxidative biomarkers (C-reactive

protein and myeloperoxidase) may accompany these conditions (and therefore are

associated) [53, 54]. Diabetes (among the 10 leading causes of death in the United

States [55]) has been induced through introduction of oxidizing-toxins in laboratory

conditions [56, 57]. Furthermore, increases in systemic markers of oxidative stress have

been associated with diabetes [58, 59]. Reactive species and compounds that can

generate reactive species have been shown to generate more rapidly during times of

carcinogenesis and are increased in tumors [60, 61]; cancer is another condition among

the leading causes of death in the US [55]. Research has established that free radical

production plays a role in aging. Researchers are working to establish the potential

relation between varying free radical and antioxidant concentrations as they relate to

cell life and longevity [62-65].

Oxidation and free radical production cannot be avoided entirely, as organisms are both exposed to them from exogenous sources and through endogenous production. Simple things like air pollution, physical activity, inactivity, food, obesity, and UV-light exposure can cause oxidative damage. Furthermore, things like the mitochondria and phagocytes within the body can cause free radical production [66, 67]. Production of antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) occurs endogenously to maintain a balanced redox system [68]; Mn-SOD is endogenous in the mitochondria while Cu-Zn SOD is in the cytoplasm, also endogenous. It has also been established that in certain biochemical/cellular situations, free radicals and reactive species are necessary for development and function such as in cell growth/proliferation, cell signaling, gene activation, and gene messaging just to name a few [69, 70].

2.2.3    The Relationship between Inflammation and Oxidative Stress

Pathogens, trauma, and infections are common offenders which can cause the body to become inflamed. Inflammation is the mechanism used to restrict any sort of possible spreading, remove the debris from the damage, and repair the damaged area caused by the offender (71). The first response to an offending agent such as a pathogen is to quickly mobilize defending leukocytes (such as monocytes and neutrophils) by vasodilation via cytokines. Cytokines such as interleukins and tumor necrosis factors will formulate to signal surrounding sensors to begin the necessary mechanisms for repair and restoration (72); these are common inflammatory biomarkers. The next response is for leukocytes and monocytes to engulf debris that might result from the damage. Neutrophils will also catalyze a reaction known as a respiratory burst in order to neutralize the offender. Superoxide radical, hydrogen peroxide, and hypochlorite are the neutralizing compounds produced by the respiratory burst (73, 74); these

are also reactive and non-reactive species of oxidative stress. The final response of inflammation includes cleanup by monocytes (and eventually macrophages) via phagocytosis (75) and then repair. The known signs of inflammation include redness, swelling, and pain. Inflammation and the aforementioned process are normal in acute situations, much like redox reactions.

It is when chronic inflammation and its mechanism arise, so do dangerous problems. The acute inflammatory response differs from the chronic inflammatory response. Cytokines such as interleukins and tumor necrosis factors tend to overproduce possibly due to an over-abundance of adipose tissue (76). Metabolic functions will then be disturbed. A variety of diseases have been linked to inflammation from this including obesity and diabetes (77), cardiovascular disease (78), and cancer (79).

Both acute inflammation and chronic inflammation will affect, and be affected by, redox components. It has been suggested reactive species (especially 8OHdG) damaging DNA might be the main culprit in developing metabolic diseases such as cancer (80). Cancer tumors exhibit an inflammatory response via increases in cytokines such as NF-κB and TNFα (2), as do situations in cardiovascular disease, diabetes, and hypertension with reactive species being possible initiators (81). Oxidative stress might even be a secondary process to chronic inflammation (82). Production of reactive species via inflammatory response (such as superoxide radical, hydrogen peroxide, and hypochlorite) will only exacerbate with chronic inflammation. Myeloperoxidase is another oxidizing agent linked to neurodegenerative and vascular diseases (83, 84). This has begun a trend in researching antioxidants as therapeutic remedies for these serious metabolic diseases (85, 86).

2.2.4    Exercise-induced Oxidative Stress

Exercise can be very beneficial to health [87-89], but may also produce dangerous compounds. Davies was able to measure free radicals in isolated rat muscles via electron paramagnetic resonance following an endurance-exercise bout. Exercise intensity increased with time and resulted in an increase in free radicals [14]. Originally, researchers hypothesized that exercise could produce free radicals in one of two ways: the electron transport chain or ischemia-reperfusion.

The electron transport chain is a biological mechanism that occurs in the mitochondria. As the name implies, electrons are transported down a gradient via a series of redox reactions in order to generate the main energy source of adenosine triphosphate (ATP) in mammalian-biological systems. As mentioned earlier, oxygen is used as the final electron acceptor within this mechanism and can be sourced from respiration. Electrons can be "leaked" from this gradient leaving oxygen unpaired and oxidized into the reactive oxygen species, superoxide, or other free radicals [90]. During exercise, respiration can intensify as a direct response to activity levels, resulting in increased electron transport chain activity. Ultimately, this increase in exercise intensity may lead to more free radical production because of increased oxygen consumption, especially in the lungs [91, 92]. This oxygen increase is thought to increase approximately 10-20-fold throughout the whole body [93] and 100-200-fold within the isolated, involving muscles [94]. Only about 0.15% of this oxygen, however, can be used to produce a reactive species [95]. Complex III of the mitochondria is required for signaling via ROS [2].

Another process that can introduce free radical production is ischemia-reperfusion. When working muscles undergo exercise intensities that meet maximal levels of oxygen

consumption ($VO_{2max}$), their oxygen concentrations decrease. During the post-exercise period, these muscles will experience a rapid increase in oxygen to make up for the lower levels experienced during the $VO_{2max}$ period. At this time, reactive species are produced [96, 97]. If antioxidant defense systems are not capable of reducing such compounds to balance the redox-reaction's equation, oxidative damage can occur.

It would appear at first that regular exercisers and athletes could be at risk of oxidative damage from exercise-induced free radical influx, especially at intense levels and increased duration [98-101]. It could be argued that regular exercisers and athletes adapt to such an influx over time, making them less susceptible to oxidative damage; individuals who do not exercise regularly experience oxidative damage but can become trained and develop a resistance to such damage [102]. The theorized mechanisms explaining why regular exercise improves adaption to oxidative stress include the upregulation of nuclear factor kappa beta (NF-κB) and of peroxisome proliferator-activated receptor gamma coactivator (PGC-1α). It appears both compounds depend on reactive species for activation of their signaling pathways that essentially lead to gene expression, phosphorylation, cell growth, and adaptations in the muscle potentially associated with improvements in exercise and sport performance [103-107]. Most research in exercise-induced oxidative stress has focused on aerobic exercise (e.g. running and bicycling), since oxygen is a major contributor to the synthesis of a reactive species. There is less research, however, regarding anaerobic exercise in the pool of exercise-induced oxidative stress.

2.2.5    Analyzing Exercise-induced Oxidative Stress

One challenge in researching exercise-induced oxidative stress and oxidative stress in general, is deciding which biomarker is most appropriate and if this biomarker is causing

substantial oxidative damage to the organism. It can also be difficult to choose which

biospecimen (e.g. blood and saliva) to use for biomarker measurement. Blood is the most

common for exercise-induced oxidative stress and biomarker extraction. Biomarkers present in

blood include protein carbonyls [108-110], total antioxidant capacity [111, 112], $F_2$-isoprostanes

[113, 114], MDA [115, 116], and TBARS [117, 118]. The presence of such blood biomarkers does

not necessarily indicate cellular/tissue damage in the surrounding areas; such research in this

area is thus warranted. MDA can be considered, however, a secondary compound to tissue

damage that will continue destroying surrounding materials [2]. Free MDA has been proposed

as the preferred method to assess lipid peroxidation status due to its direct relation to oxidative

stress and damage along with its specificity. TBARS is often used to assess lipid peroxidation in

said situations despite its lack of specificity and sensitivity.

It has been proposed that saliva could be potentially useful for measuring exercise-

induced oxidative stress [119, 120], but research in this area is limited. The TBARS assay, MDA,

advanced oxidation protein products, along with the ferrous oxidation-xylenol orange assay,

which measures all peroxides but can be altered for lipid-peroxidation specificity, have been

used as biomarkers of oxidative stress in anaerobic and aerobic exercise environments, without

significant increases in free radical concentration after exercise bouts [121, 122]. Uric acid,

however, has consistently increased in select studies after a variety of exercise bouts [39, 121-

123]. Uric acid has been cited as a potential antioxidant rather than a pro-oxidant [124]. It has

been suggested that the substantial increase was due to the reduction of lipid peroxides. Free

radicals, antioxidants, and all compounds in-between should be studied to fully understand the

balance in redox reactions and how it relates to health. Future research should have biomarkers

that are on both ends of the redox spectrum for full assessment; for example TBARS or MDA as oxidative-stress biomarkers and vitamin C as an antioxidant biomarker.

## 2.3    Vitamin C

2.3.1    Vitamin C as a Salivary Biomarker

Vitamin C, also known as ascorbic acid, is an essential micronutrient in that it is a necessity for certain species to live including humans. Scurvy (vitamin C deficiency) has been reported as early as 1550 B.C. [125] and led to vitamin C's discovery in the early 1900s. It is also a reducing agent involved in a variety of hydroxylation reactions for the synthesis of cartilage, cortisol, and epinephrine. Vitamin C is further involved in tyrosine and fatty acid metabolism.

Not long after its discovery was vitamin C found to be in saliva. In 1935 vitamin C was measured in saliva at a 2.5 µg per 1 mL concentration [126]. Other salivary vitamin C experiments using salivary vitamin C as an index of vitamin status helped in our understanding of vitamin C requirements [126-132]. Mäkilä and Kirveskari [127] are the researchers cited for the recorded range of salivary vitamin C concentration at 0.07-0.09 mg per 100 grams of wet tissue (0.7-0.9 µg per 1 mL saliva); 0.07 mg came from the mixture of whole saliva while the 0.09 mg came strictly from the parotid gland's saliva.

Saliva has been difficult to use as a biospecimen due to the failure in showing consistency of correlation with systemic biomarker levels, vitamin C falls under this category as well [129, 134]. When systemic levels of vitamin C fall to a deficient state, however, its salivary component is undetectable. This is likely a function of limited instrumental detection. But there has been some success in showing a relationship of vitamin C intake and salivary vitamin C when intakes were supplemented [127]. Research has repeatedly confirmed that other biospecimens

(e.g. blood, serum, and plasma) and vitamin C concentrations relate to dietary vitamin C intake [135]. Some diseases and disorders have also had significantly lower salivary vitamin C levels such as tuberculosis, parodontopathy, periodontitis, cancer, and leprosy [133, 136-140].

There has been a lack of consistency in the assays used for determining salivary vitamin C status; the assays that have been used are derived from one of two methods: the dichlorophenolindophenol method or the dinitrophenylhydrazine method [141]. Some methods even use a specific kit that entails dropping a reagent on the tongue [134]. These methods utilize ascorbic acid's antioxidant capabilities (that is to say it can be reduced and oxidized easily) for colorimetry readings such as by a spectrophotometer. Currently, there is no research examining both exercise and salivary vitamin C.

2.3.2   Systemic Vitamin C

Vitamin C can be actively located in a variety of tissues at a variety of approximate concentrations [141]. Some examples of said tissues include the adrenal gland, plasma, and leukocytes. Plasma is often used as a biospecimen to evaluate systemic vitamin C status due to its response to the body's systemic concentrations; however, leukocytes are the better tissue in regards to accurate measurements of bodily stores. Symptoms of vitamin C deficiency often occur when leukocyte and plasma concentrations are 10 $\mu g/10^8$ or less and 0.2 mg/dL or less, respectively [135, 142]. Scurvy symptoms tend to arise when total body vitamin C pools are 300 mg or less [143, 144]. Physiological changes related to scurvy can emerge when intakes are as little as 10 mg per day for a month. The Estimated Average Requirement (EAR) is set at 60 mg per day and 75 mg per day for females and males, respectively; this estimated requirement is set and recommended with the prevention of scurvy in mind. Roughly 37% of Americans are not taking in their respective groups' EAR [145]. The suggested requirements are still debated upon.

It is starting to become more theorized that problems might be developing due to the low intakes of vitamin C (and other essential nutrients). These problems include but are not limited to: harmful nitrosamine formation, low density lipoprotein oxidation, fatigue, and irritability [143].

Vitamin C intakes of 1.25 grams (supplemented) per day fully saturate the blood plasma but roughly 50% of the vitamin is excreted in the urine [146]; this suggests vitamin C intake has a direct relationship with its secretion (an increase in vitamin C intake will lead to an increase in vitamin C excretion). Around 200 mg is roughly the intake needed for full-body saturation with limited excretion. Doses larger than 500 mg will mostly be excreted [146]. Some of the foods that are higher in vitamin C include: peppers at 125-200 mg; kale at 120-180 mg; collard greens at 100-150 mg; broccoli at 90-150 mg; spinach at 50-90 mg; strawberries at 40-90 mg; cauliflower at 60-80 mg; and citrus fruits at 50mg (contents of mg/100g) [147]. Scurvy as a vitamin C deficiency has not been completely eradicated despite its simplicity and known manifestations [148-150]. Some states that induce oxidative stress will also create lower plasma concentrations of the vitamin such as with smoking and diabetes [151-156]. These and similar findings have generated the discussion of other situations which require additional vitamin C from exogenous sources e.g. food stuff and supplementation.

Vitamin C's location in the body relates to its activity, as there are a variety of processes requiring the vitamin to act as a cofactor, a co-substrate, and as an antioxidant in different tissues. It acts as a cofactor in the hydroxylation reactions which synthesize collagen, a protein found in a variety of tissues (skin, cartilage, ligaments, and tendons) necessary for structural and connective purposes. Proline and lysine residues utilize iron for the formation of 3-

hydroxyproline, 4-hydroxyproline, and hydroxylysine [157-159]; iron must be in its reduced state which is completed by vitamin C. Another hydroxylation reaction in which iron must be in its reduced state is the synthesis of carnitine, a compound required for fatty acid transportation into the mitochondria for energy utilization. The enzymes necessary for this reaction to occur are trimethyllysine hydroxylase and γ-butyrobetaine hydroxylase [160]; vitamin C is the reducing agent which reduces iron back to its ferrous state for these reactions. The other metal that is often required in its reduced state during physiological processes is copper; vitamin C will reduce cupric ions to cuprous ions during norepinephrine synthesis. Norepinephrine is a catecholamine and neurotransmitter that has functions in an assortment of sympathetic and central nervous system processes. To exploit these processes, its hydroxylation involving vitamin C is required [161]. Vitamin C is also involved in tyrosine metabolism which requires both iron and copper in their reduced states for the enzymes phenylalanine monooxygenase and 4-hydroxyphenylpyruvate hydroxylase, respectively [162].

### 2.3.3    Vitamin C Supplementation in Exercise

Supplements in sports nutrition is an ever-growing market in which vitamin C's role still remains unknown. Research considering vitamin C intake and exercise performance was conducted as early as the 1930s [163, 164]. With vitamin C being an antioxidant and exercise being an inducer of oxidative stress, it is still one of the most popular supplements utilized making it relevant for further research [165]. Evidence confirming vitamin C supplementation as beneficial, detrimental, or indifferent in exercise is unclear due to confounding results.

Cigarette smoke is a well-known oxidizer that of which decreases systemic vitamin C in humans [151-154, 166]. Exercise may create a similar situation. While systemic vitamin C will

quickly increase immediately after an exercise bout [167-169], a negative feedback loop occurs within a couple of days after the exercise bout and results in a decrease in systemic concentrations [170-173]. Daily training, as seen in athletes and avid exercisers, might then cause a continuous decrease in systemic vitamin C leading to possible deficiency. This might generate a need for an increase in exogenous sources since low vitamin C is related to fatigue. Whether this negative feedback loop normalizes or if specific exercise/sport intensities create different systemic vitamin C levels should be looked into further.

Vitamin C is present in a variety of tissues that undergo exercise-induced oxidative stress. Such oxidative stress then can potentially be reduced from the antioxidant. There are conflicting results in regards to this issue. Results have come about to suggest that vitamin C can attenuate oxidative biomarkers that are increased via exercise [174-177]. Whether these attenuations are necessary or even desired has yet to be determined since exercise adaptations might be reliant upon oxidative stress on some sort of level. Reducing such biomarkers might be to the contrary of the overall goal of improvement. Studies have examined vitamin C supplementation alongside a variety of other antioxidants and their effects on oxidative stress on a cellular level (NF-κB and PGC-1α) [178-180]. Vitamin C on its own has not been studied in humans and should be examined further as it has been examined in rats; results suggest vitamin C can attenuate the exercise-adaptation process on a cellular level [181].

Exercise performance can be examined in a variety of different measures: $VO_2$ max, work capacity, distance for time, overall distance, muscle force, delayed fatigue, and muscle function to name a few. Vitamin C doses from 500 mg to 1500 mg have reported an increase in exercise performance in regards to timed exercise [182-184], delayed fatigue [185], and $VO_2$ max [186]. Vitamin C might possibly compromise such performance measures, even at varying

doses of 400 mg and 1000 mg [181, 187]. Vitamin C might have no effect on performance, either

positively or negatively at doses of 1000 mg per day [188, 189]. These inconsistent results at

similar doses warrant future research on vitamin C in exercise. Individuals who had low intakes

of vitamin C consistently resulted in decreased exercise performance and higher amounts of the

biomarkers used to measure oxidative stress [190-195]. This is the only definitive situation in

which vitamin C has been suggested for supplementation.

## 2.4    **Strength and Resistance Exercise**

### 2.4.1    The Basics

Fitness can be measured in aerobically, such as $VO_2max$, and anaerobically, such as

strength. A common way to improve strength is in the form of resistance exercise, also known as

weight lifting. Resistance training and overall strength can be beneficial to athletes and

untrained individuals alike [196]. Athletes use resistance training to enhance performance and

reduce the chance of injury. Resistance exercise can benefit the average individual as it can

positively affect health status; it is very common to see improvements in blood pressure, insulin

resistance, LDL and HDL cholesterol, and bone density from resistance training [196].

### 2.4.2    Measuring Strength

Some methods to measure muscular strength are dynamometry (i.e. Biodex System 3)

via peak force, peak torque, and total work. More commonly recognized measurements of

strength include one-repetition maximum via the bench press, the squat, and the deadlift [197].

Dynamometers utilize compression and speed control to determine external force. Modern

dynamometers have computers attached that quantify such performance measures in a simple

and precise manner. Dynamometers allow for participants to learn and perform exercise

movements easily. The one-repetition maximum bench press relies on form and thus can hinder

results if form were to break-down. However, the gold standard for evaluating muscular

strength is the free-weight 1-repetition maximum for all free-weight exercises [197].

2.4.3    Vitamin C and Resistance Exercise

An area of exercise that is lacking in oxidative stress and vitamin C research is resistance

training. Common resistance-training exercises that focus on muscular force include the bench

press, back row, lateral pull-down, squat, and deadlift; such exercises have induced oxidative

stress [98]. A current combination for research and resistance training is supplementing vitamin

C along with other antioxidants to reduce oxidative stress and increase exercise performance

[198-200]. Vitamin C as the major and lone antioxidant supplement for exercise performance

has had limited research exposure and should be warranted to do so. A substantial reduction in

muscle soreness has been witnessed with vitamin C supplementation at high doses [201]. A

dynamometer, specifically the Biodex System 3, was used to determine muscle function in the

form of torque without any changes after vitamin C supplementation at high dosages [201].

Oxidative stress might influence muscular force in a negative manner as it can inhibit force on a

cellular level [202-204]. This warrants a more thorough investigation of oxidative stress' effect

on physiological parameters (i.e. muscle force via resistance exercise).


**3    Purpose**


**3.1    <u>Salivary Vitamin C</u>**

The purpose of this experiment was to examine salivary vitamin C as a noninvasive

biomarker of vitamin intake. Salivary vitamin C was examined before and after vitamin C

supplementation as well as before and after exercise. Dietary vitamin C intakes and salivary vitamin C patterns were assessed for correlation. It was hypothesized that salivary vitamin C would react to changes in vitamin C intake.

## 3.2   Salivary Biomarkers of Lipid Peroxidation

The purpose of this experiment was to examine changes in exercise-induced, salivary lipid peroxidation. The included conditions were: before and after resistance-exercise, as well as before and after antioxidant supplementation. It was hypothesized salivary biomarkers for lipid peroxidation would increase after exercise; it was further hypothesized said biomarkers would be reduced after antioxidant supplementation. Free salivary malondialdehyde (MDA) and salivary TBARS were the used biomarkers.

## 3.3   Resistance Exercise Performance

The purpose of this experiment was to examine peak pushing force after a resistance-exercise bout meant to induce oxidative stress. Peak pushing force was examined before and after 4 weeks of vitamin C supplementation. It was hypothesized that peak pushing force would increase after the vitamin C supplementation period in conjunction with the reduction of oxidative stress.

## 4   Methods and Procedures

## 4.1   Participants

Participants were gathered from the campus of the University of Nevada, Reno following a protocol approved by the Institutional Review Board. Male and female participants were both allowed to take part in the experiments. Participants could drop-out of the study at any time. A

total of nine participants were included in the study; seven were male, two were female. Based on Power Test using Mäkilä and Kirveskari [127], n of nine was adequate for statistical power of salivary vitamin C post vitamin supplementation.

The inclusion criterion for the salivary experiments was as follows: participants were not to have previously taken vitamin C supplements regularly. Any kidney problems (i.e. kidney stones) and/or iron metabolism problems (i.e. iron overload) excluded potential participants from involvement due to vitamin C's adverse effects on such situations [206]. Smoking and illnesses (such as the common cold) can alter saliva content and thus excluded potential participants [207].

The inclusion criteria for the peak pushing force experiment were as follows: participants were not to have previously taken vitamin C supplements regularly; participants were not to have trained/performed resistance exercise regularly. Any sort of pushup exercises excluded participants from the study; participants were further asked not to perform such exercises during the study. Participants were asked not to change any dietary/nutritional habits, specifically in regards to weight loss [205]; any report of weight loss would result in exclusion from the experiment. A total of nine participants were accepted.

## 4.2  Dietary Vitamin C Control

Dietary vitamin C intakes were evaluated via the Automated Self-Administered 24-Hour Recall (ASA24). Participants were informed to do a total of three 24-hour dietary recalls using the ASA24 program; two recalls on days during the week and 1 recall on a day during the weekend. Participants were asked to complete each 24-hour recall at their leisure; no specific times/dates were set. Vitamin C intakes were averaged from the completed recalls and

compared to salivary vitamin C for correlation; increases in salivary vitamin C (delta) were used for correlation assessment with average vitamin C intakes. Average vitamin C intakes from participants who did not complete three total recalls were included.

ASA24 is a free computer program provided by the United States Department of Health and Human Services. Participants were given a username and password to sign-on to the computer program; a 24-hour dietary recall was then performed. The program used the multiple-pass method; literature suggests this method to be effective and unbiased [208-210]. The multiple-pass method consisted of 5 steps: 1) the quick list in which the participant logged the foods and beverages consumed the day before; 2) the forgotten foods in which the program queried about foods and drinks often forgotten; 3) time and occasion food was consumed; 4) the detail cycle in which the program queried about descriptions and amounts of the logged foods; 5) the final probe review which asked if anything was forgotten.

Each participant supplemented 500 mg of vitamin C per day for a total of 4 weeks; the 4 weeks of supplementing vitamin C was considered the supplementation period. Participants were asked to not supplement vitamin C any further other than for what was provided; food stuff did not count as supplementation. Participants consumed 250 mg caplets every 12 hours to fully saturate systemic stores. Participants were informed not to consume the entire daily dose of 500 mg in one dose to limit the rate of excretion [135, 143, 146]. The chosen supplement was an over-the-counter supplement available in most local grocery stores. The potency of multiple over-the-counter vitamin C supplements were tested against reagent-grade ascorbic acid (vitamin C) used for creating the standards; price and potency were considered when choosing

the vitamin C supplement. The average potency ($\frac{\text{suppelement concentration}}{\text{reagent-grade concentration}}$) of the chosen

vitamin C supplement was 96% that of the laboratory-grade vitamin C.

**4.3      Saliva Collection and Storage**

Mixed, whole saliva as a biospecimen was collected from fasted participants using the

methods described by the researcher K.W. Stephen (1976). Participants were asked to tilt their

head towards the floor with closed lips where unstimulated saliva would accumulate [211]. The

collected and prepared saliva was frozen via Revco -80° freezer until biomarkers were examined.

Saliva samples from each participant were collected at the same time of day throughout the

study to limit possible circadian differences in concentrations.

The exercise room and the storage freezer were located on north end of campus and

south end of campus, respectively; pre-exercise saliva samples were immediately frozen while

post-exercise saliva samples were put on ice during the duration of travel prior to being frozen.

The travel time from the exercise room to the freezer in the laboratory was 15 to 20 minutes.

4.3.1     Salivary Vitamin C

Saliva collected for vitamin C analysis was collected before and after the vitamin C

supplementation period. Furthermore, saliva was collected for examination before and after

each resistance-exercise bout. The accumulated, unstimulated saliva was collected and pipetted

into a test tube at 1:1 saliva to 10% TCA ratio, respectively, for preservation. Samples were

vortexed on high for 10 seconds prior to freezing. Frozen samples were thawed at room

temperature, approximately 23°C, prior to examination.

4.3.2    Salivary Biomarkers of Lipid Peroxidation

Saliva samples were collected before and after each resistance-exercise bout; once

before the supplementation period, and once after the supplementation period. Samples

collected for free malondialdehyde (MDA) analyses were spat into a tube containing 5%

butylated hydroxytoluene (BTH) and EDTA disodium salt (1:1 BTH to EDTA, respectively). The

added compounds were used to prevent MDA deformation [212, 213]. Samples were vortexed

for 10 seconds and flushed with nitrogen gas prior to freezing in the -80° C freezer. Samples

collected for TBARS analyses were flushed with nitrogen gas and frozen via -80° C freezer.

Samples were thawed at room temperature prior to free MDA and TBARS examination.

**4.4     Salivary Biomarker Examination**

4.4.1    Vitamin C Examination

4.4.1.1    Chemicals and Reagents

Chemicals used were as follows: trichloroacetic acid (TCA); 2,4-dinitrophenylhydrazine;

thiourea (thiocarbamide); copper (cupric sulfate pentahydrate); sulfuric acid (36 N); and L-

ascorbic acid. Five percent and 10% w/v TCA solutions were made in deionized water to

preserve ascorbic acid in standards and saliva samples, respectively. The acidic environment

stabilizes the compound for examination. Copper was used as the oxidizing agent that formed

dehydroascorbic acid from ascorbic acid [141]; TCA aided in the oxidation process [214].

Thiourea was added to create a mildly reducing medium in an effort to react ascorbic acid and

dinitrophenylhydrazine. A 2,4-dinitropheynylhydrazine/thiourea/copper (DTC) reagent was

made by mixing 0.4 grams of thiourea, 0.05 grams of copper, along with 3 grams of 2,4-

dinitrophenylhydrazine and diluted with 100mL of 9 N sulfuric acid. Sixty-five percent sulfuric

acid, a strong acid, was used to provide acidic conditions for the reaction [214]. Dehydroascorbic

acid reacted with 2,4-dinitrophenylhydrazineto form the red osazone derivative, bis-2,4-

dinitrophenylhydrazone, that was read for absorbance. Absorbance was read via the SynergyHT

microplate reader from BioTek Instruments, Inc. at a wavelength of 520 nm.

4.4.1.2    Sample Preparation and Assay

Thawed saliva samples were centrifuged at 3500 rpm for 15 minutes to separate out the

pellet from the supernatant. One mL of the supernatant was pipetted from each separated

sample for the reaction with 0.2 mL of DTC. Solutions were vortexed on high for 10 seconds and

incubated for three hours at 37°C in a Lab-Line incubator; 1.5 mL of 65% sulfuric acid ($H_2SO_4$) was

added to the solution after the incubation period. Solutions were vortexed and left at room

temperature for another 30 minutes, allowing the reaction to occur. Final saliva solutions were

pipetted into a 96-well plate at 0.2 mL for examination; three samples from each saliva solution

were taken for the absorbance reading. Absorbance was read at a wavelength of 520 nm.

Absorbance readings from each saliva sample were averaged; averages were used to calculate

salivary vitamin C. Salivary vitamin C was assessed prior to supplementation (baseline

concentration) and after 4 weeks of supplementing vitamin C. Salivary vitamin C was further

assessed before and after each resistance-exercise bout.

The actual compound of vitamin C was further examined in saliva to ensure specificity

and reproducibility. Two different saliva samples were treated per the following descriptions:

spiked with 12 μg ascorbic acid per 1 mL 5 % TCA, and 24 μg ascorbic acid per 1 mL 5% TCA;

ratios were 1:1 prepared saliva to ascorbic acid solution, respectively. An untreated saliva

sample was prepared as well. Expected and found concentrations were used to determine

percent recovery. Percent vitamin C recovered (% recovery) was calculated to determine the

assay's reproducibility as described by Kahn et. al. [215]. The following equation was used to

determine percent recovery ($C_e$ = expected concentration; $C_f$ = found concentration):

$$\% \text{ recovery} = \frac{|C_e - |C_e - C_f||}{C_e} \times 100$$

A similar experiment that was performed by Mäkilä and Kirveskari [127] was conducted

to confirm previous results of salivary vitamin C after consuming 500 mg vitamin C; salivary

vitamin C was examined before and at hours 6 and 24 post-supplement. The experiment was

performed to examine immediate changes in salivary vitamin C after supplementation.

### 4.4.1.3    Standards Preparation and Assay

Vitamin C standards were prepared with laboratory grade ascorbic acid (vitamin C) at

concentrations ranging from 20 µg/mL to 0.3125 µg/mL; 20 µg/mL stock was prepared while

serial dilution was used to create the standards that followed. A blank was prepared without

ascorbic acid. Beer's Law was used to estimate a curve and its equation as seen in Figure 1; the

equation was used to estimate salivary vitamin C. New vitamin C standards were prepared each

time salivary vitamin C samples were assessed.

### 4.4.2    Salivary Biomarkers of Lipid Peroxidation

### 4.4.2.1    Free salivary MDA

The method described by Karatas et. al. was used to examine free salivary MDA [216];

the method was developed to exclude TBA from the MDA assay for specificity purposes.

Hydroperoxides and conjugated aldehydes react with TBA to restrict said specificity. A strong

acid can release MDA from binding proteins, creating free MDA for a sensitive and specific

assessment. An experienced professional with HPLC expertise is required, though the

preparations of standards and samples are simple.

4.4.2.1.1 Chemicals and Reagents

Chemicals used were as follows: 0.1 M perchloric acid ($HClO_4$); 1,1,3,3-

tetraethoxypropane (TEP); methanol; monopotassium phosphate ($KH_2PO_4$); and 0.1 M

hydrochloric acid (HCl).Perchloric acid was used to release bound MDA from proteins in saliva

for measurement. Tetraethoxypropane as a metabolite of MDA will react with acid in boiling

water to produce free MDA to be used for the preparation of standards.

4.4.2.1.2 Sample Preparation and Assay

Fifty μL (0.05 mL) of each saliva sample was added to 0.25 mL 0.1M $HclO_4$ and 0.7 mL

deionized water. Prepared samples were filtered through a tip (GE Healthcare Life Sciences)

prior to HPLC analysis. Free salivary MDA was assessed before and after each exercise-bout as

well as before and after the 4-week supplementation period.

4.4.2.1.3 Standards Preparation and Assay

An MDA stock of 2.92 μg/mL was prepared for the beginning standard; standards were

made from this stock via serial dilution and ranged from 2.92 μg/mL to 0.292 μg/mL. The peak

heights from the standards were used to create the calibration curve. Peak heights from

prepared saliva samples were inputted into the curve's equation for free salivary MDA

estimation.

Free MDA percent recovery (% recovery) was calculated as described by Karatas et. al.

[216]. The found concentration of the stock solution was inputted into the curve formulated

from the expected concentrations of the standards. The percent recovery was calculated at

97%. The curve used to calculate MDA concentrations was y = 30.406x + 5.2749 and is displayed in Figure 2.

#### 4.4.2.1.4 HPLC

Hewlett-Packard Series 1100 was the HPLC used to determine free MDA. A C18 reversed-phase column (25 cm, 4.6 mm i.d.; 5-μm particles) was used at ambient temperature. Thirty mM $KH_2PO_4$-methanol (65 + 35 v/v%) was used for the single mobile phase. The flow rate was 1.5 mL/minute. Chromatograms were monitored at 254 nm and injection volume was 20 μL. The retention time was 1.88-1.91 minutes [216].

### 4.4.2.2 Salivary TBARS

TBARS were examined via Yagi's method [217].

#### 4.4.2.2.1 Chemicals and Reagents

Chemicals used for TBARS examination were as follows:  sulfuric acid (36 N $H_2SO_4$); phosphotungstic acid; 2-thiobarbituric acid (TBA); 1-butanol; 1,1,3,3-tetramethoxypropane (TMP); and glacial acetic acid.

Sulfuric acid was diluted with deionized water to create N/12 sulfuric acid; phosphotungstic acid was diluted with deionized water to create a 10% phosphotungstic acid reagent. These two reagents mixed together will precipitate lipids used to measure lipid peroxidation. Glacial acetic acid and deionized water were mixed at 1:1 ratio with TBA, thus creating the TBA reagent; the TBA reagent was 0.67% TBA.  Acid hydrolysis of TMP, an MDA metabolite, created the compound MDA-TBA[2]. Sialic acid, a compound found in saliva, will not react with TBA if acetic acid is present [217]; this is a common concern of the TBARS assay. The

precipitated lipids were extracted with n-butanol. Absorbance was measured at a wavelength of 532 nm.

### 4.4.2.2.2   Sample Preparation and Assay

Saliva was centrifuged at 3000 rpm for 10 minutes and 0.5 mL of the supernatant was transferred to a separate test tube in addition to 4 mL of N/12 sulfuric acid and 0.5 mL of 10% phosphotungstic acid. Solutions were mixed gently and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded while the pellet was mixed with 2 mL N/12 sulfuric acid and 0.3 mL 10% phosphotungstic acid before centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded while the pellet was mixed with 4 mL deionized water and 1 mL TBA reagent. The samples were placed in a boiling water bath for 60 minutes. Samples were placed in tap water bath to cool, added to 5 mL n-butanol, vortexed for 10 seconds, and centrifuged at 3000 rpm for 15 minutes; 0.2 mL of the n-butanol layer (top layer) from each sample was pipetted into a 96-well plate. Three samples from each prepared saliva sample were read for absorbance; the average of the 3 was used as the TBARS measurement. Absorbance of samples was measured via BioTek microplate reader at 532 nm. Salivary TBARS were assessed before and after each exercise bout.

### 4.4.2.2.3   Standards Preparation and Assay

Tetramethoxypropane (TMP) as a MDA metabolite was diluted with deionized water and reacted with TBA reagent to prepare the 16 nM TBARS stock solution. Serial dilution was used to create the standards from the stock solution; standards ranged from 16 nM to 0.125 nM. A blank was prepared without TMP. Solutions were prepared in same procedure as samples. Absorbance readings of the standards were used to create the curve for salivary TBARS analysis. TBARS samples were assessed before and after one resistance exercise bout.

**4.5**     **Resistance Exercise**

4.5.1     Biodex System 3

The Biodex System 3 was the apparatus used to perform the resistance exercises. The

closed-kinetic-chain (CKC) attachment was used to create the push-pull exercises. The push-pull

exercises were set-up to have concentric contractions for the pushing and the pulling portions of

each repetition. Participants were introduced to the apparatus at least three days prior to the

first experimental exercise bout. The setup for each participant was created during this

introduction. Setup; which consisted of attachment height, seat placement, hand position, CKC

tilt, shoulder angle, handle-extension travel, and handle-retraction travel; were created as

recommended by the manual's instructions and performed by Riemann et. al. [218]. The CKC

neutral hand position was used during the exercises; apparatus' tilt and shoulder angle were set

at the positions labeled as neutral. The neutral shoulder angle for the apparatus was 25°. Full

handle-retraction was set for the participant's elbow to create a 90° angle at his/her side at the

end of travel. Full handle-extension was set for the arm to fully extend at the end of travel,

creating a straight arm. Height was measured so the hand was directly in-line with the shoulder

at full extension. Participants were secured in the apparatus by a shoulder strap. A foot

attachment was placed for participants to stabilize with while performing the exercises. The

parameters created from this setup were used for both exercise bouts.

4.5.2     Resistance Exercise Bout

A resistance-exercise-induced oxidative stress exercise (REIOS) and a maximal-effort set

were performed by each participant during each exercise-bout. Two exercises were performed

during each REIOS: one exercise consisting of push-ups and one exercise consisting of isokinetic

push-pull repetitions. The set and repetition scheme, with three sets of 10 repetitions, utilized in

Deminice's experiment experienced an influx of oxidative stress biomarkers after the resistance-exercise bout [39]. Exercises involving pushing and pulling repetitions were included in the previous study; the three sets of 10 repetition scheme were used in the present experiment via isokinetic-resistance exercise. An isokinetic-maximal-effort set was then performed to assess maximal-effort peak pushing force while in an oxidative state. Saliva samples was immediately taken after the maximal-effort set. Participants were to not have performed any sort of push exercises (i.e. pushups) during the duration of the study; this would suggest any experienced gain in strength would not be a result of any training adaptation.

Velocities, angles, and repetitions performed on the Biodex for all exercises were chosen from studies that performed test-retest experiments with no significant differences in the quantified peak pushing force between the two exercise bouts [218, 219].

4.5.2.1   Resistance-Exercise-Induced Oxidative Stress (REIOS) Exercise

 Participants were asked to complete as many push-up repetitions as they could; technique and performed repetitions varied amongst each participant creating a limitation. Three sets of 10 isokinetic-push-pull repetitions were performed 90 seconds after the push-up exercise. No verbal cheering was given throughout the exercise. The Biodex System 3 was set at a velocity of 120°; a 10 second rest period was given between each performed set. The rest period came standard within the Biodex program.  The peak pushing force from each of the three performed sets were averaged and assessed before and after the supplementation period.

4.5.2.2   Maximal-effort Set

A 90 second rest period was given after the REIOS exercise before performing the maximal-effort set on the Biodex System 3. Participants performed one set of five isokinetic-

push-pull repetitions at a velocity of 60° for their maximal effort measure. The slower speed was

to ensure accuracy of peak force quantification [218]. No cheering was given throughout the

exercise. Peak pushing force of the maximal-effort set was quantified before and after the 4

week supplementation period.

## 5     Statistical Analysis

Figure 1 (pg. 38) displays the times when the experiment's measures were taken. All data

was put into GraphPad Prism5 for statistical analysis. A repeated samples t-test was used to

analyze statistically-significant differences in salivary vitamin C, free salivary MDA, salivary

TBARS, peak pushing force, and average peak pushing force at their respective times. Salivary

vitamin C before and after vitamin C supplementation as well as before and after resistance

exercise was compared. Free salivary MDA was compared three different ways: before and after

each resistance-exercise bout; within exercise conditions, before and after exercise one (before

supplementation) and before and after exercise two (after supplementation); and before

supplementation overall and after supplementation overall. Maximal-effort peak pushing force

before and after the supplementation period was compared. The average peak pushing force

quantified from each of the 2 REIOS exercises, before and after the supplementation period,

was also compared. Change in salivary vitamin C content was correlated with average dietary

vitamin C intake via the Pearson Coefficient.

## 6     Results

### 6.1     <u>Salivary Vitamin C</u>

Figure 2 (pg. 39) displays a curve created by the absorbance of the vitamin C standards via

serial dilution. Triplicates were made for each standard's concentration (20 µg/mL – 0.3125

µg/mL) and averaged for the curve's equation. Linear regression was used to formulate the equation. The equation, $y = 74.225x - 0.6059$ ($R^2 = 0.9899$), was used to determine the vitamin C content in saliva at the respective times of sample collection. The R-square suggests accuracy in the serial dilution process. Standards were remade for each salivary vitamin C assay.

Figure 3 (pg. 40) displays the salivary vitamin C results from the prepared saliva as it was untreated, treated with 12 µg ascorbic acid per 1 mL 5% TCA, and treated with 24 µg ascorbic acid per 1 mL 5% TCA. Expected and quantified concentrations were used to determine the percent recovery and reproducibility of the vitamin C assay; the percent recovery was calculated at 104% using the curve's equation created by the standards, $y = 74.225x - 0.6059$.

Figure 4 (pg. 41) displays the results from the recreation of Mäkilä's and Kirveskari's experiment [127]; in the present experiment, salivary vitamin C was examined prior to vitamin C supplementation along with 6 and 24 hours after 500 mg dose of vitamin C supplementation. Salivary vitamin C prior to vitamin C supplementation was 0.12 µg/mL; salivary vitamin C 6 hours after 500 mg vitamin C supplementation was 0.22 µg/mL; salivary vitamin C 24 hours after 500 mg vitamin C supplementation was 0.32 µg/mL.

Table 1 (pg. 42) displays the results of the salivary vitamin C content prior to and after the 4-week supplementation period is displayed for each participant. The absorbance of each participant's saliva sample was read in triplicates and averaged. Mean salivary vitamin C pre and post supplementation period, standard deviations (SD), and 95% confidence intervals (95% CI) are also displayed. The mean salivary vitamin C content prior to the supplementation period was 0.47 µg/mL; the mean salivary vitamin C content after the 4-week supplementation period was 1.22 µg/mL. A paired t-test was used to analyze the results for statistical differences; the

experienced increase in salivary vitamin C after the supplementation period was statistically significant (p-value<0.0001). Figure 5 (pg. 43) is the bar graph displaying salivary vitamin C means prior to and after the 4-week supplementation period resulting in 0.47 µg/mL and 1.22 µg/mL, respectively. Standard errors of the means (SEM) are displayed via error bars.

Figure 6 (pg. 44) is a bar graph displaying salivary vitamin C means prior to and after one resistance-exercise bout. Mean salivary vitamin C content prior to the resistance-exercise bout was 0.52 µg/mL (SD=0.52); mean salivary vitamin C content after the resistance-exercise bout was 1.0 µg/mL (SD=0.58). The absorbance of each participant's saliva sample was read in triplicates and averaged. A paired t-test was used to analyze the results for statistical differences; the experienced increase in salivary vitamin C after the resistance-exercise bout was statistically significant (p-value<0.0001). Error bars are displayed on the bar graph as standard errors of the means.

Table 2 (pg. 45) displays each participant's salivary vitamin C contents before and after the 4-week supplementation period. Respective change (delta) in participants' salivary vitamin C contents after the supplementation period and dietary vitamin C intakes are also displayed. The average increase in salivary vitamin C (delta) was 0.70 µg/mL. The average dietary vitamin C intake reported was 76 mg/day. The highest reported dietary vitamin C intake of 257 mg/day (1 dietary recall completed) was examined for being an outlier with results suggesting it as an outlier. The average intake of dietary vitamin C resulted in 53 mg/day when the outlier was withdrawn. Results were used to statistically analyze correlation via Pearson correlation; results suggest a significant correlation (p-value=0.0441). The Pearson R value was 0.68 (with the outlier), suggesting a strong correlation in the increase of salivary vitamin C with the average dietary vitamin C intake. Figure 7 (pg. 46) displays the linear regression graph created with the

resulted values. Salivary vitamin C prior to the supplementation period did not correlate with dietary vitamin C intake; salivary vitamin C after the supplementation period did not correlate with dietary vitamin C intake, either.

## 6.2    **Salivary Biomarkers of Lipid Peroxidation**

Figure 8 (pg. 47) displays the curve created by the free MDA standards, ranging from 2.92 µg/mL to 0.292 µg/mL, and their respective peak heights found via HPLC. Peak heights were used to calculate the curve's equation, $y = 30.406x + 5.2749$ ($R^2 = 0.9939$); the equation was used to determine free MDA in saliva samples collected at the respective times. The found concentration of the stock was inputted into the curve's equation to determine percent recovery (% recovery) as described by Karatas et. al. [216]; % recovery= 97%. Serial dilution was used to create the standards from the stock; the $R^2$ was greater than 0.95 suggesting the standards were accurately made.

Table 3 (pg. 48) displays participants' free salivary MDA values before and after exercise bouts one and two; exercise bouts one and two are separated by the four-week supplementation period. Means, standard deviations (SD), and 95% confidence intervals (95% CI) are also displayed. Average free salivary MDA prior to supplementation/exercise-bout 1 was 0.37 µg/mL (SD = 0.014, 95% CI±0.009); average free salivary MDA after exercise-bout 1 was 0.39 µg/mL (SD = 0.017, 95%±CI 0.011). Average free salivary MDA after the 4-week supplementation period and before exercise-bout 2 was 0.36 µg/mL (SD = 0.008, 95%±CI 0.005); average free salivary MDA after exercise-bout 2 was 0.37 µg/mL (SD = 0.014, 95%±CI 0.009). Free salivary MDA values were tested for statistically significant differences via paired t-test at the following times: pre-exercise one vs post-exercise one; pre-exercise two vs post-exercise two; pre-exercise one vs pre-exercise two; post-exercise one vs post-exercise two; pre-

supplementation period vs post-supplementation period. Free salivary MDA significantly increased after exercise-bouts one and two, p-values were respectively less than 0.01. Free salivary MDA significantly decreased from pre-exercise one to pre-exercise two, from post-exercise one to post-exercise two, and from pre-supplementation period to post-supplementation period (respective p-values were 0.01, 0.03, and 0.0007). Figure 9 (pg. 49) displays a bar graph of the data means for free salivary MDA; 0.37 μg/mL, 0.39 μg/mL, 0.36 μg/mL, 0.37 μg/mL; at their respective times. Displayed error bars are standard errors from the mean.

Figure 10 (pg. 50) displays a bar graph of the results for salivary TBARS before and after exercise. There were no significant differences in the average of salivary TBARS before and after a single exercise-bout amongst the participants; salivary TBARS before exercise averaged 11.24 nM/mL (SD= 4.36; 95% CI +/- 2.14) while after exercise averaged 13.57 nM/mL (SD= 5.12; 95% CI +/- 2.52). Paired t-test p-value was calculated at 0.14.

**6.3    Resistance Exercise Performance**

Table 4 (pg. 51) displays participants' individual maximal effort peak pushing force before and after the supplementation period. The table displays the means, the standard deviations (SD), and the 95% confidence intervals (95% CI) calculated from the values obtained from each maximal effort set. Average maximal effort peak pushing force prior to the supplementation period was 82.8 pounds (SD=16.18, 95% CI$\pm$10.57); average maximal effort peak pushing force after the supplementation period was 91.2 pounds (SD=20.85, 95% CI$\pm$13.62). Paired t-test was used to analyze any statistical differences between the two maximal effort sets separated by the four-week supplementation period; maximal effort peak pushing force experienced a significant increase after the vitamin C supplementation period (p-value=0.0157). Figure 11 (pg. 52)

displays the bar graph which represents the data means of 82.8 pounds and 91.2 pounds for the pre and the post-supplementation maximal effort sets, respectively. Standard errors from the mean are displayed by the error bars.

Table 5 (pg. 53) displays the quantified averages of peak pushing force taken from the three isokinetic-exercise sets; an average was taken from each of the REIOS exercise bouts (prior to and after the vitamin C supplementation period). Each participant's quantified data are displayed along with the means, the standard deviations (SD), and the 95% confidence intervals (95% CI). The REIOS average peak pushing force prior to the supplementation period was 58.72 pounds (SD=12.66, 95% CI$\pm$9.73); the REIOS average peak pushing force after the supplementation period was 66.94 pounds (SD=15.49, 95% CI$\pm$11.91). A paired t-test was used to analyze any statistical differences between the exercise bouts separated by the 4-week vitamin C supplementation period. The REIOS average peak pushing force experienced a significant increase after the vitamin C supplementation period (p-value=0.0256). Figure 12 (pg. 54) displays the bar graph which represents the data means of 58.72 pounds and 66.94 pounds for the pre and the post-supplementation REIOS exercise bouts, respectively. Standard errors from the mean are displayed by the error bars.

# Pre-post Intervention Experimental Design with Respective Saliva Collection Times
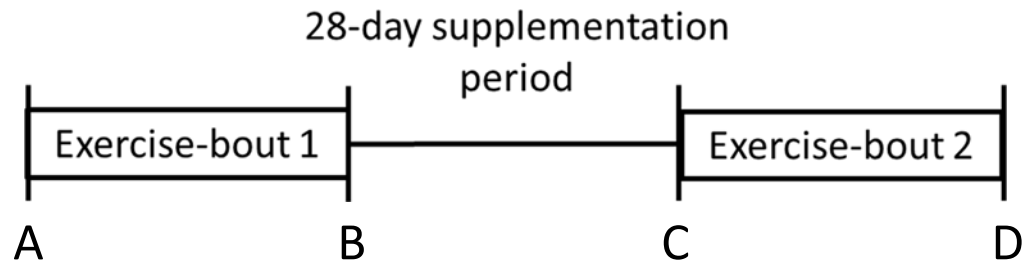


**Figure 1:** The design was a pre-post intervention experiment. Vitamin C was supplemented for 28 days for the intervention; resistance-exercise bouts, one and two, were performed before and after the supplementation period. Saliva was collected: A) before exercise-bout one before vitamin C supplementation; B) after exercise-bout one before vitamin C supplementation; C) before exercise-bout two after vitamin C supplementation; D) after exercise-bout two after vitamin C supplementation. Measures were compared via their respective times as follows:

Average peak pushing force: exercise-bout one vs exercise-bout two

Maximal peak pushing force: exercise-bout one vs exercise-bout two

Salivary vitamin C: A vs C; A vs B

Free salivary MDA: A vs B; C vs D; A vs C; B vs D; AB vs CD

Salivary TBARS: A vs B
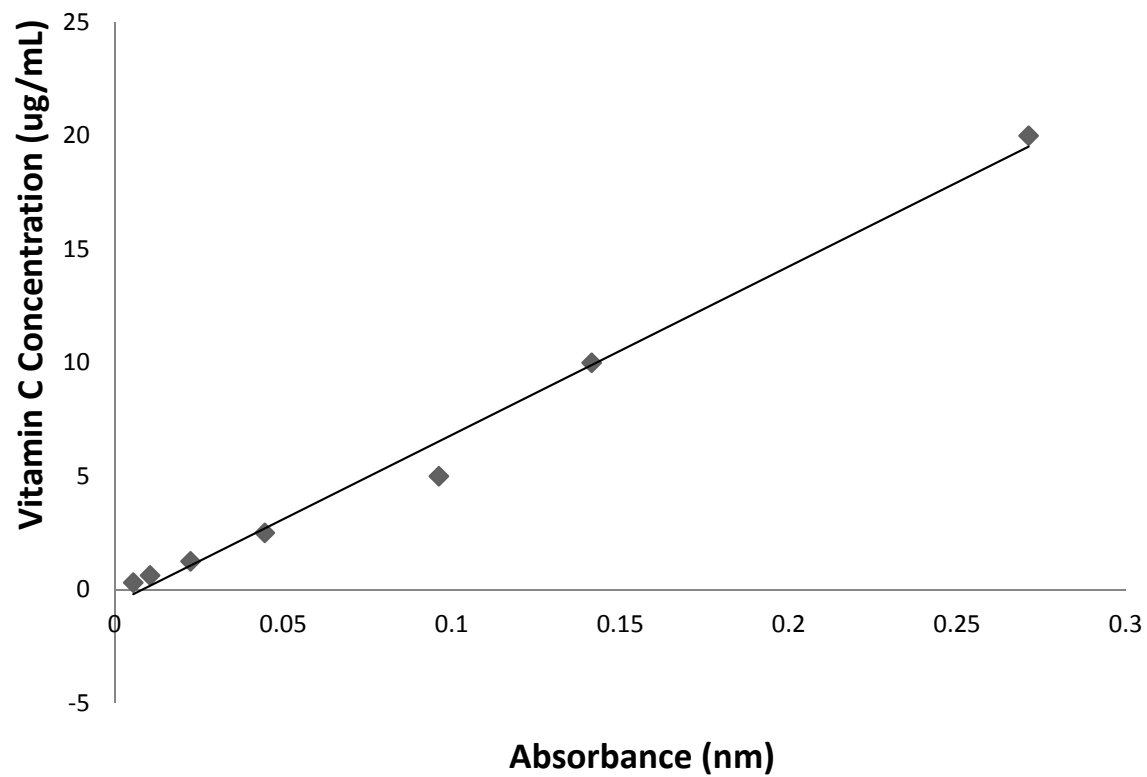
**Figure 2:** Curve from vitamin C standards, y = 74.225x – 0.6059 ($R^2$ = 0.9899); the equation was used to determine salivary vitamin C. Read in triplicates.

**Salivary Vitamin C in Untreated Saliva and
Vitamin C Treated Saliva**
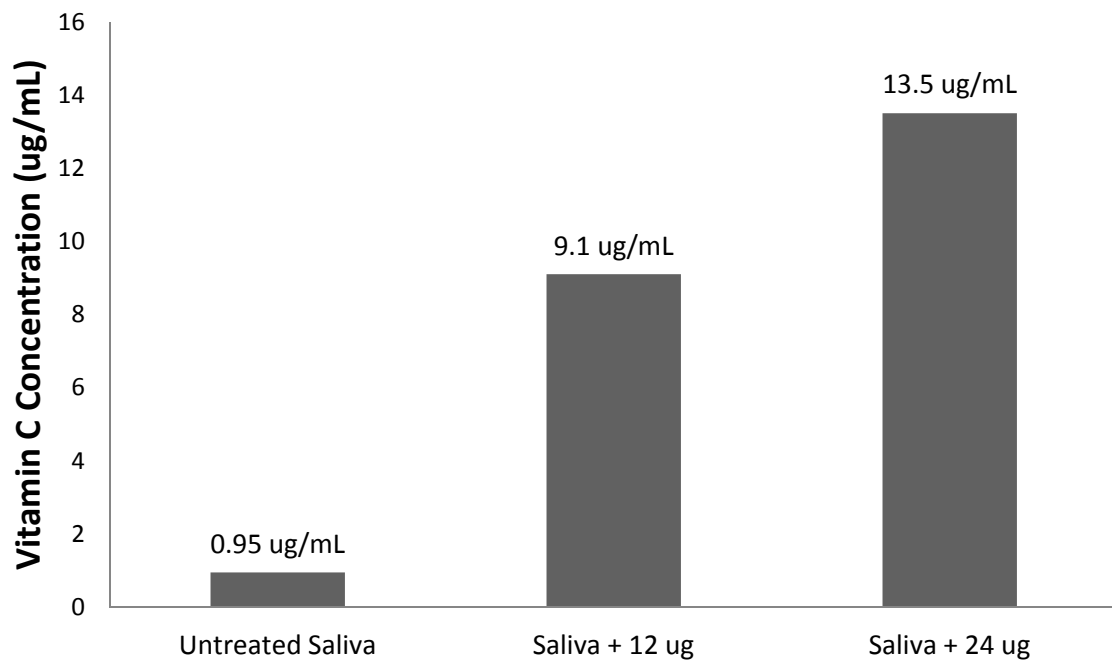


**Figure 3:** Saliva was respectively treated 2 different ways: prepared saliva treated with 12 μg per mL 5% TCA; prepared saliva treated with 24 μg per mL 5% TCA. Results are shown next to the untreated, prepared saliva which was used. Results were used to calculate percent recovery (% recovery); % recovery = 104%.

## Salivary Vitamin C at
## Select Times Post-500 mg Supplement
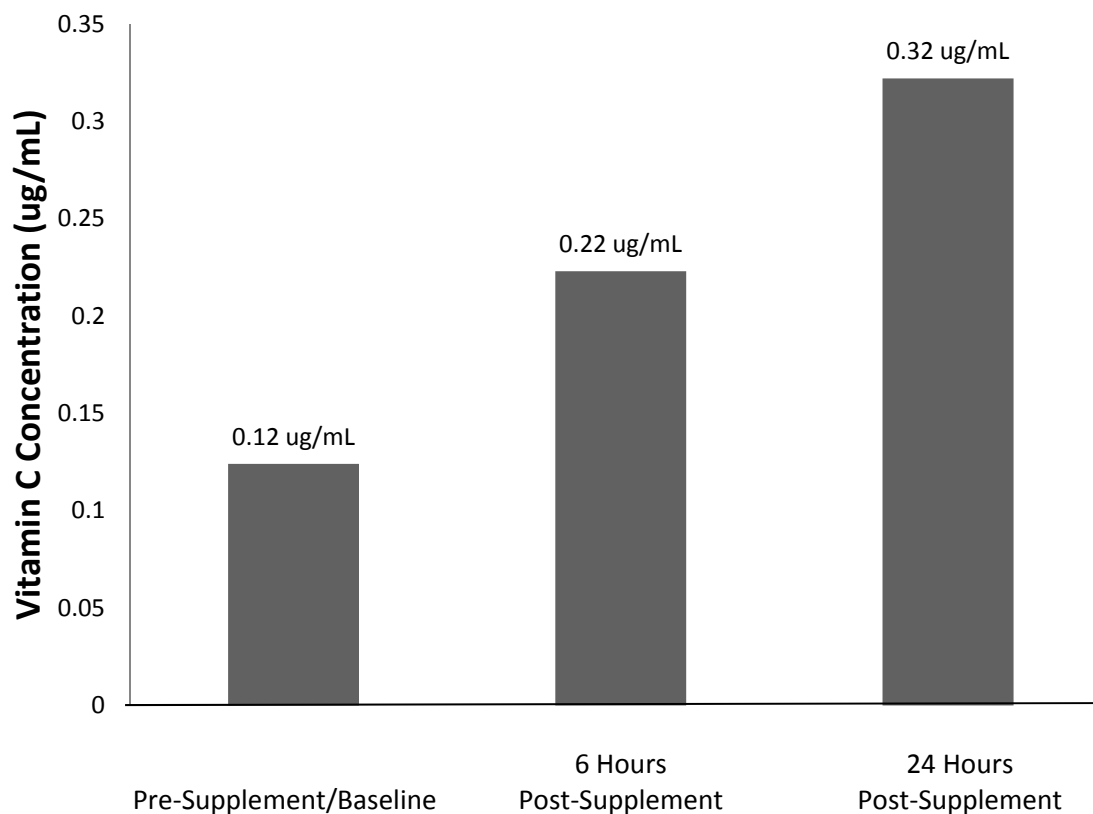


**Figure 4:** Saliva samples were taken before and at hours 6 and 24 after one dose of 500 mg vitamin C supplement to examine immediate changes in saliva. Results confirm the experiment performed by Mäkilä and Kirveskari [127]; a 97% increase in salivary vitamin C was experienced after 6 hours post 500 mg vitamin C in the previous experiment (n=9). The present results experienced an 83% increase in salivary vitamin C 6 hours after the 500 mg dose (n=1). Read in triplicates.

**Table 1:** Individual salivary vitamin C content before and after the 4-week vitamin C supplementation period. Salivary vitamin C experienced a significant increase after the supplementation period (* p <0.0001).

## Individual Salivary Vitamin C (ug/mL) Pre and Post Vitamin C Supplementation Period

| Subject | Pre-Supplement | Post-Supplement |
|---|---|---|
| 1 | 1.78 | 3.03 |
| 2 | 0.16 | 1.04 |
| 3 | 0.21 | 1.08 |
| 4 | 0.25 | 1.25 |
| 5 | 0.43 | 1.03 |
| 6 | 0.5 | 1.44 |
| 7 | 0.2 | 0.62 |
| 8 | 0.16 | 0.63 |
| 9 | 0.51 | 0.82 |
| **Mean** | 0.47 | 1.22* |
| **SD** | 0.51 | 0.73 |
| **95% CI** | 0.34 | 0.48 |

# Average Salivary Vitamin C
# Pre and Post Supplementation
# Period
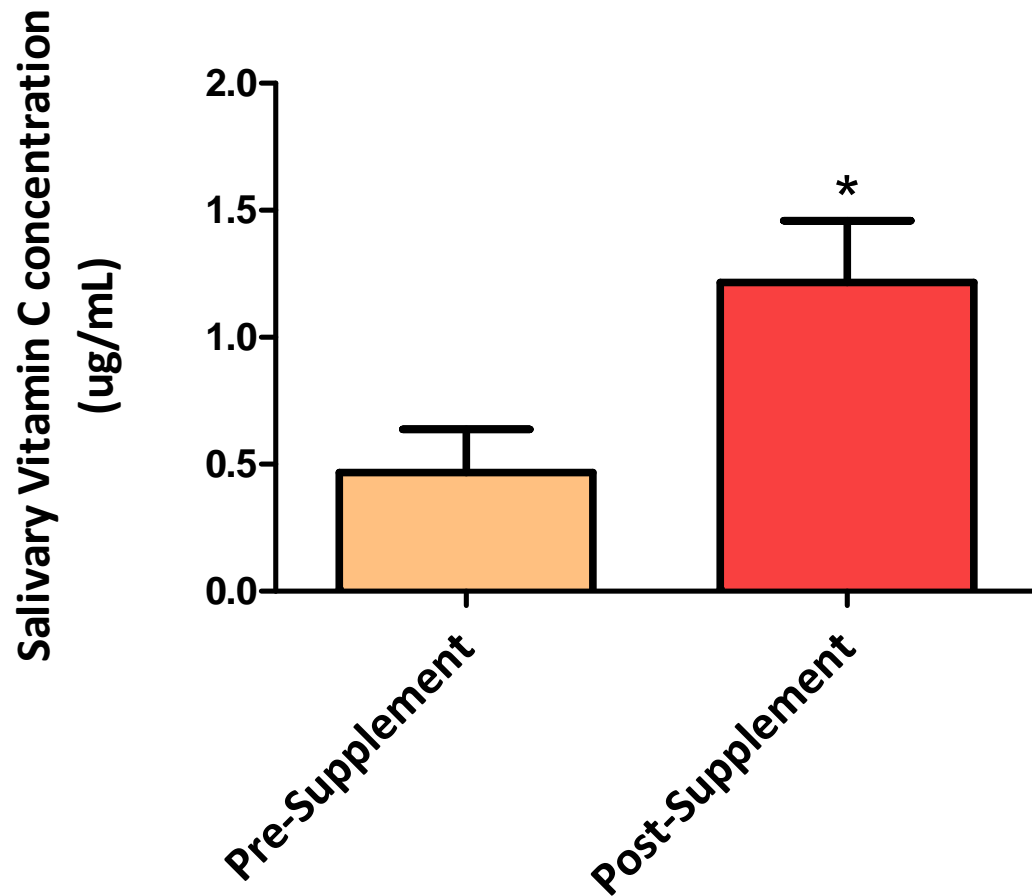


**Figure 5:** Salivary vitamin C means pre (0.47 µg/mL) and post (1.22 µg/mL) 4-week supplementation period. Salivary vitamin C experienced a significant increase after the 4-week supplementation period (* p<0.0001). Data is represented in means, error bars = SEM, n = 9.

# Average Salivary Vitamin C Concentration Pre and Post Resistance Exercise
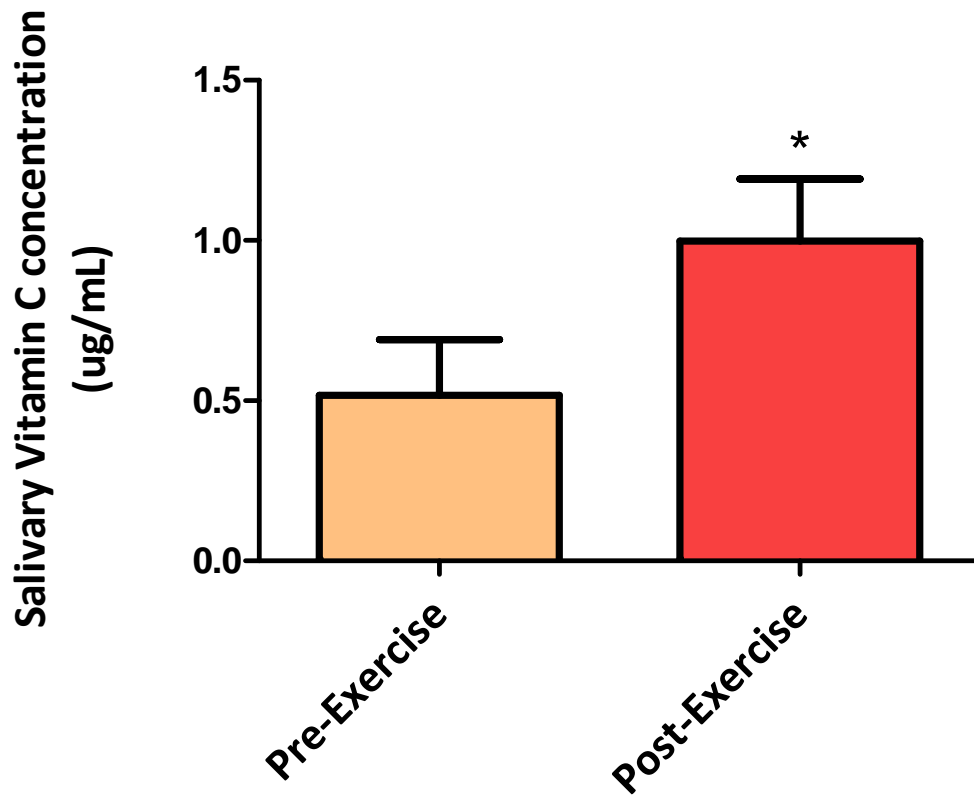


**Figure 6:** Salivary vitamin C means before (0.52 μg/mL) and after (1.00 μg/mL) 1 resistance-exercise bout. Salivary Vitamin C significantly increased after the exercise bout (* $p < 0.0001$, error bars = SEM, n = 9).

**Table 2:** Individual results of salivary vitamin C (pre-supplementation, post-supplementation, and delta salivary vitamin C) and average vitamin C intake (mg/day) from completed dietary recalls. The values of the increase in salivary vitamin C (delta) and the average dietary vitamin C intake were used to assess correlation via Pearson correlation as displayed in Figure 7.

# Delta of Salivary Vitamin C and Vitamin C Intakes

**Salivary Vitamin C (ug/mL)**

| Subject | Pre-Supplement | Post-Supplement | Delta Salivary Vitamin C | Average Vitamin C Intake (mg/day) | # of Dietary Recalls Completed |
|---|---|---|---|---|---|
| 1 | 1.78 | 3.14 | 1.36 | 257 | 1 |
| 2 | 0.16 | 1.04 | 0.88 | 20 | 3 |
| 3 | 0.21 | 0.56 | 0.35 | 18 | 3 |
| 4 | 0.25 | 1.25 | 1 | 60 | 1 |
| 5 | 0.43 | 1.03 | 0.6 | 27 | 2 |
| 6 | 0.5 | 1.44 | 0.94 | 105 | 3 |
| 7 | 0.2 | 0.62 | 0.42 | 39 | 1 |
| 8 | 0.16 | 0.63 | 0.47 | 75 | 3 |
| 9 | 0.51 | 0.82 | 0.31 | 82 | 3 |

# Correlation Between  Average  Vitamin C Intake and the Increase in Salivary Vitamin C
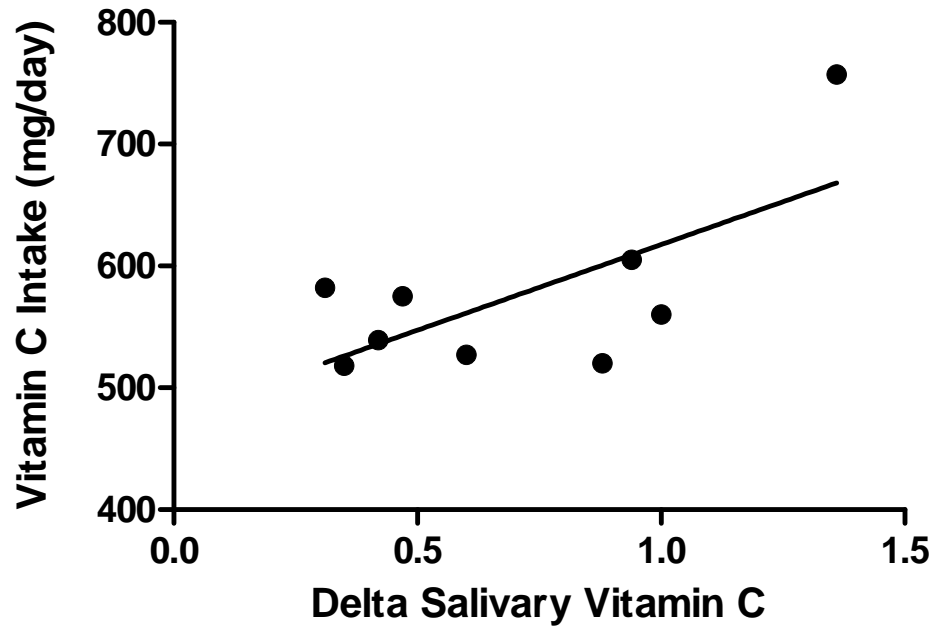


**Figure 7:** A significant correlation was experienced with the average intake of dietary vitamin C and the delta of salivary vitamin C (p-value<0.05, n = 9). Pearson R = 0.68 suggesting a strong correlation between the increase in salivary vitamin C and ; 95% CI = 0.028 − 0.926.
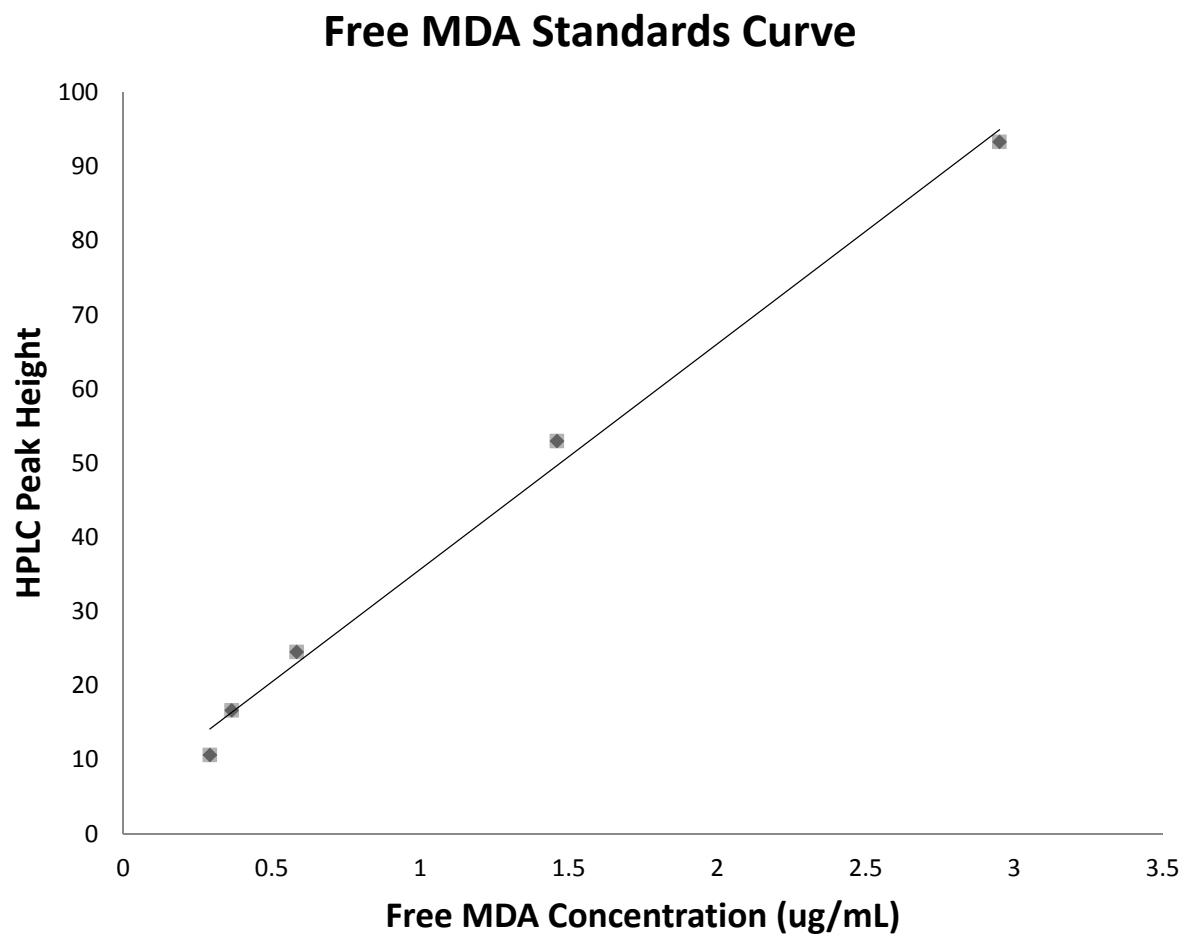
**Figure 8:** Curve from free MDA standards, y = 30.406x + 5.2749 ($R^2$ = 0.9939); equations were used to determine free MDA in saliva samples. The found concentration of the stock was inputted into the equation to determine percent recovery (% recovery) [216]; % recovery= 97%.

**Table 3:** Individual participants' free salivary MDA pre and post exercise-bouts 1 and 2, before and after 4-week vitamin C supplementation period. Free salivary MDA significantly increased after each respective exercise-bout (pre vs post, *p<0.01, n = 9); free salivary MDA was reduced from the following conditions: pre-exercise 1 vs pre-exercise 2, post-exercise 1 vs post-exercise 2 after vitamin C supplementation (# p<0.05).

## Free Salivary MDA Pre and Post Resistance Exercise, Before and After Vitamin C Supplementation (μg/mL)

| Subject | Pre-Supplementation | | Post-Supplementation | |
| --- | --- | --- | --- | --- |
| | Pre-Exercise 1 | Post-Exercise 1 | Pre-Exercise 2 | Post-Exercise2 |
| 1 | 0.36 | 0.37 | 0.36 | 0.38 |
| 2 | 0.37 | 0.39 | 0.37 | 0.37 |
| 3 | 0.36 | 0.36 | 0.35 | 0.36 |
| 4 | 0.35 | 0.37 | 0.35 | 0.36 |
| 5 | 0.38 | 0.41 | 0.36 | 0.38 |
| 6 | 0.39 | 0.4 | 0.36 | 0.37 |
| 7 | 0.38 | 0.38 | 0.35 | 0.36 |
| 8 | 0.38 | 0.4 | 0.37 | 0.4 |
| 9 | 0.39 | 0.39 | 0.36 | 0.38 |
| **Mean** | 0.37 | 0.39 * | 0.36# | 0.37*# |
| **SD** | 0.014 | 0.017 | 0.008 | 0.014 |
| **95% CI** | 0.009 | 0.011 | 0.005 | 0.009 |

# Average Free Salivary MDA Pre and Post Resistance Exercise, Performed Before and After Supplementation
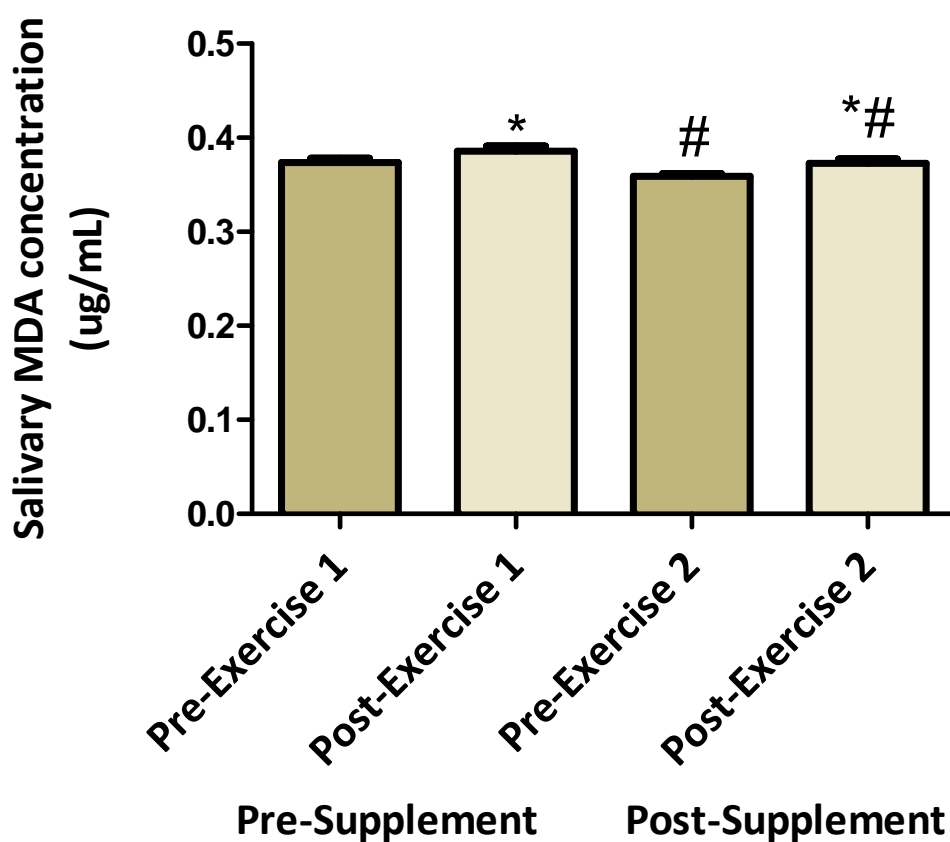


**Figure 9:** Free salivary MDA before and after the exercise bouts prior to and after the vitamin C supplementation period. Free salivary MDA experienced a significant increase after each respective exercise bout (pre vs post, *p<0.01, error bars = SEM, n = 9); free salivary MDA experienced a significant reduction after the four-week supplementation period in the following conditions: reduction in pre-exercise two from pre-exercise one, reduction in post-exercise two from post-exercise one (# p<0.05, error bars = SEM, n = 9).
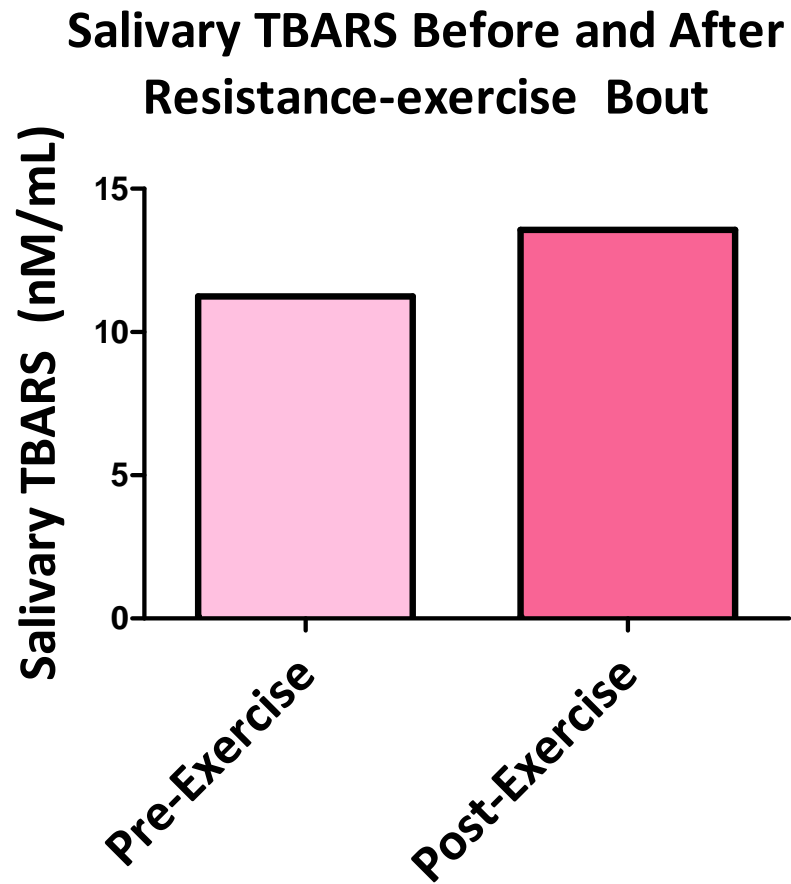
**Figure 10:** The average salivary TBARS of the participants before and after resistance-exercise bout one. No significant differences were experienced between the measures taken before the exercise bout and after the exercise bout (p=0.14, n= 9).

**Table 4:** Individual results from the maximal effort set that was performed after each REIOS exercise bout. Participants experienced a significant increase in maximal effort peak pushing force after the vitamin C supplementation period (*p< 0.05).

## Max Peak Pushing Force (pounds) Pre and Post Vitamin C Supplementation Period

| Subject | Pre-Supplement | Post-Supplement |
|---------|----------------|-----------------|
| 1 | 105.4 | 119.9 |
| 2 | 68.7 | 88.4 |
| 3 | 72.8 | 85.7 |
| 4 | 88.9 | 104.6 |
| 5 | 104.8 | 107.6 |
| 6 | 68.7 | 63.8 |
| 7 | 71 | 73.4 |
| 8 | 101 | 111.8 |
| 9 | 63.9 | 65.2 |
| **Mean** | 82.8 | 91.16 * |
| SD | 16.18 | 20.85 |
| 95% CI +/- | 10.57 | 13.62 |

# Max Peak Pushing Force Pre and Post Vitamin C Supplementation
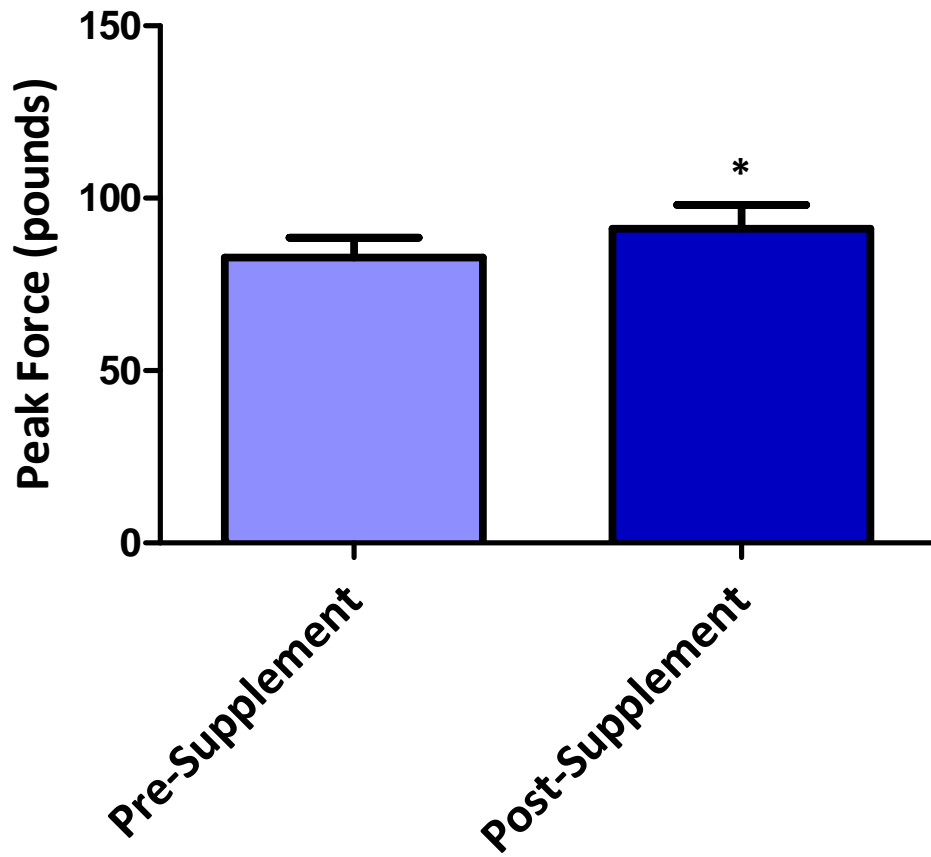


**Figure 11:** Maximal-effort peak pushing force pre and post vitamin C supplementation period. Participants experienced a significant increase in maximal effort peak pushing force after the vitamin C supplementation period (*p<0.05, n = 9).

**Table 5:** Individual results of REIOS average peak pushing force before and after the vitamin C supplementation period. Participants experienced a significant increase in the REIOS average peak pushing force after the supplementation period (* $p<0.05$).

## REIOS Average Peak Force (pounds) Pre and Post Vitamin C Supplementation Period

| Subject | Pre-Supplement | Post-Supplement |
|---|---|---|
| 1 | 67.9 | 61.2 |
| 2 | 59.1 | 49.5 |
| 3 | 63.4 | 76.7 |
| 4 | 32.1 | 37.4 |
| 5 | 69.4 | 91.6 |
| 6 | 57.1 | 70 |
| 7 | 41 | 63.2 |
| 8 | 47.9 | 61.3 |
| 9 | 61.9 | 69.4 |
| **Mean** | 55.53 | 64.48 * |
| **SD** | 12.66 | 15.49 |
| **95% CI +/-** | 9.73 | 11.91 |

# REIOS Average Peak Pushing Force
# Pre and Post Vitamin C Supplementation
# Period



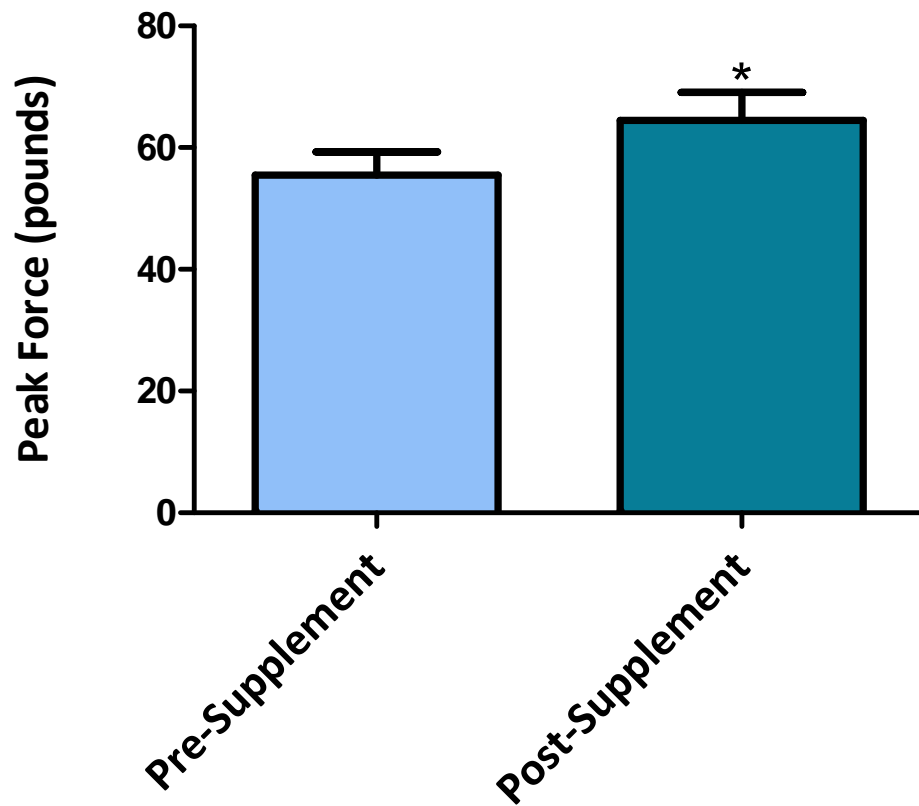**Figure 12:** The average peak force from the REIOS exercise bouts performed pre-supplementation period and post-supplementation period. Participants experienced a significant increase in the REIOS average peak pushing force after the supplementation period (*p<0.05, n = 9).

**7    Discussion**

The major aim of the present study was to use saliva to monitor vitamin C and exercise-induced oxidative stress. Saliva is a biospecimen that accumulates in the oral cavity. It is produced by three major glands: the parotid, the submandibular, and the sublingual. Because saliva is a hypotonic solution, compounds in the blood can permeate from capillaries through the three major glands and into saliva [71]. The compounds that do permeate into saliva can then be measured. Saliva is primarily (97-99.5%) water allowing water-soluble compounds, including vitamin C and MDA, to be especially prevalent [71]; both compounds can be measured in saliva.

One oral dose of vitamin C at 500 mg increased salivary vitamin C 1.83-fold in a single subject after six hours; Mäkilä and Kirveskari demonstrated a 1.96-fold increase in salivary vitamin C six hours after a 500 mg vitamin C dose. Plasma vitamin C will increase two to three-fold after oral vitamin C supplementation [146]. Such results suggest plasma and salivary vitamin C do not mirror each other in reacting to vitamin C intake. This can further be confirmed in no correlation with pre- and post-supplementation period salivary and dietary vitamin C. Participants experienced a large increase in salivary vitamin C after the four-week, 500 mg/day vitamin C supplementation period; a 2.6-fold increase was observed. Plasma vitamin C will increase an average five-fold after similar vitamin C doses (400 mg/day [135]). The present results do not confirm the results found by Leggott et. al. as vitamin C intake did not correlate with salivary vitamin C [134]. A correlation was witnessed in the present study between the increase in salivary vitamin C (delta) and the average dietary vitamin C intake from the dietary recalls completed by the participants. The pathway compounds take to permeate into saliva from the blood is not direct; the content of saliva can furthermore depend on circadian timing,

flow rates, osmotic blood pressure, and hydration. Saliva will reflect pharmaceuticals when consumed at high doses [220]; 500 mg of vitamin C can be considered a pharmaceutical dose. The dose of vitamin C in the present study was high enough and supplemented in such a way for the variables which were not controlled for (i.e. hydration) to not affect the results negatively. Results suggest saliva could be a potential biospecimen in monitoring vitamin C status.

Free MDA permeation into the saliva reflected oxidative stress. After the resistance-exercise bout meant to induce oxidative stress, free salivary MDA significantly increased. Deminice et. al.'s resistance-exercise bout had participants perform three sets of 10 repetitions [39]; plasma lipid peroxidation biomarkers experienced a substantial increase after exercise while salivary biomarkers did not. It also involved six different muscle contracting exercises. The present study was a hybrid version of the resistance-exercise bout with only three muscle contracting exercises; the REIOS exercise did include, however, two muscle contractions within one push-pull repetition. The push-ups and the maximal effort set did not equate to three sets of 10 repetitions. This is to say the intensity of the exercise bouts were relatively low; the lack of a significant increase in TBARS confirms this suggested theory. Salivary TBARS has been shown to reflect oxidative stress, but in higher intensity workouts [221]. The HPLC method of measuring free salivary MDA as a marker of lipid peroxidation was sensitive enough to reflect the minor induction of oxidative stress; this confirms the literature's suggestion that free MDA measured via HPLC is the best method in terms of sensitivity and specificity [2, 216]. MDA's half-life is short [213]; levels may have deteriorated during travel from blood in the isolated-muscle to the saliva. The level of oxidative stress near the site of muscle contraction would be substantially higher [94].

Vitamin C is an antioxidant that is capable of reducing reactive species and its products, such as lipid peroxidation. Hydrogen peroxide as a reactive species can increase lipid peroxidation and MDA formation. Vitamin C is well-known for its ability to reduce lipid peroxidation and can do so in exercise-induced conditions; results have been reflected in plasma markers [176, 223]. A reduction in free salivary MDA within the participants was witnessed after the 4-week supplementation period; this was further witnessed between the conditions as free salivary MDA was reduced from pre-exercise one to pre-exercise two, as well as from post-exercise one to post-exercise two. Similar results were observed when an aerobic exercise was used to induce oxidative stress in Popovic et. al.'s experiment [176]; plasma was the utilized biospecimen for this previous study. This further confirms saliva's ability to reflect systemic biomarkers under exercise conditions.

Systemic markers of vitamin C, and other antioxidants, will substantially increase after exercise-induced oxidative stress [170, 173, 224]. This was confirmed in the present study as salivary vitamin C significantly increased post-exercise. Redox systems are balanced in which free radical production will create an overabundance of antioxidants to reduce the sudden induction of oxidative stress. Reactive species and nonreactive species leading to lipid peroxidation can alter biological processes in ways that adversely affect muscle contraction, function, and overall force [202-204, 222].The resistance-exercise bouts in this study created an environment where vitamin C's antioxidant properties were necessary as lipid peroxidation measured by free MDA significantly increased; both systemic markers permeated into the saliva to confirm this. Saliva contains the antioxidants vitamin E and glutathione [225-227]. Vitamin C plays a major role in reducing oxidized vitamin E and glutathione. It is possible the increase in

salivary vitamin C after exercise resulted from a reduction of reactive species as well as oxidized antioxidants.

Reducing oxidative stress, which would otherwise hinder muscular force and contractibility, via antioxidants should theoretically counter the negative associated effects. The present study illustrated a substantial increase in muscular force in conjunction with the reduction of oxidative stress biomarkers, specifically lipid peroxidation via free salivary MDA, after the vitamin C supplementation period. Participants' peak pushing force increased for both the maximal-effort set and the REIOS exercise performed after the supplementation period. Push-ups were not evaluated for performance measures. The increases might have been due to either reactive/non-reactive species' effect on muscular force or their effect on fatigue; reducing oxidative stress will reduce muscle fatigue [228]. Force produced by the muscle, specifically the myofilament portion, is heavily influenced by sarcoplasmic reticulum calcium. Reactive/nonreactive species, such as hydrogen peroxide radicals, diminish myofilaments sensitivity to calcium which will ultimately reduce muscular force [202]; vitamin C is a common scavenger of such reactive/nonreactive species. Results from the present study conflict with previous studies examining vitamin C supplementation and muscular function [201, 229]. Such studies used vitamin C supplementation at higher doses making it susceptible to excretion. Vitamin C at high doses, for that matter, will exhibit pro-oxidant properties and increase hydrogen peroxide production [230] leaving muscular force susceptible to restriction. The present study utilized a technique meant to maximize bioavailability and tissue saturation [231]. By not consuming all 500 mg in one dose, the vitamin C supplement would not be fully excreted. Maximal bioavailability of vitamin C allows its properties to be utilized to their fullest.

The present study has a number of limitations. No other systemic (i.e. plasma or urinary) biomarkers for vitamin C or for free MDA were examined; this makes it difficult to suggest vitamin C was not being excreted, plasma vitamin C was substantially increased in a bioavailable form, oxidative stress was induced systemically, or damage from the induced oxidative stress was prevalent. The dietary recalls utilized in this study were another limitation, as multiple subjects did not fully complete all three; additionally, recalls were not evenly spaced, potentially affecting the accuracy of estimated average vitamin C intake. The correlation between increased salivary vitamin C and average vitamin C intake, therefore, may not be accurate. Furthermore, the intake of vitamin C supplementation was not fully controlled as participants might not have adhered to taking vitamin C every 12 hours. The population of the present study was small but diverse; the only exclusion criteria were: regular consumption of vitamin C supplements and regular performance of resistance exercise. Results may not be generalizable to those who regularly take vitamin C supplements or engage in resistance exercise. The small sample size also created a limitation. Only upper-body, bench-like movements were utilized during this experiment to measure increases in the performance; the literature on relating strength performance via isokinetic-exercises and free weight movements (i.e. the bench press) is limited. Strength values between isokinetic-exercise and free-weight do not necessarily differ [232]. The strength values did increase in the present study but no other strength assessment was utilized to confirm the results. This might be a limitation.

## 8    Future Research Suggestions

The established range of salivary vitamin C is currently 0.7-0.9 µg/mL; the nine participants involved in the present study averaged 0.42 µg/mL prior to supplementation, ranging from 0.16 to 1.78 µg/mL. To utilize saliva as a biospecimen in health status assessment,

establishment of a healthy range of salivary biomarkers would be required as the previous range and the witnessed range differed. A larger study population would be necessary to establish the ranges. No safe range has been established for free salivary MDA; a similar saliva collection study with a larger population would need to be conducted. Levels of salivary/systemic vitamin C are influenced by: vitamin C intake (including supplementation), oxidative stressors (like exercise), blood pressure, hydration, and urinary vitamin C excretion; future research in this area should control for these variables. Correlations with these variables and salivary vitamin C would need to be established. A top priority should be to establish a correlation between salivary vitamin C and free MDA with their systemic markers. To better correlate free salivary MDA with systemic MDA, variables such as exercise intensity and antioxidant status should be controlled. Variables which affect saliva and its contents, such as blood pressure and hydration, should also be controlled.

In exercise, biomarkers that reflect oxidative stress play a part in important sport and exercise adaptation. Future researchers should examine if such biomarkers reflect real damage or oxidative stress or are expressing beneficial exercise adaptations at the cellular and molecular level. The NF-κB and of PGC-1α pathways require reactive species for up-regulation leading to exercise adaptation; Vitamin C's role in redox balance should be taken into account for exercise adaptation as vitamin C supplementation will prevent this pathway [181]. There may be a certain range in which specific antioxidant and oxidative biomarkers are beneficial; falling outside of this range may be detrimental and setting an established range may alleviate this concern. A variety of different variables can affect exercise adaptation and general performance over time (i.e. diet, training intensity, training schedule, level of skill, and other performance supplementation); these variables should be controlled for in future research. Vitamin C as a

performance enhancer should be further examined under controlled conditions, specifically vitamin C dose, supplementation duration, and the level of exercise experience. The present results suggest exercisers might benefit from vitamin C supplementation at a certain dose during the beginning of the exercise adaptation process. Such benefits might plateau as one develops experience in exercise and sport.

**9      Conclusions**

Participants' salivary vitamin C increased after the vitamin C supplementation period; vitamin C was supplemented twice daily for a total of 500 mg per day for 28 days. Similar increases in systemic vitamin C markers have been observed with vitamin C supplementation. Salivary vitamin C increased substantially after each isokinetic-resistance exercise bout; this will occur systemically as well further suggesting an association between salivary and systemic vitamin C. Free salivary MDA was examined via HPLC and significantly increased after the performed exercise-bouts. Participants' free salivary MDA reduced after the 28-day vitamin C supplementation period, both overall and between exercise conditions. Systemic MDA reacted to exercise and vitamin C supplementation in similar manners. The present results suggest saliva to be a potential biospecimen in monitoring vitamin C and exercise-induced oxidative stress. Salivary TBARS was not affected however, by the exercise bout; this might have been due to the exercise bout lacking intensity and/or the assay lacking sensitivity or specificity. An increase in peak pushing force measurements was observed after the vitamin C supplementation period; this occurred in conjunction with a decrease in free salivary MDA, an oxidative stress biomarker. Vitamin C supplementation was able to increase peak muscular pushing force by reducing oxidative stress in untrained individuals, and could potentially be a useful performance enhancing supplement.

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