Effect of Ursolic Acid Consumption from Apple Wax on Cytokine Levels in the Blood and Handgrip Strength

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Abstract

The wax of apples contains ursolic acid, a triterpenoid that has been shown to have anti-inflammatory, anticarcinogenic, and antioxidant properties, as well as inducing a decrease in muscle atrophy and an increase in hypertrophy. Cytokines are a group of proteins released into the blood that have pro- and anti-inflammatory functions. Purpose: To determine the effect of consuming an apple with the peel, without the peel, or no apple, once a day, on pro- and anti-inflammatory cytokine levels and handgrip strength. Methods: Participants were divided into three groups (No Apple, Peeled Apple, and Whole Apple) and consumed their required amount of apple once a day each weekday for three weeks. Blood samples, handgrip strength and anthropometric measurements (height, weight, blood pressure) were taken from the participants (N=32, age = 52±8, BMI = 31.2±5.5) at the beginning and end of the three-week intervention. The blood samples were analyzed for cytokines using a bead-based multiplex assay. Results: There was a significant main effect of time on dominant handgrip strength, where dominant handgrip strength decreased in all three groups over time (35.283 kg average handgrip strength pre-intervention, 33.753 kg average post-intervention; p = 0.015). There was also a significant main effect of time on IL-8 levels, where IL-8 levels decreased significantly post-intervention in the Whole Apple group (150.250 vs. 113.833 pg/mL; p=0.034), and tended to decrease in the Peeled Apple (162.364 vs. 129.564 pg/mL; p=0.078) and No Apple (156.778 vs. 122.556 pg/mL; p=0.081) groups. There were no significant changes in IL-1β, IL-6, IL-10, or TNF-α post-intervention. Conclusions: Aside from IL-8, no significant changes could be attributed to ursolic acid consumption, and IL-8 levels tended to decrease in all groups, so those results may also not be due to ursolic acid consumption. This study was limited by its short intervention period, small number of participants, and small concentration of ursolic acid consumed, all factors that should be adjusted in future studies.

Introduction

Obesity is currently a national health problem of epidemic proportions; two-thirds of adults in America are overweight and one-third are classified as obese (defined as a BMI greater than 30) (Executive Summary). Obesity is characterized by excess adipose tissue, which causes chronic inflammation (Gregor). Chronic inflammation has been shown to increase the risk of developing many lifestyle-related diseases, including metabolic syndrome, cardiovascular disease, type II diabetes, colon cancer, and breast cancer (Weisberg). Chronic inflammation also causes skeletal muscle dysfunction and atrophy (Londhe). These diseases are costly, both to the physical health of those who develop them and the cost to America's healthcare system (Executive Summary). On average, obese individuals incur 27% higher outpatient healthcare costs, 46% higher inpatient healthcare costs, and 80% higher prescription drug costs than non-obese individuals (Executive Summary). In 2008, obesity-related medical costs in the United States accounted for $147 billion, and many of these diseases, such as cardiovascular disease, account for the leading causes of death in the United States (Executive Summary).

Certain lifestyle choices, such as diet and exercise, have been shown to have protective effects against lifestyle related diseases, both by decreasing adiposity and decreasing the chronic inflammation associated with these diseases (Gregor). A diet high in fruits and vegetables has been shown to be especially effective in decreasing chronic inflammation (Hyson). Apples are one of the fruits that have been widely studied and have a strong positive correlation with decreased inflammation (Hyson).

One theory for the reason apples have this anti-inflammatory effect is due to a group of phytochemicals called triterpenoid acids, including ursolic acid, betulinic acid, and oleanolic acid, that are present in the cutaneous wax of apples. Ursolic acid is the triterpene present in largest amounts in apples, and it is also found in blueberries, olives, grapes, and tomatoes. Ursolic acid has been shown to have many biological effects, including anti-inflammatory, anti-carcinogenic, and antioxidant effects, as well as activating thermogenesis, decreasing muscle atrophy and increasing muscle protein synthesis (Katashima). Intraperitoneal injections of ursolic acid (25 mg/mL) have been shown to decrease muscle atrophy and increase hypertrophy by increasing insulin-like growth factor (IGF-1), growth hormone, and insulin secretion in mice (Katashima). Increased muscle hypertrophy due to ursolic acid supplementation (0.14% diet) has been shown to increase grip strength, decrease resting heart rate, and improve exercise capacity in mice (Kunkel). Skeletal muscle is also responsible for the majority of metabolic processes in the body, so increasing skeletal muscle hypertrophy could help decrease metabolic diseases. Ursolic acid has been shown to increase insulin sensitivity, which increases glucose transported into the muscle for metabolism and decreases blood glucose, and could be protective for those with Type 2 Diabetes. (Katashima).

Thermogenesis is the process of breaking down food into heat energy as a byproduct of energy production. Consuming a diet high in saturated fat has been shown to stimulate inflammatory pathways, which causes a breakdown in thermogenic signaling hormones, such as leptin and thyroid hormone, which in turn can increase body fat mass (Katashima). Ursolic acid is thought to increase thermogenesis by activating the uncoupling protein 1 (UCP1) and AMP-activated protein kinase (AMPK) in adipose tissue, thus increasing the metabolic activity of adipose tissue (Katashima). In one study, mice treated with 0.5% ursolic acid in their diet for six weeks showed weight loss and increased energy expenditure (Katashima). Ursolic acid has also been shown to increase brown fat, a type of fat that undergoes thermogenesis to maintain body temperature (Kunkel). The fact that ursolic acid leads to an increase in skeletal muscle and brown fat, two of the most metabolically active tissues in the body, is a possible explanation for why ursolic acid increases energy expenditure and promotes weight loss.

Cytokines are a group of proteins that are released into the blood by a variety of tissues in the body, including immune cells, which have many wide-ranging effects. They can be pro-inflammatory or anti-inflammatory, and have possible immune and metabolic functions (Wu and Ballantyne). There are many different types of cytokines, but those examined in this study were interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) due to their effects on metabolism and immune function.

IL-1β is a pro-inflammatory cytokine that is stimulated by nutrient excess. Long-term elevated levels of IL-1β result in chronic inflammation (Gregor). Elevated levels of IL-1β are also associated with increased risk of cancer (Garza-Gonzalez).

IL-6 is the most highly studied cytokine, and it has many wide-ranging effects in the body. During acute inflammation, IL-6 acts in an anti-inflammatory role by decreasing the amount of pro-inflammatory cytokines and activating other anti-inflammatory cytokines, thus acting protectively to control the immune response (Gabay). Acute treatment with IL-6 has also been shown to increase insulin sensitivity, increase glucose uptake by myocytes, increase lipolysis and fatty acid oxidation in myocytes and adipocytes, and increase muscle hypertrophy (Wu and Ballantyne). On the other hand, in states of chronic inflammation such as are associated with obesity, IL-6 has been found to play a pro-inflammatory role, mediating the removal of neutrophils from and the arrival of monocytes to the site of inflammation, a key difference between acute and chronic inflammation (Gabay). IL-6 also increases insulin resistance in states of chronic inflammation (Wu and Ballantyne).

IL-8 is a pro-inflammatory cytokine that activates neutrophils and attracts them to areas of inflammation to help promote the immune response (Bickel). IL-8 also stimulates angiogenesis in muscular tissue (Pedersen). It is upregulated from exercise and resting levels are elevated in in individuals with obesity and type 2 diabetes (Wu and Ballantyne).

IL-10 is one of the original cytokines that was discovered, and it possesses many anti-inflammatory and immune functions in the body (Mosser). Recently, IL-10 has been associated with tumor progression by suppressing the immune system (Mosser). Research is currently investigating possible therapeutic uses of IL-10 suppression to treat cancer and IL-10 injection to treat diseases of the immune system, such as psoriasis. There is hope that one day IL-10 levels will be able to be manipulated to control immune response, but that will be difficult to achieve without causing immunosuppression and affecting other immune system functions (Mosser).

TNF-α is a pro-inflammatory cytokine that is elevated during states of chronic inflammation, such as obesity (Wu and Ballantyne). It also induces insulin resistance by inhibiting insulin sensitivity and glucose uptake, which can lead to the development of Type 2 Diabetes in a state of chronic inflammation (Wu and Ballantyne). Understanding how these cytokines function within the body and how they may be altered by diet and exercise is of critical importance, because cytokine immunotherapy could potentially be a future way to fight diseases of chronic inflammation such as type 2 diabetes and the development of some types of cancer.

The purpose of this study was to determine the effect of consuming an apple with the peel, without the peel, or no apple, once a day, on pro- and anti-inflammatory cytokines and handgrip strength. This study was a pilot study, so it was also intended to see if a larger study of this design would be possible to conduct. I hypothesized that consuming apples with the peel would result in increased anti-inflammatory cytokines, decreased pro-inflammatory cytokines, and increased grip strength compared to the other groups, due to the presence of ursolic acid in apple peels, although I did not think that a three-week intervention would be long enough to see significant changes in any of these variables.

Methods

Participants in this study were volunteers who work at a local furniture factory in western North Carolina. Inclusion criteria were that the participants had to be over the age of thirty-five and have a BMI greater than 25. Exclusion criteria were that participants could not have diabetes. Participants were recruited through an invitation sent to 270 factory workers. Of the 270 workers, 80 volunteered to participate, 39 were selected to participate (based on inclusion and exclusion criteria), and 32 completed the study in its entirety. Participants were randomly assigned to three groups and were required to either: a) consume one whole sliced apple with the peel, b) consume one whole sliced apple without the peel, or c) consume no apples each weekday (Monday – Friday) for three straight weeks. The study began with n=13 in each group. Apples were processed and hand delivered to participants each day by study personnel. Contract nurses drew blood samples and took anthropometric measurements including BMI, waist circumference, and blood pressure at the beginning and end of the three-week intervention period.

Handgrip strength was measured with a dynamometer at the beginning and end of the three-week intervention period for both the subject's dominant and non-dominant hands. The dynamometer was placed in position 2. Participants were directed to hold their arm at a 90º angle, with the wrist at a neutral 0º angle. Three trials were conducted with each hand, alternating hands, and the best of the three trials was used for analysis.

Cripp's Pink apples were the variety of apples used. The peels from these apples contained approximately 10.33 mg/g dry weight of ursolic acid, and 12.30 mg/g dry weight of total triterpenoid acids. They were prepared after 12:00 pm the day before they were consumed by peeling them if necessary, quartering them, and soaking them in a 0.1% ascorbic acid solution (0.5 g ascorbic acid: 5 liters water) for 10-15 minutes to preserve freshness. Apples were delivered to the factory each morning and handed out to participants to consume during their morning break, at approximately 9:00 am each workday.

Serum samples were analyzed for cytokines with a magnetic bead-based multiplex assay (MILLIPLEX® Human Cytokine Panel) using the MAGPIX instrument and MILLIPLEX® Analyst Software according to manufacturer's specifications. The samples were run in duplicate, and the average of the two values was taken.

A 2x3 (time x treatment) two-way repeated measures ANOVA was utilized to analyze handgrip and cytokine data. Following a significant F-ratio, a Bonferroni post hoc analysis was used to determine specific treatment effects using SigmaPlot 12.5 statistical analysis software (Systat Software, Inc.). A priori significance was set at a p-value of α=0.05. Data are expressed as Mean ± SEM.

Results

The average age of the participants was 52±8, BMI was 31.2±5.5, 78% of the participants were female, 40% were on blood pressure medication, 13% were on cholesterol medications, 72% were post-menopausal, 36% had a family history of diabetes, 18% had a family history of stroke, and 41% had a family history of cardiovascular disease.

There was no significant difference between the different subject groups over time, but there was a significant main effect of time on dominant handgrip strength (Figure 1). In all groups (overall n = 32), dominant handgrip strength decreased significantly over time, from an average of 35.2 kg pre-intervention, to an average of 33.8 kg post-intervention ($p = 0.015$). No significant differences were present for non-dominant handgrip strength over time (overall p-value = 0.389; Figure 2).

No significant differences were observed in IL-1β levels (overall p-value = 0.312; Figure 3). Upon conducting a power analysis, it was determined that 33 subjects per group would be required to observe any significant difference in IL-1β levels in this experiment (power = 0.8; $\alpha = 0.05$).

No significant differences were observed in IL-6 levels (overall p-value = 0.454; Figure 4). Upon conducting a power analysis, it was determined that 66 subjects per group would be required to observe any significant difference in IL-6 levels in this experiment (power = 0.8; $\alpha = 0.05$).

No significant differences were observed overall in IL-8 levels (overall p-value = 0.989; Figure 5), but there was a significant main effect of time on IL-8 levels. IL-8 levels decreased significantly over time, from an average of 156.5 pg/mL pre-intervention, to

121.9 pg/mL post-intervention ($p = 0.002$). In the No Apple group ($n = 12$), IL-8 levels trended towards significance, decreasing from 156.8 pg/mL pre-intervention to 122.6 pg/mL post-intervention ($p = 0.081$). In the Peeled Apple group ($n = 9$), IL-8 levels also trended towards significance, decreasing from 162.4 pg/mL pre-intervention to 129.6 pg/mL post-intervention ($p = 0.078$). In the Whole Apple group ($n = 11$), IL-8 levels decreased significantly over time, from 150.3 pg/mL pre-intervention to 113.8 pg/mL post-intervention ($p = 0.034$).

No significant differences were observed in IL-10 levels (overall p-value = 0.454; Figure 6). Upon conducting a power analysis, it was determined that 33 subjects per group would be required to observe any significant difference in IL-10 levels in this experiment (power = 0.8; $\alpha = 0.05$).

No significant differences were observed in TNF-α levels (overall p-value = 0.915; Figure 7). Upon conducting a power analysis, it was determined that 77 subjects per group would be required to observe any significant difference in TNF-α levels in this experiment (power = 0.8; $\alpha = 0.05$).

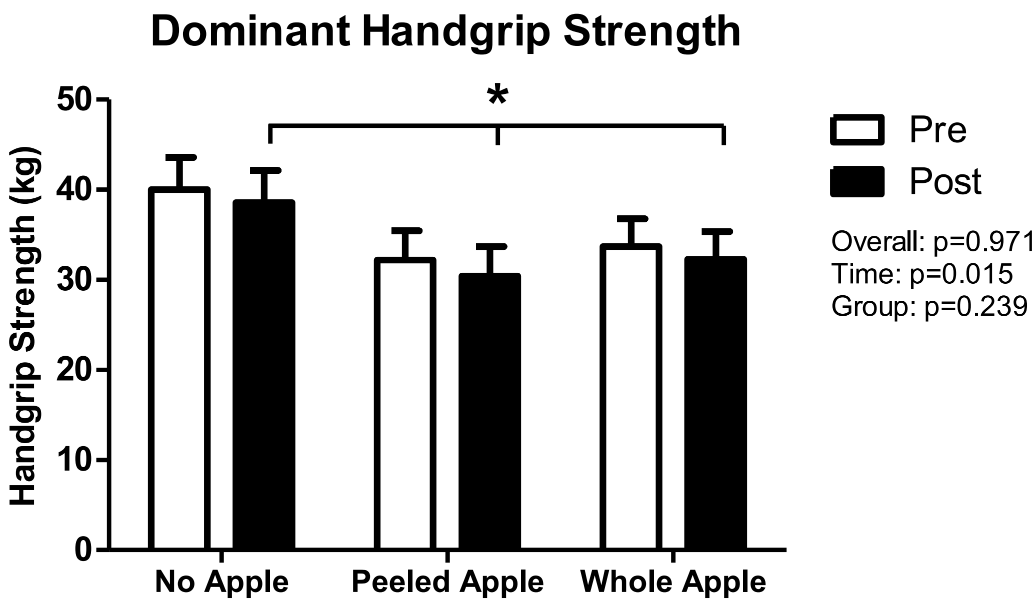

Figure 1. Handgrip strength of the dominant hand before (Pre) and after (Post) the 3-week intervention period. Dominant handgrip strength was significantly lower after the 3-week intervention period, compared to before (significant main effect of time; p=0.015); however, no significant differences were revealed between groups. Data are displayed as Mean ± SEM. * - indicates significantly different than Pre within the same treatment group.

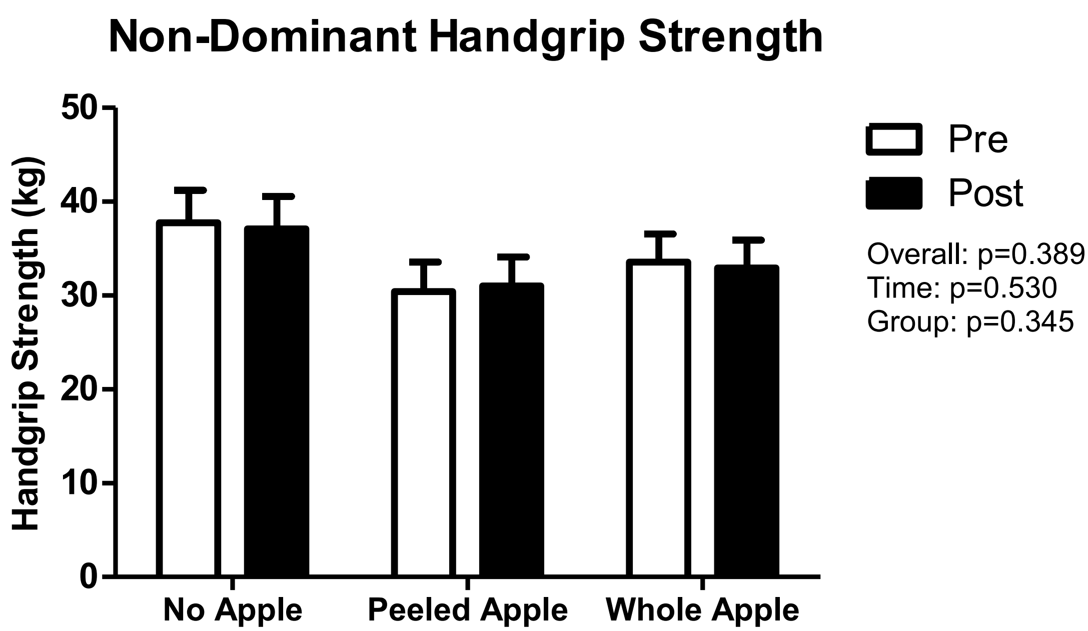

Figure 2. Handgrip strength of the non-dominant hand before (Pre) and after (Post) the 3-week intervention period. There were no significant differences in non-dominant handgrip strength with treatment. Data are displayed as Mean ± SEM.

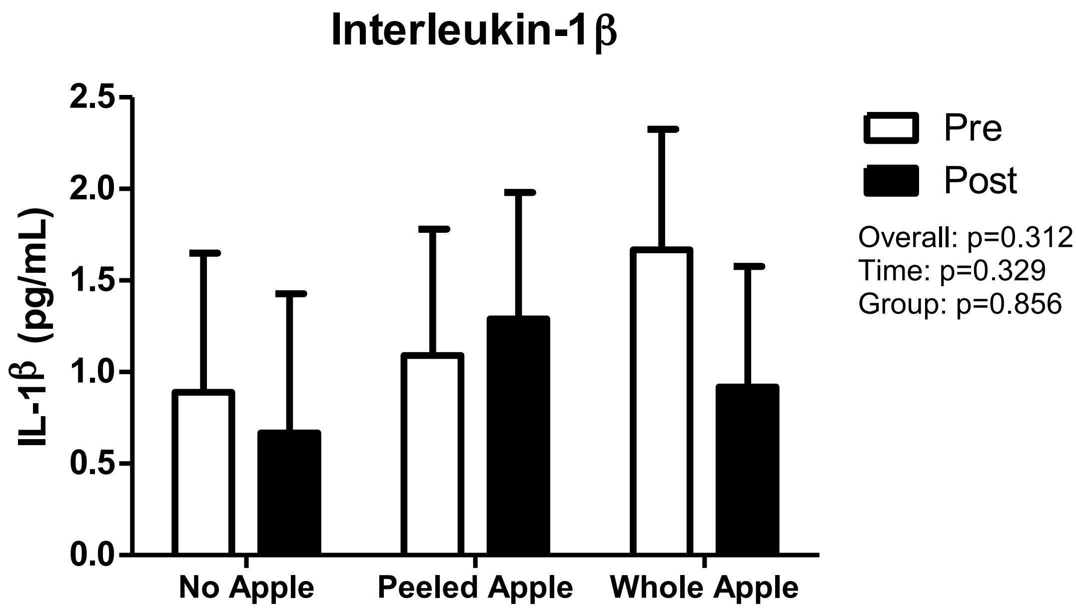

Figure 3. Interleukin (IL)-1β before (Pre) and after (Post) the 3-week intervention period. There were no significant differences in IL-1β with treatment. Data are displayed as Mean ± SEM.

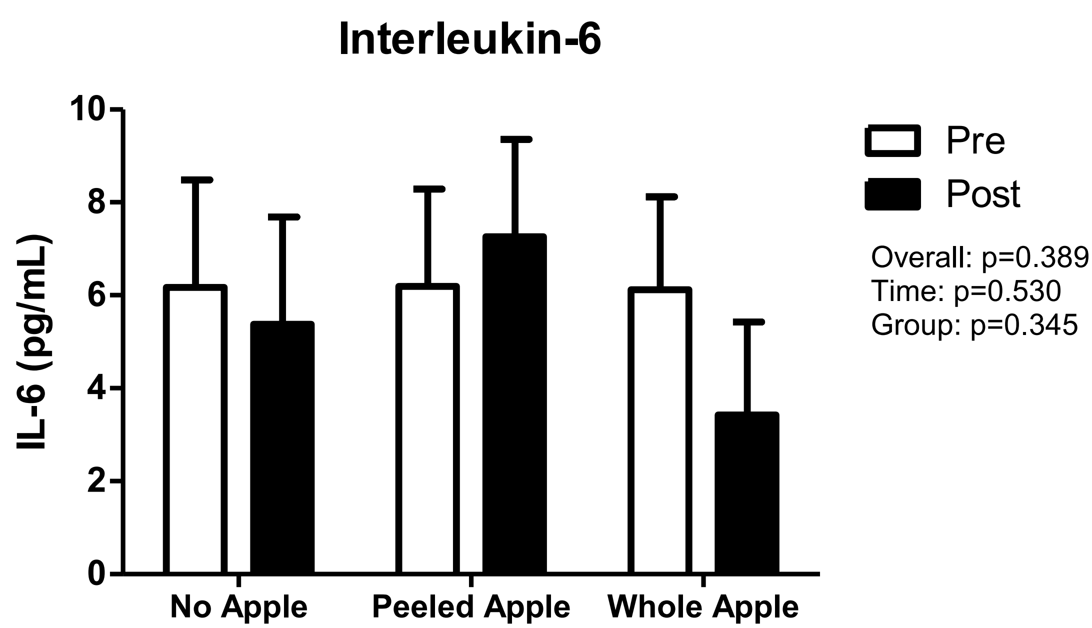

Figure 4. Interleukin (IL)-6 before (Pre) and after (Post) the 3-week intervention period. There were no significant differences in IL-6 with treatment. Data are displayed as Mean ± SEM.

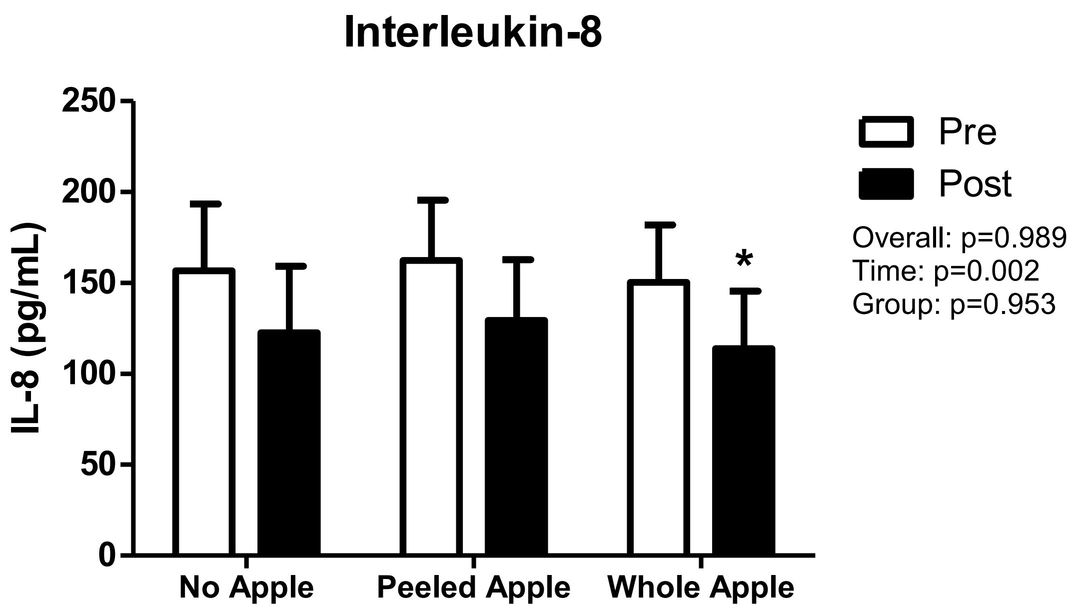

Figure 5. Interleukin (IL)-8 before (Pre) and after (Post) the 3-week intervention period. There was a significant main effect of time, revealing that IL-8 was significantly lower after the 3-week intervention period, compared to before, in the Whole Apple group only, (p=0.034). IL-8 tended to be lower after the 3-week intervention period, compared to before, in the No Apple and Peeled Apple groups, (p=0.081 and p=0.078, respectively). Data are displayed as Mean ± SEM. * - indicates significantly different than Pre within the same treatment group.

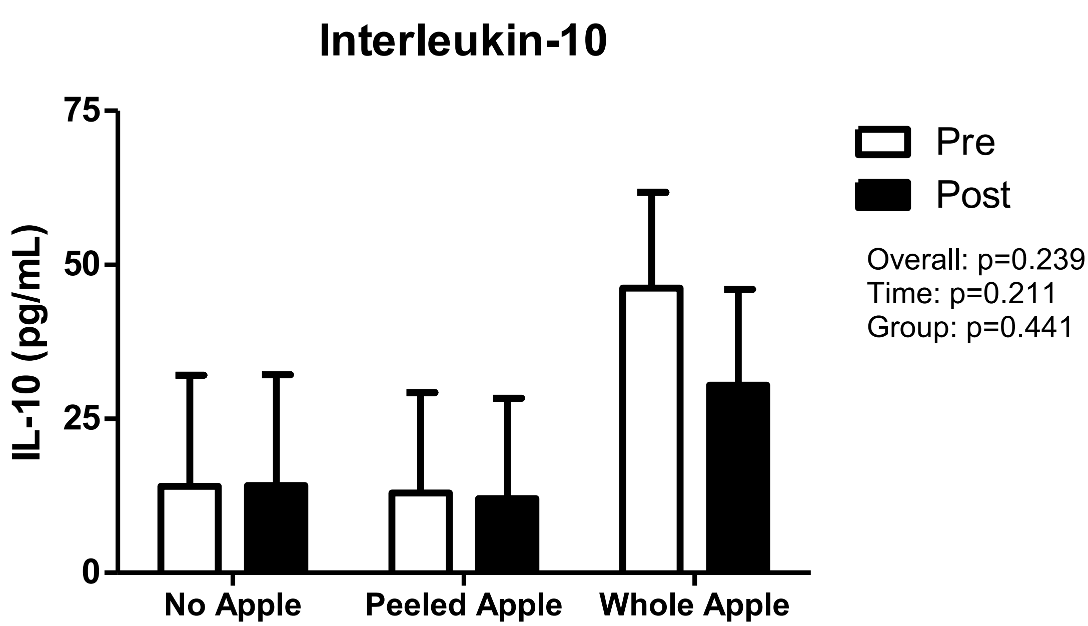

Figure 6. Interleukin (IL)-10 before (Pre) and after (Post) the 3-week intervention period. There were no significant differences in IL-10 with treatment. Data are displayed as Mean ± SEM.

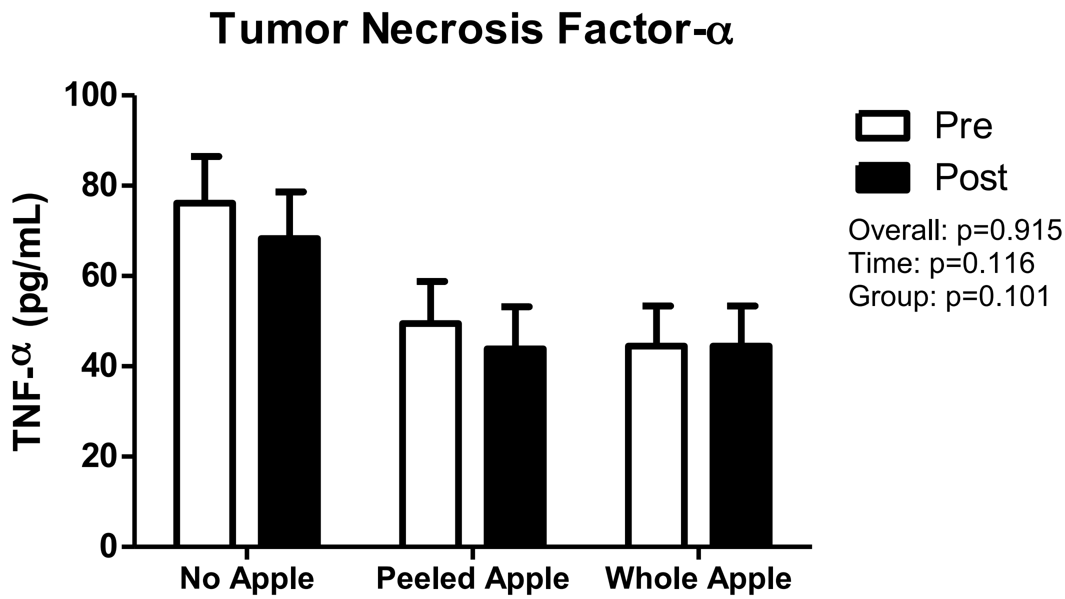

Figure 7. Tumor Necrosis Factor (TNF)-α before (Pre) and after (Post) the 3-week intervention period. There were no significant differences in TNF-α with treatment. Data are displayed as Mean ± SEM.

Discussion

The purpose of this study was to investigate whether an apple a day, with or without the peel, for three weeks had any influence on handgrip strength or circulating inflammatory markers in the blood. I hypothesized that consuming apples, with the peel, would result in increased anti-inflammatory cytokines, decreased pro-inflammatory cytokines, and increased grip strength compared to the other groups. Contrary to my hypothesis, dominant handgrip strength decreased significantly from pre-intervention to post-intervention for all groups. This was unexpected, because the participants in the Whole Apple group were consuming ursolic acid, which has been shown to increase muscle hypertrophy and decrease muscle atrophy in mice (Katashima). A possible reason that handgrip strength did not increase is that a three-week intervention is not a long enough time to result in a significant change in body composition due to ursolic acid consumption. Another potential reason that the results were not consistent with the results of previous studies is that the mice in the studies were consuming much higher concentrations of ursolic acid than the people in this study. The mice in one study consumed 0.14% or 0.27% ursolic acid diets (Kunkel). The people in the Whole Apple group for this study consumed one apple with 10.33 mg/g ursolic acid per day, which is about a 0.00344% ursolic acid diet, assuming a 2,000-calorie diet. This amount is nearly 100 times less than that consumed by mice in the aforementioned study.

In another study, mice had intraperitoneal injections of 25 mg/mL ursolic acid, a higher concentration than in the apples that was also injected directly as opposed to consumed through food (Katashima). The interventions were also longer, at 6-17 weeks as opposed to just 3 weeks in this study (Katashima). All of these differences could explain why handgrip strength did not increase post-intervention. A possible explanation for the decrease in handgrip strength could be due to whether the participants had a strenuous day at work shortly before the post-data was collected, or if they were tired of the study by the end of it.

The other main purpose of the study was to investigate whether inflammatory cytokines were influenced by consuming an apple a day. One significant result of this study is that IL-8, a pro-inflammatory cytokine, decreased post-intervention. It was in accordance with the hypothesis that IL-8 levels would decrease in the Whole Apple group post-intervention, due to the anti-inflammatory effects of ursolic acid. It was not expected that IL-8 levels would decrease in all groups post-intervention, because the No Apple and Peeled Apple groups were not consuming ursolic acid. Therefore, the decrease in IL-8 levels post-intervention cannot be attributed to increased apple consumption, per se. It is unclear what could have caused this decrease in IL-8 in all groups, but it could be due to other dietary or lifestyle factors that were not regulated by the study. Perhaps the participants began consuming healthier food in general because they were being recorded, and these foods included more anti-inflammatory factors than previously, when they were not enrolled in the study.

There were no significant changes in levels of IL-1β or TNF-α, the other pro-inflammatory cytokines measured for pre- and post-intervention. This was contrary to the hypothesis, which predicted that levels of these cytokines would decrease post-intervention.

There was also no significant change in levels of the anti-inflammatory cytokine IL-10 post-intervention. This result was contrary to the hypothesis that IL-10 levels would increase post-intervention in the Whole Apple group due to ursolic acid's anti-inflammatory properties.

No significant change was measured in IL-6 levels post-intervention either. IL-6 is a cytokine that has an anti-inflammatory role during acute inflammation, but a pro-inflammatory role during states of chronic inflammation such as obesity. It was expected that IL-6 levels would decrease post-intervention in the Whole Apple group due to the decreased state of chronic inflammation caused by ursolic acid in the apple peels.

Overall, very few significant results were observed in this study. One large reason for this is probably because the intervention period was too short to cause any significant biological changes. The participants also were not consuming large amounts of ursolic acid, and were only consuming apples five days a week, as they did not consume them on the weekends. Consuming fifteen apples over three weeks is probably not enough to have a significant impact on one's health. Another limiting factor of the study is that the participants' activity level and diet outside of the study were not monitored, both of which play a great role in cytokine production and handgrip strength. Another factor that could have influenced the results of the study is that the participant's diets before the study were not monitored. If the participants were already eating one or more apples a day, their anti-inflammatory cytokine levels may not change throughout the three-week study, or may even decrease post-intervention if they were consuming less ursolic acid than before.

There were also only 32 participants, separated in three groups, who had pre- and post-intervention blood samples, which made it difficult to achieve significant results. Therefore, this study was likely under powered.

There were many limitations to this study, but as a pilot study it served its purpose of demonstrating to the investigators that it is possible to conduct a study of this design in a factory setting. Future studies should be conducted with more participants over a longer period of time, and also have the participants consume apples on Saturday and Sunday, as well, to achieve significant results.

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