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Effects of a 21-Day Daniel Fast with and without Krill Oil Supplementation on Blood Lipids and Lipid Peroxidation

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EFFECTS OF A 21-DAY DANIEL FAST WITH AND WITHOUT KRILL OIL SUPPLEMENTATION ON BLOOD LIPIDS AND LIPID PEROXIDATION

by

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DEDICATION

TO MY FATHER, WHO TAUGHT ME TO OBSERVE WHILE OTHERS MERELY LOOK.

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I would like to acknowledge Dr. Richard Bloomer, who took me under his wing during my tenure at the University of Memphis. A fantastic scientist, Dr. Bloomer is an even better person.

ABSTRACT

Aims: The purposes of this investigation were 1) to replicate or improve upon the findings of the initial study of the Daniel Fast, while 2) maintaining or improving high-density lipoprotein cholesterol (HDL-C) via krill oil supplementation.

Methods: 39 subjects (12 men and 27 women) completed a 21-day Daniel Fast. During the fasting period, 19 subjects were randomly assigned to consume krill oil (2 grams per day in capsules), while the other 20 subjects consumed placebo capsules. Blood samples were collected immediately prior to and following the fast and assayed for blood lipids and malondialdehyde.

Results: Krill oil supplementation had no effect on blood lipids or malondialdehyde ($p > 0.05$). Total cholesterol, HDL-C, and low-density lipoprotein cholesterol each decreased significantly from pre to post fast ($p < 0.05$).

Conclusion: Although the findings of the initial Daniel Fast study were largely replicated, krill oil supplementation does not further improve the blood lipid profile or result in a decrease in malondialdehyde.

Key Words: Daniel Fast, blood lipids, cholesterol, oxidative stress, malondialdehyde

PREFACE

This thesis was written in article format for submission to *The American Journal of Clinical Nutrition* following defense. The content and organization of this thesis represent and fulfill the requirements for submission to this journal.

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
SUBJECTS AND METHODS	3
Subjects and Screening	3
Heart Rate and Blood Pressure	5
Anthropometric Variables	6
Blood Collection and Biochemical Variables	6
Conditions (Supplementation)	7
Dietary Records and Physical Activity	7
Post-Fast Questionnaire	8
Statistical Analysis	8
RESULTS	8
Compliance, Mood, and Satiety	9
Body Weight and Body Fat	9
Blood Lipids and Malondialdehyde	9
Dietary Data	9
DISCUSSION	10
CONCLUSION	15
REFERENCES	16
APPENDICES	
A. Tables	19
B. Extended Literature Review	24

C. Institutional Review Board Approval 65

INTRODUCTION

Hundreds of scientific investigations have examined the health-related benefits of fasting. The fasts featured in these studies can be divided into two broad groups: those that restrict energy intake but do not restrict food choices, and vice-versa. Within the latter group, a religiously-motivated fast that has only recently been scientifically examined (1, 2) is the Biblically-based Daniel Fast. This fast proscribes the intake of animal products, refined foods, white flour, preservatives, additives, sweeteners, flavorings, caffeine, and alcohol. Consequently, only fruits, vegetables, whole grains, nuts, seeds, and oil are allowed during the Daniel Fast; although energy intake is unrestricted.

The initial investigation of a 21-day Daniel Fast noted favorable changes in a variety of health-related biomarkers (1, 2). Specifically, systolic and diastolic blood pressure (BP), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), hydrogen peroxide, and malondialdehyde (MDA) each decreased from pre to post fast, while Trolox Equivalent Antioxidant Capacity and nitrate/nitrite both increased. Also, C-reactive protein, serum insulin, and the homeostatic model of insulin resistance were each lowered to a clinically meaningful, albeit statistically insignificant, extent. Unfortunately, this investigation also noted a 14% reduction (55.65 ± 2.50 vs. 47.58 ± 2.19 $mg \cdot dL^{-1}$) in high-density lipoprotein cholesterol (HDL-C) (1).

High-density lipoprotein cholesterol protects against cardiovascular disease mainly via its role in reverse cholesterol transport. Through this process, HDL-C travels throughout the bloodstream and gathers cholesterol from areas of overabundance, including atherogenic foam cells (3). High-density lipoprotein cholesterol then transports this cholesterol to the liver, where it is converted into bile and excreted (4). In addition to its role in reverse cholesterol transport,

HDL-C helps protect against inflammation, LDL oxidation, and thrombosis (4). Also, HDL-C increases nitric oxide production and inhibits the expression and migration of atherosclerotic adhesion molecules (3). Given that a 1 $mg \cdot dL^{-1}$ increase in HDL-C lowers the risk of developing cardiovascular disease by 2% and 3% in men and women, respectively (5), a 14% reduction in this lipoprotein is not ideal if the Daniel Fast is to be regarded as a dietary regimen that promotes optimal cardiovascular health.

Theoretically, including an intervention known to raise HDL-C could counterbalance a fast-induced lowering of this lipoprotein. Three interventions that have been scientifically demonstrated to increase HDL-C are the performance of regular exercise, the use of prescription drugs, and the intake of selected nutrients. A recent meta-analysis found that aerobic exercise training increases HDL-C by an average of 2.53 $mg \cdot dL^{-1}$ (6), which is insufficient to offset an 8.07 $mg \cdot dL^{-1}$ decrease that was reported in the initial study of the Daniel Fast (1). Moreover, although several pharmaceuticals (statins, niacin, fibrates, thiazolidnediones, glitazars, cholesteryl ester transfer protein inhibitors, and recombinant HDL infusion) have been shown to substantially raise HDL-C (3), use of these drugs without a medical need departs from the abstemious philosophy of a Daniel Fast. All-natural dietary components or nutritional supplements, on the other hand, appear to be ideal intervention candidates.

A promising candidate in this regard is krill oil. This unique oil, which is extracted from Antarctic krill, is abundant in docosahexaenoic acid and eicosapentaenoic acid (7). Unlike most fish oils, krill oil carries the majority of these acids in a phospholipid form, which allows for better intestinal absorption and more rapid incorporation into cell membranes (7, 8). Moreover, krill oil contains astaxanthin, a potent antioxidant that has been shown to protect rats (9) and human endothelial cells (10) against lipid peroxidation. Regarding blood lipids, krill oil

supplementation at a dosage of 1-3 $g \cdot d^{-1}$ for 90 days was reported to decrease TC between 13-18% and LDL-C between 32-39% (11). The same investigation reported that HDL-C increased between 43-60% following krill oil supplementation.

However, two recent studies (12, 13) found that krill oil supplementation (1 $g \cdot d^{-1}$ for 42 days and 3 $g \cdot d^{-1}$ for 49 days, respectively) had no effect on HDL-C. These two studies also found that krill oil supplementation had little (12) to no effect (13) on biomarkers of lipid peroxidation, which runs counter to findings that astaxanthin attenuates this type of oxidative stress (14).

The heterogeneity of findings pertaining to krill oil's effects on blood lipids and lipid peroxidation provide an impetus for further investigation. Thus, the main purposes of the present study were 1) to attempt to replicate or improve upon (via krill oil supplementation) the findings of the initial study of a 21-day Daniel Fast in terms of improving the blood lipid profile and lowering MDA; and 2) to determine if HDL-C can be maintained or even improved during a 21-day Daniel Fast via krill oil supplementation.

SUBJECTS AND METHODS

Subjects and Screening

40 subjects (12 men and 28 women) were initially enrolled in this study. 1 subject withdrew from the study due to illness and a family emergency. The subjects had a mean age of 34 ± 2 years, with a range of 19-65 years. 6 subjects had elevated BP upon screening (≥140/90 mmHg), seven subjects had elevated TC (>200 $mg \cdot dL^{-1}$), and 2 were diabetic (type 2). One man used a statin, and one used a benign prostatic hyperplasia medication. One woman used a statin, two used allergy medications, three used anti-depressants, one used an anti-inflammatory, three

used oral contraceptives, two used hormone replacement therapy, one used a stimulant/appetite suppressant, one used an antibiotic, one used insulin, one used a sleep aid, one used migraine medication, one used acne medication, one used hyperthyroid medication, and two used hypothyroid medication. All subjects were nonsmokers and were not consuming antioxidant supplements from two weeks prior to commencing the Daniel Fast until the conclusion of the fast. Subjects' baseline characteristics are presented in Table 1.

As with our initial investigation, no restrictions were placed on subjects regarding body mass index (BMI) necessary for enrollment. This was due to the fact that individuals in all weight categories, regardless of body mass at entry, responded well to the fast (1, 2). Therefore, the BMI of subjects ranged from 18 $kg \cdot m^{-2}$ to 37 $kg \cdot m^{-2}$; with 22 subjects classified as normal weight (BMI <25 $kg \cdot m^{-2}$), 9 classified as overweight (BMI 25-29.9 $kg \cdot m^{-2}$), and 8 classified as obese (BMI ≥30 $kg \cdot m^{-2}$). Of the 39 subjects that completed the investigation, 33 were classified as exercise-trained (defined as performing a combined minimum of 2 hours per week of anaerobic and aerobic exercise of moderate to high intensity). Specifically, subjects performed 2.1±0.2 hours of anaerobic and 4.7±0.4 hours of aerobic exercise per week for the past 6.8±1.1 and 8.3±1.0 years, respectively. Eligibility and classification were determined via completion of questionnaires pertaining to health history, physical activity, and drug and dietary supplement usage. Prior to participation, each subject was informed of all procedures, potential risks, and benefits associated with the study in both verbal and written form in accordance with the approved procedures of the University Institutional Review Board for Human Subjects Research (H11-14). Subjects signed an informed consent form prior to being admitted as a subject.

During the initial visit to the laboratory, subjects completed all paperwork, were provided detailed instructions pertaining to the Daniel Fast, were given food logs for dietary recording and

reviewed food models in order to improve the accuracy of recording (as described below), and were provided a calendar outlining their full participation. Subjects were also provided a detailed outline of foods that are allowed as well as commonly consumed foods that are not allowed. A recipe guide was also provided. It should be noted that subjects purchased and prepared all of their food. Subjects returned to the laboratory 1-2 weeks later to have baseline assessments performed and to begin the 21-day fast. The outcome variables described below were measured before (baseline: day 1 of the fast) and after the fast (day 22). All data collection was done in the morning hours (5:00-11:00 am) while subjects were in a 12-hour post-absorptive state.

Heart Rate and Blood Pressure

Upon arrival at the laboratory, subjects were asked to void. Women performed a urine pregnancy test to confirm that they were not pregnant, as pregnant women were not allowed to participate in this study due to potential fetal radiation exposure during a dual energy x-ray absorptiometry (DEXA) scan. Subjects were then seated in a chair with a blood pressure cuff placed on their left arm. After resting for 10 minutes, subjects' heart rate was measured via palpation for 60 seconds using the radial artery by two trained technicians – one on each wrist. Blood pressure was then measured via auscultation using a calibrated manometer and a dual earpiece stethoscope that allowed two technicians to listen simultaneously. The average of all measures was used in data analysis. If values deviated by more than 5 beats per minute for heart rate or 5 mmHg for blood pressure, an additional measure was taken and the two closest measures were used for analysis.

Anthropometric Variables

Subjects' height was measured using a stadiometer, and body weight was measured using a calibrated medical scale. Body mass index was calculated as weight (kg) divided by height squared (m^2). Waist and hip circumference measurements were obtained using a tension-regulated measuring tape, with subjects wearing "spandex-like" shorts. Body composition was determined via DEXA (Hologic QDR-4500W) using a 4-minute fan array. Specifically, total and regional (trunk-specific) body fat was measured. The assessment was performed by a licensed technician.

Blood Collection and Biochemical Variables

Venous blood samples were taken from subjects' antecubital vein via needle and Vacutainer™ by a trained phlebotomist. Following collection, the blood samples were processed accordingly, and a portion of the plasma/serum were sent to Laboratory Corporation of America (LabCorp) for analysis of the lipid panel using enzymatic procedures (Roche/Hitachi Modular). The remaining blood samples were stored at -70°C until they were analyzed for MDA following the procedures of Jentzsch et al. (15) using reagents purchased from Northwest Life Science Specialties (Vancouver, WA). Specifically, 75µL of plasma was added to microcentrifuge reaction tubes with the addition of 3µL of butylated hydroxytoluene in methanol to minimize *ex vivo* lipid peroxidation. 75µL of 1M phosphoric acid and 75µL of 2-thiobarbituric acid reagent were added to each reaction tube and mixed thoroughly. Samples and reagents were incubated for 60 minutes at 60°C. Following incubation, tubes were removed, and the reaction mixture was transferred to a microplate. The absorbance was read using a spectrophotometer at both 535 and 572nm to correct for baseline absorption. Malondialdehyde equivalents were calculated using the difference in absorption at the two wavelengths. Quantification was performed with a

calibration curve using tetramethoxypropane in a stabilizing buffer. The detection limit for MDA, as per the manufacturer, is 0.1µM.

Conditions (Supplementation)

On a double-blind basis, subjects were randomly assigned to consume one of the following for the entire duration of the 21-day fast: 1) 19 subjects consumed krill oil capsules (NOW Foods, Bloomingdale, IL: 2 grams per day in two daily dosages of 1 gram); and 2) twenty subjects consumed placebo capsules (coconut oil; NOW Foods, Bloomingdale, IL: 2 grams per day in two daily dosages of 1 gram). Specifically, half of the male subjects and half of the female subjects were placed in the group supplementing with krill oil, and the remaining subjects were placed in the group supplementing with a placebo. The krill oil provided approximately 2-3 $mg \cdot d^{-1}$ of esterified astaxanthin. Subjects were instructed to consume one capsule in the morning (with breakfast) and one capsule in the evening (with dinner) every day for the 21-day period. For both conditions, capsule counts upon bottle return determined compliance to the supplementation.

Dietary Records and Physical Activity

All subjects were instructed to maintain their normal diet until they began the fast and to record on food logs all food and beverage consumed during the 7 days immediately prior to the start of the fast. Subjects were also instructed to record food and beverage intake during the final seven days of the fast. A technician reviewed in detail the food logs with each subject upon receipt. Records were analyzed by using Food Processor SQL, version 9.9 (ESHA Research, Salem, OR). Regarding physical activity, subjects were instructed to maintain their normal habits during the entire study period with one notable exception: subjects were instructed not to perform strenuous exercise during the 48 hours immediately preceding the two assessment days.

Subjects were also instructed to refrain from alcohol consumption during the fast, as well as during the 48 hours that preceded day 1.

Post-Fast Questionnaire

During their final visit to the laboratory, subjects completed a questionnaire pertaining to their experience with the fast. Subjects rated their compliance to the fast, their overall physical health/vitality during the fast, their overall mental mood during the fast, and their overall satiety during the fast.

Statistical Analysis

All outcome measures were analyzed using a 2 (condition) x 2 (pre/post fast) analysis of variance. However, data for both conditions were subsequently collapsed and analyzed using a paired t-test. Analyses were performed using JMP statistical software (version 4.0.3, SAS Institute, Cary, NC). Statistical significance was set at $P \leq 0.05$. The data are presented as mean ± SEM.

RESULTS

The outcome measures of main interest in the present study were blood lipids and MDA. Although we hypothesized that these values would improve to a greater extent in subjects supplementing with krill oil, this was not confirmed by our statistical analyses. In fact, subjects in the krill oil and placebo groups had nearly identical pre- and post-fast blood lipid and MDA values. A summary of our findings is presented in Table 2. Based on these analyses, data for all variables were pooled and are simply presented as pre and post fast. As such, all data were analyzed using a paired t-test.

Compliance, Mood, and Satiety

Thirty-nine subjects (12 men and 27 women) completed the 21-day fast as well as the pre- and post-fast assessments. Compliance to the fast was 97.8 ± 0.4%. On a scale from 0-10, subjects rated their "physical feeling of health and vitality" (8.3 ± 0.2), "mental outlook and mood" (8.6 ± 0.2), and "level of satiety" (8.0 ± 0.2) during the fast.

Body Weight and Body Fat

Although not a main outcome measure, we recorded both bodyweight and body fat percentage pre and post intervention. Bodyweight was significantly ($p < 0.0001$) lower post fast (71.53 kg) compared to pre fast (74.14 kg). Body fat percentage was also significantly ($p = 0.0028$) lower post fast (28.80%) compared to pre fast (29.39%).

Blood Lipids and Malondialdehyde

Significant ($p < 0.05$) reductions from pre to post fast were noted in several parameters of the lipid panel, which can be viewed in Table 3. Malondialdehyde did not change to a significant extent ($p = 0.79$; Table 3).

Dietary Data

Several differences existed in dietary intake from pre fast to the final week of the fast. These included decreases in total kilocalories ($p < 0.01$), protein grams ($p < 0.01$), the percentage of protein ($p < 0.01$), fat grams ($p = 0.02$), saturated fatty acid (SFA) ($p < 0.01$), trans fat ($p < 0.01$), and cholesterol ($p < 0.01$). Increases in the percent of carbohydrate intake ($p < 0.01$), fiber ($p < 0.01$), polyunsaturated fatty acid (PUFA) ($p = 0.01$), vitamin C ($p < 0.01$), vitamin E ($p = 0.03$) and vitamin A ($p = 0.02$) were also noted. Data are presented in Table 4.

DISCUSSION

Although the initial investigation of the Daniel Fast reported favorable changes in many health-related biomarkers, a 14% decrease in HDL-C was also noted (1). Thus, the main purpose of the present investigation was to determine if krill oil supplementation at 2 $g \cdot d^{-1}$ for 21 days would maintain or even increase HDL-C in individuals partaking in a 21-day Daniel Fast. Unfortunately, as indicated in Table 2, krill oil supplementation had no effect on HDL-C, which decreased by an average of approximately 9% from pre to post fast. This finding strongly contradicts the 55% increase in HDL-C following supplementation of krill oil at 2 $g \cdot d^{-1}$ for 90 days that was reported by Bunea and colleagues (11). Rather, our findings are more similar to those of two recent investigations. Skarpańska et al. (12) and Ulven et al. (13) both reported that krill oil supplementation (1 $g \cdot d^{-1}$ for 42 days and 3 $g \cdot d^{-1}$ for 49 days, respectively) had no effect on HDL-C.

In addition to its lack of effect on HDL-C, krill oil supplementation failed to affect any other blood lipid (Table 2). Again, our findings oppose those of Bunea and coworkers (11) but support those of Skarpańska et al. (12) and Ulven et al. (13). Bunea and coworkers (11) reported that krill oil supplementation at the aforementioned dosage and duration decreased TC, LDL-C, and triglycerides (TAG) by 18%, 37%, and 28%, respectively. In contrast, Skarpańska et al. (12) and Ulven et al. (13) both reported that krill oil supplementation at the aforementioned dosages and durations had no effect on TC, LDL-C, or TAG.

Two features in the study design of Bunea et al. (11) markedly differed from the designs of the other investigations that examined the effects of krill oil supplementation on human blood lipids. First, the 90-day krill oil supplementation period of Bunea et al. (11) was considerably longer than the 49-day (13), 42-day (12), and 21-day (the present investigation) supplementation

periods of the other investigations. Second, pre-supplementation TC, LDL-C, and TAG were each substantially higher in the study conducted by Bunea et al. (11) compared to the other studies (Table 5). Clearly, individuals that have the greatest "room for improvement" and are supplementing for the longest duration are likely to experience the most robust changes in their blood lipid profile.

However, pre-supplementation HDL-C was similar across the studies: 58.0 $mg \cdot dL^{-1}$ in the study conducted by Ulven et al. (13), 61.0 $mg \cdot dL^{-1}$ in the study conducted by Skarpańska et al. (12), 55.97 $mg \cdot dL^{-1}$ in the present study, and 57.22 $mg \cdot dL^{-1}$ in subjects supplementing with krill oil at 1.0 $g \cdot d^{-1}$ (who experienced a 43.92% increase in this cholesterol following supplementation) in the study conducted by Bunea et al. (11). Thus, other than the longer supplementation duration, it is unclear why Bunea and colleagues (11) found such a dramatic increase in HDL-C compared to the other investigations.

We hypothesized that krill oil supplementation would reduce MDA due to its astaxanthin content, as this antioxidant has been shown to attenuate lipid peroxidation in rats (both *in vitro* and *in vivo*) (9) and human endothelial cells *in vitro* (10). However, krill oil supplementation had no effect on MDA in the present study, and two recent investigations have reported similar findings. The aforementioned investigation conducted by Ulven et al. (13) found no change in the lipid-specific marker F_2-Isoprostanes (F_2-IsoPs). The work of Skarpańska et al. (12) found that krill oil supplementation reduced the lipid-specific marker, thiobarbituric acid reactive substances, 24 hours following the completion of a rowing 2000 meter time trial. However, krill oil supplementation had no effect on thiobarbituric acid reactive substances immediately before or after the time trial.

Two possible explanations for the lack of effect of krill oil supplementation on MDA are worthy of mention. First, despite being shown to reduce lipid peroxidation in animals and in human cells *in vitro*, astaxanthin could potentially be ineffective at reducing lipid peroxidation in humans *in vivo*. In fact, a recent investigation found that astaxanthin supplementation at 8 $mg \cdot d^{-1}$ for 8 weeks did not affect plasma 8-isoprostane concentrations (16). Thus, it is possible that the 2-3 $mg \cdot d^{-1}$ dosage of astaxanthin provided by the krill oil in the present study was insufficient to lower MDA. Alternatively, even if such a dosage were sufficient to reduce MDA, such a reduction could be potentially masked by an increase in lipid peroxidation due to the eicosapentaenoic acid and docosahexaenoic acid contained in the krill oil. Specifically, integration of these acids into membranes and lipoproteins could increase lipid peroxidation by increasing the unsaturation index (17). Indeed, lipid peroxidation has been found to increase following intake of omega 3 fatty acids (18). However, other investigations have found that omega 3 fatty acid consumption decreases (19) or does not affect (20) lipid peroxidation. Therefore, more investigations are needed to determine the effects of krill oil supplementation on MDA and other biomarkers of lipid peroxidation.

Few investigations have compared the effects of krill oil and fish oil on blood lipids and lipid peroxidation. The aforementioned study conducted by Bunea et al. (11) reported that krill oil supplementation elicited superior changes in HDL-C, LDL-C, TC, and TAG compared to fish oil supplementation at 3 $g \cdot d^{-1}$ for 90 days. However, a recent study (13) found no difference in HDL-C, LDL-C, TC, TAG, or the HDL-C:TAG ratio between subjects supplementing with krill oil at 3 $g \cdot d^{-1}$ for 49 days and subjects supplementing with fish oil at 1.8 $g \cdot d^{-1}$ for the same time period. The same investigation found that neither krill oil supplementation nor fish oil supplementation affected F_2-IsoPs (13).

Considering our collapsed data, a 21-day Daniel Fast resulted in reductions in TC, HDL-C, and LDL-C; while TAG and very low-density lipoprotein cholesterol did not change significantly (Table 3). Similar findings were reported in the initial investigation of the Daniel Fast (1). This is not surprising given that compliance to the fast was similar between studies (97.8 ± 0.4% and 98.7 ± 0.2% in the present study and the initial study, respectively). Both the TC:HDL-C ratio and the LDL-C:HDL-C ratio decreased in the present investigation, whereas the TC:HDL-C ratio did not change in the initial investigation. (The LDL-C:HDL-C ratio was not included.) Considering that the decrease in TC was similar between studies, differences in the TC:HDL-C ratio are mainly due to the fact that HDL-C decreased to a lesser extent in the present study compared to the initial study (9% and 14%, respectively).

The Daniel Fast complies with nearly all of the dietary recommendations given by the Adult Treatment Panel III of the National Cholesterol Education Program for maintaining desirable blood lipid values (21), including the recommended intake of SFAs, PUFAs, monounsaturated fatty acids (MUFAs), total fat, fiber, protein, and cholesterol (22). Studies featuring dietary intakes similar to these recommendations, particularly those that reduce intake of SFAs and cholesterol, have been shown to reduce LDL-C by an average of 16% (23). This is similar to the reductions in LDL-C noted by the initial (1) and present investigation of a 21-day Daniel Fast (23% and 20%, respectively).

It appears that plant-based diets may be superior to diets that include animal products at reducing cholesterol concentrations. Moreover, plant-based diets that restrict animal product consumption to the greatest extent result in the largest reductions of TC and LDL-C. Ovolactovegetarian diets (allowing eggs and dairy products) and “primary” plant-based diets

(allowing eggs, dairy products, and an occasional small quantity of lean meat) decrease TC and LDL-C by 10-15%, whereas vegan diets decrease these cholesterols by 15-25% (24).

However, the most-favorable effects on blood lipids are likely observed in "portfolio" diets that emphasize the consumption of viscous fibers, plant sterols, vegetable proteins, and nuts (25). Four metabolically controlled 4-week trials have reported reductions in TC and LDL-C by 22-27% and 29-35%, respectively (26-29). When comparing the findings of these investigations to the present one, three considerations are worthy of mention: First, subjects in the dietary portfolio studies had substantially higher baseline LDL-C than subjects in the present study. Second, the dietary intervention was 33% longer in the dietary portfolio investigations than in the present one. Third, whereas the subjects in the present study purchased and prepared all of their own food, the subjects in the dietary portfolio studies had all of their food delivered to them, making them more likely to comply with their diet. In fact, subjects who purchased and prepared all of their own food in accordance with a self-selected dietary portfolio plan for one year experienced more modest reductions in TC (10%) and LDL-C (13%) (30).

Malondialdehyde remained unchanged in the present study despite an increased intake of vitamin C and vitamin E, two antioxidants that have been demonstrated to reduce lipid peroxidation (31). This finding runs counter to the 15% reduction in MDA reported in the initial investigation of a 21-day Daniel Fast (2). An important difference between the two studies that may have affected MDA values is that PUFA consumption increased by 37% from pre to post fast in the present study (6.7 ± 0.5 vs. 9.2 ± 1.0; $p = 0.01$) but remained unchanged in the initial study (8 ± 1 vs. 9 ± 1; $p = 0.47$) (2). PUFAs are more susceptible to peroxidation than MUFAs and SFAs (32), and increased consumption of PUFAs has been shown to increase lipid peroxidation (33). Thus, the increased PUFA intake in the present study may have offset the

increased antioxidant intake, leaving MDA unchanged. Other investigations featuring plant-based diets have reported minimal effects on MDA (34, 35).

The search for a non-pharmacological intervention capable of maintaining HDL-C during a 21-day Daniel Fast continues. Two potential candidates in this regard are phosphatidylinositol supplementation and increased MUFA consumption. Two weeks of phosphatidylinositol supplementation at 5.6 $g \cdot d^{-1}$ increased HDL-C by 18% (36). Unfortunately, this remains the only study to date that has examined the effect of phosphatidylinositol supplementation on HDL-C in humans. Jenkins et al. (37) recently reported that substituting 13% of total energy intake as carbohydrate with MUFAs (predominantly in the form of sunflower oil) in a portfolio diet for four weeks increased HDL-C by 12.5%. However, it should be noted that subjects in this study had much lower baseline HDL-C values (38.7 $mg \cdot dL^{-1}$) than subjects in the present investigation (55.97 ± 2.62 $mg \cdot dL^{-1}$).

CONCLUSION

A 21-day Daniel Fast is associated with reductions in TC, LDL-C, the TC:HDL-C ratio, and the LDL-C:HDL-C ratio. Unfortunately, HDL-C decreased by 9%, and krill oil supplementation at 2 $g \cdot d^{-1}$ for 21 days did not prevent this reduction, nor did it impact any other cholesterol concentration or MDA. Future investigations should examine other non-pharmacological interventions that can potentially maintain HDL-C during the fast, such as phosphatidylinositol supplementation or increased MUFA consumption. Finally, additional investigations are needed to compare the effects of krill and fish oil on blood lipids, lipid peroxidation, and other indices of health.

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APPENDIX A – TABLES

Table 1. Baseline characteristics of 39 men and women participating in a 21-day Daniel Fast

Variable	Value
Age (yrs)	33.9 ± 2.1
Height (cm)	168.9 ± 1.4
Weight (kg)	74.1 ± 2.4
BMI ($kg \cdot m^{-2}$)	25.9 ± 0.7
Waist (cm)	86.8 ± 2.0
Hip (cm)	101.2 ± 1.8
Waist:Hip	0.86 ± 0.01
Body Fat (%)	29.4 ± 1.5
Heart Rate (bpm)	66.5 ± 1.6
Systolic Blood Pressure (mmHg)	110.7 ± 2.2
Diastolic Blood Pressure (mmHg)	71.5 ± 2.3
Aerobic Exercise ($hrs \cdot wk^{-1}$)	4.2 ± 0.6
Years Aerobic Exercise	7.9 ± 1.4
Anaerobic Exercise ($hrs \cdot wk^{-1}$)	1.8 ± 0.3
Years Anaerobic Exercise	5.9 ± 1.4

Values are presented as mean ± SEM.

Table 2. Probability values for blood lipid and malondialdehyde data for subjects supplementing with krill oil and placebo during a 21-day Daniel Fast

Variable	Condition	Time*	Condition x Time
Cholesterol	0.3855	0.0005	0.9429
Triglycerides	0.7068	0.2633	0.9007
HDL-C	0.9253	0.1903	0.5860
VLDL-C	0.6839	0.2601	0.9612
LDL-C	0.2471	0.0016	0.7607
LDL-C:HDL-C	0.1925	0.2290	0.5464
TC:HDL-C	0.3211	0.3107	0.6112
MDA	0.8787	0.8709	0.9297

Note: Due to a lack of statistical significance between conditions, data are pooled and presented in Table 3.
*Time indicates pre and post fast

Table 3. Blood lipid and MDA data of 39 men and women before and after a 21-day Daniel Fast

Variable	Pre	Post	P value
Cholesterol ($mg \cdot dL^{-1}$)	173.07 ± 5.03	146.00 ± 4.82	<0.0001
Triglycerides ($mg \cdot dL^{-1}$)	82.46 ± 7.40	75.23 ± 5.72	0.18
HDL-C ($mg \cdot dL^{-1}$)	55.97 ± 2.62	50.90 ± 2.62	<0.0001
VLDL-C ($mg \cdot dL^{-1}$)	16.54 ± 1.48	15.10 ± 1.14	0.1982
LDL-C ($mg \cdot dL^{-1}$)	100.56 ± 4.31	80.00 ± 3.66	<0.0001
LDL-C:HDL-C	1.99 ± 0.15	1.71 ± 0.12	0.0006
TC:HDL-C	3.33 ± 0.18	3.05 ± 0.14	0.0023
MDA ($\mu mol \cdot L^{-1}$)	0.73 ± 0.06	0.71 ± 0.07	0.79

Values are presented as mean ± SEM.

Table 4. Dietary data of 39 men and women before and during the final seven days of a 21-day Daniel Fast

Variable	Pre	During	P value
Kilocalories	1857.6 ± 94.4	1601.7 ± 84.7	<0.01
Protein (g)	79.4 ± 6.2	53.0 ± 3.4	<0.01
Protein (%)	16.7 ± 0.7	13.2 ± 0.5	<0.01
Carbohydrate (g)	229.2 ± 12.5	240.5 ± 14.1	0.35
Carbohydrate (%)	49.5 ± 1.0	60.7 ± 1.7	<0.01
Fiber (g)	20.7 ± 1.7	40.0 ± 2.5	<0.01
Sugar (g)	81.6 ± 5.7	73.2 ± 5.2	0.15
Fat (g)	66.6 ± 3.8	54.9 ± 4.7	0.02
Fat (%)	32.2 ± 0.8	30.1 ± 1.7	0.25
SFA (g)	21.0 ± 1.3	8.4 ± 0.8	<0.01
Monounsaturated Fat (g)	13.7 ± 1.6	18.3 ± 2.7	0.07
PUFA (g)	6.7 ± 0.5	9.2 ± 1.0	0.01
Trans Fat (g)	0.9 ± 0.2	0.2 ± 0.1	<0.01
Omega 3 (g)	0.5 ± 0.1	0.6 ± 0.1	0.81
Omega 6 (g)	4.8 ± 0.4	6.0 ± 0.9	0.13
Cholesterol (mg)	224.4 ± 21.0	12.2 ± 5.1	<0.01
Vitamin C (mg)	64.5 ± 5.6	140.6 ± 11.1	<0.01
Vitamin E (mg)	5.7 ± 0.8	7.7 ± 0.8	0.03
Vitamin A (RE)	362.0 ± 42.4	485.5 ± 52.1	0.02
Selenium (µg)	46.9 ± 4.9	38.4 ± 9.1	0.35

Values are presented as mean ± SEM.

Table 5. Pre-intervention blood lipid data in investigations featuring krill oil supplementation

Variable	Bunea et al. (2004)*	Skarpańska et al. (2010)	Ulven et al. (2011)	Present Investigation
Total Cholesterol ($mg \cdot dL^{-1}$)	247.4	166.3	193.0	173.1
LDL-C ($mg \cdot dL^{-1}$)	182.9	83.9	119.0	100.6
Triglycerides ($mg \cdot dL^{-1}$)	160.4	97.0	97.4	82.5

Values are presented as mean (SEM data not available for all studies).

*Data are only in regards to subjects that supplemented krill oil at 2 $g \cdot d^{-1}$.

APPENDIX B – EXTENDED LITERATURE REVIEW

OVERVIEW OF FASTING

The pioneering work of McCay and colleagues demonstrated that restricting dietary intake without inducing malnutrition (i.e., fasting) increases longevity in rats (1). Since then, countless investigations have examined this dietary regimen as a potential treatment for extending human life and/or reducing the occurrence of human diseases. These investigations have typically manipulated dietary intake in one of two ways. The more common form of manipulation reduces energy intake without restricting the types of foods that may be consumed. Caloric restriction (CR) regimens and alternate-day fasting (ADF) regimens both fit this model. The less common form of manipulation restricts the *types* of foods that may be consumed without necessarily affecting total caloric intake. This form of manipulation is commonly referred to as dietary restriction (DR). These two forms of dietary manipulation can be observed in religiously-motivated fasts. Indeed, Islamic Ramadan can be described as a variant of ADF, while the principal fasting periods of Greek Orthodox Christianity (GOC) as well as the Biblical-based Daniel Fast can each be described as a form of DR.

Caloric Restriction

CR is by far the most commonly studied dietary regimen designed to decrease caloric consumption. Under this regimen, daily caloric intake is typically reduced by 20-40% of *ad libitum* consumption (2). CR investigations have studied species from three Kingdoms (Animalia, Fungi, and Protoctista) (3), with rodents being the most commonly studied species. Typically, investigations featuring nonhuman species initiate CR as early in life as the subjects can tolerate the reduced caloric intake without starving, and this feeding pattern continues until death. In human trials, CR is often performed for a duration ranging from 6-24 months (4).

The health-promoting effects of CR are profound and wide-ranging. In fact, CR can increase maximum lifespan in species ranging from yeast to nonhuman primates by up to 50% (5). Regarding cardiovascular health, the following changes have been noted following a CR regimen: decreases in heart rate (HR) and blood pressure (BP); increases in HR variability; and improvements in left ventricular function, post-exercise recovery of both HR and BP, and flow-mediated vasodilation (6). Regarding glucoregulatory health, CR has been shown to decrease fasting glucose and insulin levels, increase insulin sensitivity, decrease body fat percentage, and lower the incidence of diabetes (7, 8).

CR also elicits favorable changes in blood lipids. Specifically, CR reduces plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TAG), and the total cholesterol:high-density lipoprotein cholesterol (TC:HDL-C) ratio, while having no effect on plasma high-density lipoprotein cholesterol (HDL-C) (9) . Little is currently known about the effects of CR on plasma very low-density lipoprotein cholesterol (VLDL-C).

Markers of oxidative stress have been reported to decrease following a CR regimen, such as lipofuscin, malondialdehyde (MDA), superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) (10). In fact, the ability of CR to extend life is theorized by many to be mediated through its ability to attenuate oxidative stress (11).

Despite CR's favorable and multifarious effects on health, one substantial shortcoming is the austerity of this dietary regimen. Simply put, few humans are willing to reduce their caloric consumption by 20-40% for any notable length of time (2). This shortcoming has given birth to alternative dietary regimens, which are designed to replicate the health-promoting effects of CR while serving as more tolerable long-term plans.

Alternate-Day Fasting

Like CR, ADF regimens reduce caloric intake, but this reduction occurs every other day. On "feast days," subjects may consume food *ad libitum;* but on "fast days," food consumption is reduced or eliminated altogether. Two subtypes of ADF exist: "true" ADF regimens completely disallow food consumption during fast days, whereas "modified" ADF regimens typically allow for the consumption of 15-25% of baseline requirements (12). Humans and rodents are the two most commonly studied species of ADF investigations. Rodent trials typically initiate ADF 2-3 months after birth, and this feeding pattern commonly lasts between 8 and 20 weeks (13). Human trials have performed ADF for a period ranging from 2-8 weeks (14). Some investigations have reported that rodents on ADF regimens gorge themselves during feast days and therefore avoid losing bodyweight (13). In contrast, humans appear to lose weight when undergoing ADF for 3 weeks or longer (13). Due to the fact that participants are in a calorie-reduced state for only half of the time under ADF, this dietary regimen may prove to be more tolerable for humans than CR (14). However, no investigation to date has directly compared the tolerability of both dietary regimens in humans.

Initial investigations suggest that the health-related benefits of ADF are very comparable to those of CR (15, 16). ADF regimens increase longevity in some strains of mice, and this increase appears to be largely affected by genotype and the time at which ADF was initiated (13). Regarding cardiovascular health, ADF has been shown to decrease HR and BP in rats by a similar magnitude to the decrease observed in a 40% CR regimen (16). Also, ADF regimens have been reported to increase HR variability and attenuate the development of post-infarct chronic heart failure in rodents (17, 18). In humans, ADF was reported to lower systolic (but not diastolic) BP in prehypertensive individuals (14) but have no effect on either variable in

normotensive individuals (19). Regarding glucoregulatory health, ADF has been shown to decrease plasma glucose and insulin in rodents by a similar magnitude to the decrease observed in a 40% CR regimen (15). Human trials have reported that ADF does not affect plasma glucose and may or may not decrease plasma insulin (13). Alternate-day fasting may have gender-specific effects on glucoregulatory function. For example, ADF improved insulin sensitivity in men but had no effect on this variable in women (13). Furthermore, glucose tolerance was unchanged in men partaking in an ADF regimen, but women partaking in the same regimen experienced impaired glucose tolerance (13).

Like CR, ADF favorably affects blood lipids and appears to attenuate oxidative stress. ADF has been reported to decrease TC, LDL-C, and TAG, while increasing HDL-C (20). However, no study to date has examined the effects of ADF on VLDL-C, the TC:HDL-C ratio, or the LDL-C:HDL-C ratio. Regarding oxidative stress, levels of the following markers of oxidative damage have been observed to decrease on an ADF regimen: protein-bound carbonyls, nitrotyrosine, 8-isoprostane, histidine adducts, and lysine 4-hydroxynonenal adducts (21).

Dietary Restriction

As mentioned above, DR is a restriction of the types of food (typically macronutrients) that may be consumed without necessarily affecting total caloric intake. Few investigations continue to examine carbohydrate restriction or lipid restriction, because research suggests that neither form of restriction extends life (22). On the other hand, protein restriction (PR) has been reported in numerous studies to increase longevity (23). While PR regimens reduce the dietary intake of all amino acids, methionine restriction (MR) regimens only reduce the dietary intake of the amino acid methionine. Current investigations are examining whether MR can elicit comparable health-related benefit to those elicited by PR.

Protein intake has been reduced anywhere from 40-85% in PR investigations, while MR investigations often restrict methionine intake by either 40% or 80% (23). Rodents are the most commonly studied species in both PR and MR investigations; both forms of restriction are typically initiated during or immediately following the weaning phase and continue until death. PR increases lifespan by approximately 20%, suggesting that it accounts for half of the (40%) life extension commonly observed in CR regimens (23). Initial investigations of MR have found a similar extension in (22). Interestingly, one study found that a 40% restriction of all dietary amino acids except methionine had no effect on either mitochondrial reactive oxygen species generation or oxidative damage in mitochondrial deoxyribonucleic acid (DNA) (24). The authors of this investigation concluded that the reduction of methionine intake may account for all of the life extension observed in PR regimens (24).

Few (if any) investigations have examined the effects of PR or MR on blood lipids. However, both PR and MR have been shown to lower markers of oxidative stress. Specifically, PR for seven weeks has been reported to lower liver mitochondrial H_2O_2 production, free radical leak, and 8-oxo-7,8-dihydro-2'-deoxyguanosine in both nuclear and mitochondrial DNA (25). Also, 6-7 weeks of MR has been reported to lower heart and liver mitochondrial H_2O_2 production, free radical leak, 8-oxo-7,8-dihydro-2'-deoxyguanosine in mitochondrial DNA, glutamic semialdehyde, aminoadipic semialdehyde, N^{ε}-(carboxylmethyl)lysine, N^{ε}-(carboxylethyl)lysine, and N^{ε}-(MDA)lysine (26).

Religious Fasting

Religious fasts are partaken chiefly for spiritual betterment, but they can also substantially impact physical health (27). During the past 20 years, investigations have examined the health-related effects of the following fasting periods: 1) Islamic Ramadan, 2) the

three principal fasting periods of GOC, and 3) the Biblical-based Daniel Fast. Ramadan and the fasting periods of GOC will both be discussed in this section. However, because the Daniel Fast is the main focal point of this text, this specific form of fasting will be discussed at the conclusion of this literature review.

Ramadan

Muslims abstain from consuming food or drink from sunrise (Sahur) to sunset (Iftar) during the holy month of Ramadan, which lasts between 28 and 30 days. The first day of Ramadan advances 11 days each year due to the fact that the Islamic lunar calendar (Hijra) is not synchronized with the Gregorian calendar. As a consequence, Ramadan falls on different parts of the year over a 33-year cycle. Ramadan can be considered to be a variant of ADF, because it alternates periods in which food consumption is allowed *ad libitum* with periods in which food consumption is completely disallowed. The feast and fast periods of Ramadan are each 12 hours in length on average (28), equating to half of the length of the 24-hour periods featured in ADF regimens. However, daily fasting time can vary considerably from investigation to investigation, as it is highly dependent upon a location's latitudinal distance from the equator as well as the part of the seasonal year that Ramadan occurs.

A common dietary practice during Ramadan is to consume a large meal at dusk and a smaller meal before dawn (29), although some Muslims consume an additional meal before sleeping (30). These meals can be described as celebratory feasts that are often partaken in the company of family and close friends (31). A greater variety of foods is consumed during these meals (32), and sugary foods are consumed more frequently (33).

All healthy, adult Muslims are required to fast during Ramadan. Individuals who are sick, traveling, pregnant, breast-feeding, menstruating, or debilitated are exempt from fasting

(34). These individuals perform a compensational fast if and when they are able to do so. However, many Muslims who are eligible for exemption decide nonetheless to fast during Ramadan due to social pressure and the desired camaraderie of fasting with family and friends (34).

The collective body of research on Ramadan fasting has noted mostly heterogeneous findings regarding dietary intake and health-related outcomes, making it difficult to draw definitive conclusions about the health-related effects of this fast (27). Much of the heterogeneity of findings can likely be attributed to differences between investigations in daily fasting time as well as differences in the following subject characteristics: food choices, smoking status, and oral medication consumption (27).

Only one investigation to date has examined the effects of Ramadan fasting on markers of oxidative stress. The authors of this investigation reported reduced levels of MDA in erythrocytes, although no changes were noted regarding serum MDA or plasma protein-bound carbonyls (29).

Greek Orthodox Christianity

The three principal fasting periods of GOC have similar food proscriptions to one another. During the Nativity fast (40 days), dairy products, eggs, and meat are proscribed every day, while fish and olive oil are also forbidden on Wednesdays and Fridays. During Lent (48 days), dairy products, eggs, and meat are proscribed. Olive oil may be consumed only on weekends during this period, and fish may be consumed only on March 25th and Palm Sunday. During the Assumption (15 days), dairy products, eggs, and meat are proscribed. Olive oil may be consumed only on weekends during this period, and fish may be consumed only on August 6th. A faster's diet is comprised chiefly of bread, fruits, legumes, nuts, seafood, snails, and

vegetables during these periods (35). Thus, each principal fasting period can be described as a variant of vegetarianism and a form of DR.

Only six investigations to date have directly examined the health-related effects of the GOC fasts. Three studies examined the effects of all three principal fasting periods (35-37), two studies examined the Nativity fast only (38, 39), and one study compared a fasting week during Lent with a non-fasting week (40). Clearly, more research remains to be performed, but the results thus far have been promising.

Little is known about the effects of GOC fasting on either cardiovascular or glucoregulatory health. GOC fasting may or may not increase systolic BP and may or may not decrease diastolic BP (37, 40). In addition, heterogeneous findings have been reported regarding plasma glucose levels (35, 39, 40). Regarding blood lipids, it appears that TC and LDL-C decrease during the fasting periods, but the LDL-C:HDL-C ratio does not appear to change (35, 39, 40). Also, conflicting findings have been reported regarding both HDL-C and the TC:HDL-C ratio as well as TAG (35, 39, 40). Although no study to date has examined oxidative stress in response to GOC fasting, a recent study measured serum antioxidant levels before and after the Nativity fast (39). This investigation found that serum retinol, the retinol:TC ratio, serum α-tocopherol, and the α-tocopherol:TC ratio each decreased following this fast.

OVERVIEW OF BLOOD LIPIDS

A lipid panel is one blood test that is used to partially assess an individual's likelihood of developing cardiovascular disease (CVD). In particular, lipid panels commonly measure serum levels of LDL-C, HDL-C, VLDL-C, TC, and TAG. Each of these variables contributes either favorably (in the case of HDL-C) or unfavorably (in the case of the other variables) to the

promotion of cardiovascular health. This section of the review will discuss each aforementioned lipid panel component, as well as non-HDL cholesterol (non-HDL-C), the TC:HDL-C ratio, and the LDL-C:HDL-C ratio.

Low-Density Lipoproteins

The main function of LDL-C is to transport cholesterol from the liver and intestines to areas of need (41). However, LDL-C does not always reach its intended target and instead becomes trapped in the arterial intima, an early step in the development of atherosclerosis (42). Atherosclerosis eventually leads to the formation of a thrombus, which can result in the development of several cardiovascular events, including myocardial infarction and stroke (43).

It is of no surprise then that elevated LDL-C levels are associated with an increased risk of atherosclerotic heart disease and an increased occurrence of cardiovascular events (44, 45). Accordingly, the Adult Treatment Panel III of the National Cholesterol Education Program (NCEP) recommends that serum LDL-C be less than 100 $mg \cdot dL^{-1}$ for optimal cardiovascular health (46). However, the mean level of serum LDL-C among U.S. adults is 120.8 $mg \cdot dL^{-1}$, and 70.9% of this population has serum LDL-C levels above the recommended 100 $mg \cdot dL^{-1}$ (47). Lowering LDL-C in individuals with elevated values could substantially reduce the risk of developing a cardiovascular-related disorder. In fact, a 38.67 $mg \cdot dL^{-1}$ decrease in LDL-C was demonstrated to reduce the risk of coronary heart disease (CHD) death by 19% and all-cause mortality by 12% (45).

Several pharmaceutical options for lowering LDL-C are currently available. HMG-CoA reductase inhibitors (18-55% reduction), better known as "statins," lower the body's endogenous production of cholesterol (48). Bile acid sequestriants (15-30% reduction) prevent intestinal absorption of bile acids, which causes the liver to increase the conversion of cholesterol to bile

acids (48). Cholesterol absorption inhibitors (18% reduction) block the intestinal absorption of dietary and biliary cholesterol (49). Although its mechanism of action is currently unclear, niacin has been shown to lower LDL-C between 5 and 25% (48). The mechanism of action for fibric acid derivatives is also currently unknown, but these pharmaceuticals have been reported to reduce LDL-C between 5 and 20% (48).

Multiple non-pharmacological interventions have been shown to moderately decrease LDL-C. Intake of the following foods has been associated with a decline in LDL-C: polyunsaturated fats, omega 3 fatty acids, low-glycemic fruits and vegetables, soluble-fiber (5-10% reduction), phytosterols (up to 13%), nuts (2-19%), and soy protein (6%) (50, 51). In addition, supplementation of the following nutraceuticals has been demonstrated to lower LDL-C: tocotrienol (8-27% reduction), pantethine (20-27%), red-yeast rice (22-32%), ginseng (10%), probiotics (5-8%) and sesame oil (10%) (50). Resistance exercise appears to have a minimal effect on LDL-C, while aerobic exercise can reduce this cholesterol by approximately 5% (52).

High-Density Lipoproteins

The ability of HDL-C to protect against atherosclerosis is mainly mediated through its role in reverse cholesterol transport. Through this process, HDL-C travels throughout the bloodstream and gathers cholesterol from areas of overabundance, including atherogenic foam cells (53). HDL-C then transports this cholesterol to the liver, where it is converted into bile and excreted (54). In addition to its role in reverse cholesterol transport, HDL-C helps protect against inflammation, LDL oxidation, and thrombosis (54). Also, HDL-C increases nitric oxide production and inhibits the expression and migration of atherosclerotic adhesion molecules (53).

As would be expected, HDL-C levels are inversely correlated with the risk of CHD (46). In fact, a 1 $mg \cdot dL^{-1}$ increase in HDL-C lowers the risk of developing CHD by 2% and 3% in men

and women, respectively (55). For optimal cardiovascular health, HDL-C should be ≥40 mg·dL^{-1}, but approximately one-third of men and one-fifth of women have HDL-C levels below this threshold (46).

The pharmacological increase in HDL-C has been the subject of numerous investigations over the past decade. Statins raise HDL-C by 5-15%, while niacin increases this cholesterol by 15-35% (53). Fibrates (10-15% increase) activate peroxisome proliferator-activated receptor (PPAR)-α, which in turn up-regulates hepatic HDL synthesis (53). Thiazolidnediones increase HDL-C in insulin-resistant individuals by activating PPAR-λ (53). The two forms of thiazolidnediones currently in use are pioglitazone and rosiglitazone. Pioglitazone has been reported to increase HDL-C by 4.6 mg·dL^{-1}, while rosiglitazone has been shown to increase HDL-C by 2.7 mg·dL^{-1}. However, rosiglitazone may or may not significantly increase the risk of myocardial infarction (53). Glitazars combine the effects of fibrates and thiazolidnediones by activating both PPAR-α and PPAR-λ. These drugs have been demonstrated to increase HDL-C by 13-31%, but the following side effects have been noted: edema, anemia, leucopenia, and weight gain (53). Also, increased occurrences of death, cardiovascular events, and congestive heart failure have been noted with these drugs (53). Cholesteryl ester transfer protein (CETP) inhibitors increase HDL-C by suppressing the transfer of cholesteryl esters from HDL to intermediate low-density lipoprotein, LDL, and VLDL (56). Torcetrapib, dalcetrapib, and anacetrapib are the three forms of CETP inhibitors that have been investigated to date. Despite raising HDL-C by 72.1%, torcetrapib has been shown to increase all-cause mortality and the occurrence of cardiovascular events (53). In two separate trials, dalcetrapib has been reported to increase HDL-C by 28% and 34% (53). Anacetrapib has been shown to elevate HDL-C by 129% in one study and >130% in another (53).

HDL infusion is one of the more promising treatments aimed at increasing HDL-C. This treatment has been demonstrated to increase nitric oxide bioavailability, stimulate reverse cholesterol transport, reduce inflammation, and improve glycemic control (53). Also, recombinant apolipoprotein A-I Milano infusion has been found to reduce coronary atheroma burden (53).

Some non-pharmacological interventions have had moderate success at elevating HDL-C. Diets featuring plentiful amounts of unsaturated fats (particularly omega 3 fatty acids) and low glycemic index carbohydrates may increase HDL-C levels (53). In fact, a recent investigation reported that substituting 13% of total energy intake as carbohydrate with monounsaturated fat (predominantly in the form of sunflower oil) in a "portfolio" diet for four weeks increased HDL-C by 12.5% (57). Aerobic exercise without weight loss may or may not minimally increase HDL-C; however, weight loss induced by both aerobic exercise (4.6 ± 1.2 mg·dL^{-1}) and diet (5.0 ± 1.2 mg·dL^{-1}) have been shown to raise this cholesterol (58, 59). Cigarette smoking cessation appears to raise HDL-C by 4 mg·dL^{-1} (53). Increases in HDL-C have been noted following the supplementation of the following nutraceuticals: phosphatidylinositol (up to 17.8%) (60); soy lecithin (4.4-14%) (61, 62); a calcium and vitamin D combination (~5%) (63); red-yeast rice (5.8 mg·dL^{-1}) (64); a combination of vitamin C, vitamin E, coenzyme Q10, and selenium (65); policosanol (8-29%) (66); and krill oil (67). Regarding policosanol, numerous investigations conducted by one research group have reported HDL-C raising benefits, but the majority of external research groups have been unable to replicate these findings (68).

Krill oil deserves special mention as a potential HDL-C-raising supplement. This nutrient, which is extracted from Antarctic krill, contains a plentiful amount of omega 3 fatty acids. These omega 3 fatty acids exist in a phospholipid form as opposed to a TAG form, which

allows them to be better-absorbed by the intestine and more readily incorporated into cell membranes (69). Krill oil supplementation at a dosage of 1-3 $g \cdot d^{-1}$ for 90 days was reported to increase HDL-C between 43-60% (67). However, two recent studies (70, 71) found that krill oil supplementation (1 $g \cdot d^{-1}$ for 42 days and 3 $g \cdot d^{-1}$ for 49 days, respectively) had no effect on HDL-C.

Triglycerides, Very Low-Density Lipoproteins, and Non-High-Density Lipoproteins

Elevated TAG increases the risk of CVD via direct and indirect means. Directly, TAG partners with VLDL-C and CETP to make LDL-C more atherogenic and HDL-C less anti-atherogenic (72). Specifically, VLDL (with the aid of CETP) exchanges its TAG-rich lipoprotein particles for cholesterol esters from LDL-C and HDL-C. Once these lipoproteins encounter hepatic lipase, they are stripped of their TAG particles, rendering them much smaller than normal. This removal of TAG makes it easier for LDL-C to enter the arterial wall and lowers the amount of cholesterol that HDL-C can transport back to the liver. Also, postprandial hypertriglyceridemia promotes CVD (as well as type 2 diabetes mellitus [T2DM]) by elevating postprandial oxidative stress (73), a phenomenon that will be discussed later in this review. Indirectly, hypertriglyceridemia has high correlations with several risk factors of CVD, including obesity, metabolic syndrome, biomarkers of inflammation and thrombosis, and T2DM (74).

The Adult Treatment Panel III of the NCEP recommends that serum TAG equal 150 $mg \cdot dL^{-1}$ or less (46). Unfortunately, 30.0% of Americans aged 20 or older are estimated to have serum TAG > 150 $mg \cdot dL^{-1}$ (75). This prevalence increases to 42.8% in Americans aged 50 or older (76). A meta-analysis of 21 investigations found that an 89 $mg \cdot dL^{-1}$ increase in serum TAG was associated with a 32% and 76% increased risk of CHD in men and women, respectively (77). Moreover, this association remained statistically significant (12% and 37%

increased risk in men and women, respectively) even after controlling for TC, LDL-C, HDL-C, body mass index, and diabetes (78).

Many of the pharmacological options for lowering LDL-C have also been demonstrated to lower TAG. Indeed, statins (7-30% reduction), fibrates (20-50%), niacin (20-50%), and cholesterol absorption inhibitors are effective TAG-lowering options (79). In addition, several non-pharmacological interventions have TAG-reducing effects. Many dietary regimens with varying macronutrient compositions have been proposed to reduce TAG (reviewed in [79]). Dietary regimens rich in omega 3 fatty acids as well as monounsaturated and polyunsaturated fats appear to be particularly effective in this regard. Decreases in TAG have been reported following supplementation of the following: fish oil (up to 50% reduction), soy protein (10.5%), dietary fiber, and red yeast rice (up to 34.1%) (79). In addition, garlic supplementation may or may not lower TAG (79). Weight loss and regular exercise of moderate intensity (4-7 $kcal \cdot min^{-1}$) are also recommended as TAG-reducing interventions (46).

As mentioned above, VLDL-C teams with TAG and CETP to promote CVD. For individuals with serum TAG < 400 $mg \cdot dL^{-1}$, VLDL-C is often estimated by dividing TAG levels by 5 (46). This method becomes inaccurate once serum TAG levels exceed 400 $mg \cdot dL^{-1}$, necessitating that VLDL-C be measured directly (46). VLDL-C should be $\leq$ 30 $mg \cdot dL^{-1}$ for optimal cardiovascular health (46). However, VLDL-C is not frequently considered by clinicians as an independent risk factor for developing CVD; instead, non-HDL-C, which is the sum of VLDL-C, LDL-C and all other apolipoprotein B-containing proteins, is more commonly used to predict CVD risk. Indeed, non-HDL-C has been shown to be a better predictor of coronary mortality than LDL-C (46). For optimal cardiovascular health, non-HDL-C, which is calculated as TC minus HDL-C, should be < 130 $mg \cdot dL^{-1}$ (80). Also, each 30 $mg \cdot dL^{-1}$ increment

in non-HDL-C is associated with a 19% and 15% increased risk in cardiovascular mortality in men and women, respectively (80). Interventions that have been demonstrated to lower LDL-C and/or TAG are excellent options for lowering non-HDL-C. In particular, statins, which have been demonstrated to substantially lower LDL-C and VLDL, are an outstanding first line of defense (80).

Total Cholesterol and Cholesterol Ratios

Recommendations state that TC, the sum of all lipoproteins, should be < 200 $mg \cdot dL^{-1}$ for optimal cardiovascular health (46). Moreover, epidemiological studies have found that a 1% reduction in TC is associated with a 2% reduction in CHD risk (46). However, TC is a flawed predictor of an individual's risk of CVD, because it does not differentiate between atherogenic lipoproteins (non-HDL-C) and anti-atherogenic lipoproteins (HDL-C). In fact, 35% of CHD patients have a TC below 200 $mg \cdot dL^{-1}$ (81).

By contrast, cholesterol ratios such as the TC:HDL-C ratio and LDL-C:HDL-C ratio take atherogenic and anti-atherogenic components into account. Consequently, these ratios serve as better predictors of CVD than TC, LDL-C, and HDL-C alone (82). When compared to each other, the TC:HDL-C ratio and LDL-C:HDL-C ratio are equally effective at predicting CVD (82). This should come to no surprise, as LDL-C is highly correlated with TC (46). However, in individuals with serum TAG > 300 $mg \cdot dL^{-1}$, the TC:HDL-C ratio is a better predictor of cardiovascular events than the LDL-C:HDL-C ratio, because LDL-C cannot be calculated reliably (82). For men and women, desirable values for the TC:HDL-C ratio are < 4.5 and < 4.0, respectively. Regarding the LDL-C:HDL-C ratio, desirable values for men and women are < 3.0 and < 2.5, respectively (82).

In sum, HDL-C promotes cardiovascular health, while LDL-C, HDL-C, VLDL-C, TC, and TAG promote CVD. Non-HDL-C, the TC:HDL-C ratio, and LDL-C:HDL-C ratio are superior predictors of a cardiovascular event to many of the individual lipoproteins that comprise a lipid panel. While blood lipids are clearly well-defined clinical measures of CVD risk, one emerging risk factor of this disease is oxidative stress.

OVERVIEW OF OXIDATIVE STRESS

"Reactive oxygen and nitrogen species" (RONS) is an umbrella term that encompasses oxygen radicals, nitrogen radicals, and their nonradical derivatives (83). Although RONS can perform useful functions such as initiating cellular signaling and defending against harmful agents, an overexpression of this species can cause damage to lipids, protein, DNA, and other molecules (84). To prevent this, organisms are equipped with antioxidant defense systems that scavenge RONS. However, the balance between RONS expression and antioxidant scavenging can become disturbed if RONS production is increased and/or the antioxidant defense system is weakened (85). This undesirable phenomenon, which is referred to as "oxidative stress," has been linked to over one-hundred diseases as well as the aging process (84, 86). This section of the review will summarize the current knowledge pertaining to the relationship between oxidative stress and human health. In particular, the following will be discussed: 1) RONS production, 2) postprandial oxidative stress, 3) implications for health and disease, 4) measuring oxidative stress, and 5) attenuating oxidative stress.

RONS Production

Radicals

Within an atom, electrons spin around the nucleus in defined regions known as orbitals. For most atoms and molecules, each orbital contains a pair of electrons that spin in opposite directions, which cancels each electron's magnetic field and maintains stability within the atom or molecule; such species are said to be nonradicals (87). A free radical (hereafter referred to as a radical), then, is any species capable of free existence that contains one or more electrons that spin alone and unopposed in an orbital. Radicals are denoted with a superscript dot ($^{\bullet}$), such as nitric oxide ($NO^{\bullet}$).

The presence of one or more unpaired electrons creates instability within a radical. In an effort to regain stability, the radical typically reacts with another molecule (often a nonradical) in one of two ways: 1) by donating its lone electron(s) to the other molecule or 2) by extracting one or more electrons from the other molecule to pair with its lone electron(s). Thus, when radicals react with nonradicals, the nonradical either gains or loses an electron and converts to a radical. This cycle continues indefinitely until a termination reaction occurs, which can happen in one of two ways: 1) two radicals react with each other or 2) a radical reacts with an antioxidant.

Superoxide, Hydrogen Peroxide, and the Hydroxyl Radical

If radicals can generate new radicals, then how is the first radical produced? The answer to this question can be found in the mitochondrion (reviewed in [88]). During cellular respiration, electrons move throughout the transport chain and provide energy to pump protons from the mitochondrial matrix to the intermembrane space. At certain sites within the transport chain, carriers are only able to transport one electron at a time. An estimated 1-3% of these single electrons "leak" to oxygen and form $O_2^{\bullet -}$ (84).

Considered to be the "primary" RONS, $O_2^{\bullet-}$ generates "secondary" RONS through its interaction with other molecules (84). For example, $O_2^{\bullet-}$ undergoes dismutation via superoxide dismutase to form H_2O_2. By themselves, $O_2^{\bullet-}$ and H_2O_2 are not particularly toxic. In fact, neither species is able to damage DNA or lipids (83). Nonetheless, $O_2^{\bullet-}$ and H_2O_2 pose danger due to their respective abilities to form more toxic RONS. While a complete listing of all of the RONS that are generated from these two species is beyond the scope of this review, the highly reactive hydroxyl radical ($OH^{\bullet}$) is worthy of mention.

$O_2^{\bullet-}$ and H_2O_2 produce $OH^{\bullet}$ *in vivo* mainly via the Haber-Weiss reaction. First, $O_2^{\bullet-}$ reduces ferric iron to ferrous:

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad [1]$$

The newly formed ferrous iron is then combined with H_2O_2 in the Fenton reaction to form $OH^{\bullet}$:

$$Fe^{2} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+} \qquad [2]$$

Thus, the net reaction is:

$$O_2^{\bullet-} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + O_2 \qquad [3]$$

$OH^{\bullet}$ is the most reactive oxygen species in existence. In fact, $OH^{\bullet}$ reacts with the first thing it comes into contact with immediately after it is formed (89). Consequently, whether $OH^{\bullet}$ attacks DNA, lipids, protein, or something else depends entirely on what is immediately proximal to this radical upon its formation. Few (if any) antioxidants can prevent $OH^{\bullet}$ from reacting with another molecule, which means that damage is virtually unavoidable once this radical has been formed (83).

Postprandial Oxidative Stress

RONS formation can be acutely increased in response to several stressors, including physical exercise (73) as well as exposure to chemicals and environmental pollutants such as

cigarette smoke, radiation, and ozone (83). Another stressor known to increase RONS production that has recently received considerable interest from the scientific community is the metabolism of a consumed meal. Indeed, RONS production has been demonstrated to increase following the postprandial metabolism of carbohydrate, lipid and protein ingested either alone or combined with other macronutrients (73). The excessive RONS formation and subsequent oxidative damage caused by this postprandial metabolism is termed postprandial oxidative stress.

Postprandial oxidative stress is a cyclical phenomenon (reviewed in [73]). That is, the postprandial elevation of RONS activates certain pathways – such as cellular adhesion, gene transcription, and inflammation – that in turn lead to additional RONS generation. This cycle is believed to initiate in response to the gathering of substrates (i.e., glucose and/or free fatty acids [FFA]) within the peripheral tissues and/or vasculature. For both sites of RONS production, the metabolism of a high-calorie meal rich in carbohydrate and/or lipid hastens the processing of these substrates through the electron transport chain. Given that electron leakage and subsequent $O_2^{\bullet-}$ production occur even during basal metabolism, the accelerated postprandial metabolism is thought to lead to even greater levels of $O_2^{\bullet-}$ production (73). This accelerated production of $O_2^{\bullet-}$ is exacerbated in the vasculature, because the endothelium lacks the ability to restrict the entry of substrate, particularly glucose (73). It follows that excessive intake of high-energy foods rich in dietary fat and carbohydrate may exacerbate the production of RONS and lead to increased oxidative stress over time. To the contrary, restriction of dietary energy and/or certain macronutrients (e.g., fat) may be associated with a decrease in RONS production and subsequent oxidative stress.

Implications for Health and Disease

As mentioned above, oxidative stress can inhibit the regular function of lipids, protein, and DNA (84). Such inhibition can lead to deleterious effects, including the pathogenesis of various diseases. The roles of oxidative stress in the pathogenesis of diabetes, CVD, and the aging process merit further discussion.

Diabetes and Cardiovascular Disease

The "common soil" hypothesis postulates that T2DM and CVD share common antecedents, particularly postprandial oxidative stress (reviewed in [90]). When an excessive amount of $O_2^{\bullet-}$ is produced in response to the metabolism of a high fat and/or high carbohydrate meal, the body inhibits beta-oxidation in order to curb additional $O_2^{\bullet-}$ production. Such an inhibition increases intracellular FFA, which in turn reduces glucose transporter type 4 translocation. The reduction in this translocation may lead to impaired glucose uptake into tissue and may possibly lead to insulin resistance, which can be viewed as a defense mechanism against additional oxidative stress.

As a response to insulin resistance, the pancreas releases greater levels of insulin in an effort to maintain normal glucose tolerance. However, impaired glucose tolerance eventually occurs once insulin resistance increases to a level that can no longer be compensated for by additional insulin secretion (90). This pre-diabetic condition is characterized by postprandial hyperglycemia: fasting serum glucose is normal, but postprandial serum glucose is elevated.

Postprandial hyperglycemia combined with increased levels of FFA can result in increased RONS production. This poses a particular threat to β cells, which have low levels of antioxidant enzymes to scavenge these RONS (90). Consequently, the oxidative stress caused by postprandial hyperglycemia and elevated FFA levels damages β cells and impairs insulin

production, content, and secretion (90). Impaired insulin secretion, coupled with insulin resistance, eventually leads to the development of overt T2DM.

Type 2 diabetes mellitus, which is characterized by fasting and postprandial hyperglycemia, elevates RONS production through two mechanisms: 1) via the exposure of endothelial cells to glucose and FFAs, and 2) via the production of advanced glycation end products (AGE) (reviewed in (91). The additional RONS promote endothelial dysfunction by inactivating endothelial nitric oxide and inhibiting nitric oxide formation, endothelial nitric oxide synthase, and prostacyclin synthetase. Such dysfunction eventually causes atherosclerosis and CVD via the following: impaired endothelium-dependent vasodilation, increased adhesion molecule expression, increased adhesion of monocytes and platelets, and increased LDL oxidation.

To summarize, the consumption of a high fat and/or high carbohydrate meal elevates RONS production, which in turn damages endothelial cells and β cells. Damage to β cells leads to overt T2DM, which in turn may cause further elevations in RONS production and greater damage to endothelial cells. Damage to the endothelium may eventually cause atherosclerosis and CVD. Therefore, oxidative stress (postprandial in particular) may be viewed as the common soil of T2DM and CVD.

Aging

The Oxidative Stress Theory of Aging (OSTA) postulates that organisms age due to the accumulation of oxidative damage over time. Three main predictions are associated with this theory (reviewed in [92]):

I. Oxidative damage will increase with age. This increase could result from elevated RONS production, decreased antioxidant activity, and/or decreased repair/turnover of oxidative-damaged molecules.

II. Interventions that increase lifespan will reduce oxidative damage. This reduction could result from decreased RONS production, increased antioxidant activity, and/or increased repair/turnover of oxidative-damaged molecules

III. Attenuating oxidative stress will increase lifespan. Conversely, increasing oxidative stress will decrease lifespan. Alterations in oxidative stress can be achieved by manipulating RONS production, antioxidant levels, and or the repair/turnover of oxidative-damaged molecules.

Thus, the validity of this theory rests on the evidence in support of these three predictions.

The majority of evidence supports the first prediction of the OSTA. Indeed, lipid peroxidation, protein oxidation, and DNA oxidation have been observed to increase with age in rats, mice, flies, monkeys, and human tissue (92). In contrast, some investigations have reported no age-related change in DNA oxidation in rodent tissues (93). However, it is currently believed that artifactual DNA oxidation arising during the isolation and analysis of DNA samples is the reason why no age-related change was observed in some studies (92). It should be noted that even though the evidence currently supports the first prediction, this does not necessarily prove the validity of the OSTA.

Regarding the second prediction, CR and certain genetic manipulations, both of which have been shown to reduce oxidative stress, increase lifespan. The scientific literature suggests that CR reduces lipid peroxidation, protein oxidation, and DNA oxidation in rodents. Specifically, this reduction in oxidative damage appears to be due to decreased RONS production and/or increased repair/turnover of oxidative-damaged molecules, because CR appears to have little to no effect on antioxidant activity (92). Even though the collective body of CR literature supports the second prediction, it is currently unknown whether the life extension caused by CR is mediated through attenuated oxidative damage; indeed, other biological pathways that are affected by CR could conceivably be responsible for some or all of the observed life extension (92). Regarding genetic manipulations, mutant strains of *C. Elegans*,

Drosophila, and mice have been reported to live longer and experience attenuations in oxidative stress. However, studies with *C. Elegans* and *Drosophila* have yet to definitively demonstrate the existence of a physiological mechanism that links attenuated oxidative stress with increased lifespan (92).

While the first two predictions of the OSTA attempted to demonstrate correlation between oxidative stress and aging, the third prediction attempted to demonstrate causation. Early investigations tried to prove the third prediction by administering antioxidants to animals. In theory, consuming exogenous antioxidants would increase tissue levels of these molecules, which in turn would decrease the age-related accumulation of oxidative damage and therefore increase lifespan (92). The majority of studies noted no change in lifespan following the consumption of exogenous antioxidants, which seemed to disprove the OSTA. However, these studies failed to prove that the exogenous antioxidants did indeed attenuate oxidative damage in tissues. Without this proof, it could not be claimed that the attenuation of oxidative stress fails to extend life. Recent work has utilized recombinant gene technology to alter the expression of either antioxidant enzymes or enzymes involved in the repair/turnover of oxidative-damaged molecules. Many of these studies reported that overexpression of antioxidant enzymes increases lifespan and that reduced expression of repair enzymes lower lifespan, thus supporting the third prediction (92). However, other studies noted that altering the expression of either type of enzyme has no effect on longevity (92). Unfortunately, few of these investigations examined whether altering the expression of these enzymes had any effect on the accumulation of oxidative damage. Again, knowledge of an intervention's effects on oxidative damage is absolutely critical to proving or disproving the third prediction. Consequently, although plentiful evidence

suggests that organisms age due to the accumulation of oxidative damage over time, more work remains to be performed before this belief is accepted as fact.

Measuring Oxidative Stress

The analysis of certain biomarkers can provide insight regarding the localization and effects of oxidative damage (86). These biomarkers can be assayed from a variety of human tissues or biological fluids (most commonly blood and urine). Biomarkers of oxidative stress can be categorized into three groups: 1) direct indicators of RONS activity; 2) indirect indicators of RONS activity; and 3) the total antioxidant capacity (TAC) (94).

Direct Indicators of RONS Activity

Electron spin resonance (ESR) spectroscopy is currently the only available direct method for detecting RONS. This technique determines the presence of unpaired electrons by measuring the magnetic properties of a tissue sample. However, even during conditions of oxidative stress, these electrons are often too low in concentration and too unstable to normally be detected by ESR spectroscopy *in vivo* (95). Fortunately, more stable and more easily detectable radical adducts can be formed by adding exogenous "spin traps" to the sample. Nevertheless, ESR spectroscopy is labor intensive and requires expensive equipment; as a consequence, it is not commonly used in research settings.

Indirect Indicators of RONS Activity

RONS are typically too unstable and short-lived to be measured directly without utilizing techniques such as ESR spectroscopy. Instead, the more stable molecular end products of oxidative damage more commonly serve as biomarkers of oxidative stress (86). End products of lipid peroxidation, protein oxidation, and DNA oxidation are the most frequently assayed biomarkers of oxidative stress.

Lipid peroxidation involves the abstraction of a hydrogen atom from a fatty acid or a fatty acyl side chain (reviewed in [96]). Most of the time, a newly formed lipid radical will react with O_2 to form a peroxyl radical. Peroxyl radicals can react with one another, oxidize membrane proteins, or abstract hydrogen from adjacent fatty acid side chains, thus persisting lipid peroxidation (96). The two most commonly measured biomarkers of lipid peroxidation are MDA and F_2-Isoprostanes (F_2-IsoPs). Malondialdehyde is a byproduct of the peroxidation of arachidonic acid, eicosapentaenoic acid, and docosahexanoic acid. This biomarker is typically measured via the thiobarbituric acid reactive substances (TBARS) assay, which has the advantages of being relatively inexpensive and easy to perform. To perform a TBARS assay, the sample is heated with thiobarbituric acid under acidic conditions, and a fluorescent red derivative (which allegedly represents a $[TBA]_2$-MDA adduct) is spectophotometrically measured by its absorbance at 532 nm (96). However, the TBARS assay has been widely criticized due to the fact that several biological molecules – such as amino acids, bile acids, carbohydrates, nucleic acids, and certain antibiotics – can react with thiobarbituric acid and produce false-positive readings (95). In an attempt to improve the measurement of MDA (or TBARS), alternative procedures have been developed which require a dual wavelength read (97). F_2-IsoPs are prostaglandin-like compounds generated *in vivo* via nonenzymatic peroxidation of arachidonic acid (86). Several methodologies of measuring F_2-IsoPs currently exist, including gas chromatography-mass spectrometry (MS), liquid chromatography (LC)-MS, enzyme immunoassays, and radioimmunoassays. F_2-IsoPs are considered some of the most reliable biomarkers of oxidative stress for the following reasons: 1) they can be measured accurately down to picomolar concentrations; 2) they are stable in both urine and exhaled breath condesates, which allows for an accurate, noninvasive measurement of lipid peroxidation; 3)

they do not exhibit diurnal variations, nor are they affected by lipid dietary intake; and 4) they are present and detectable in all human tissues and biological fluids, thus providing a reference of comparison between oxidatively damaged and non-oxidatively damaged samples (86).

Proteins are some of the main targets of oxidative modification *in vivo*, because they are abundant in living organisms, and because they have a primary role in most cellular processes (86). RONS can attack protein directly, or they can react with molecules such as lipids and sugars to form new products that attack protein (98). Some oxidative modifications of protein cleave the peptide bond, while others modify sidechains. Oxidative damage to protein is typically irreparable and can lead to downstream impairments of a variety of cellular functions (86). The two most commonly measured biomarkers of protein oxidation are protein-bound carbonyls and nitrotyrosine. Several physiological phenomena can result in protein-bound carbonyl formation, including oxidation of several amino sidechains, formation of Michael adducts, and glycation/glycoxidation of lysine (86). Protein-bound carbonyls are an attractive choice for laboratory measurement, because they remain stable for up to ten years when stored at -80°C (86). These biomarkers are commonly measured via spectrophotometric 2,4-dinitrophenylhydrazine assay, spectrophotometric 2,4-dinitrophenylhydrazine assay coupled to protein fractionation by high-performance liquid chromatography, and one- or two-dimensional electrophoresis and Western blot immunoassay (99). Unfortunately, these methods lack the specificity to identify which amino acids were oxidatively attacked as well as which proteins were oxidative modified (99). Regarding nitrotyrosine, its presence was initially thought to be ironclad evidence of peroxynitrite formation *in vivo*. However, recent work has demonstrated that nitrotyrosine can be generated via multiple pathways, some of which do not involve peroxynitrite (95). Several methods can be used to quantify this biomarker, including enzyme-

linked immunosorbent assay, high-performance liquid chromatography with electrochemical detection, MS-based assays, LC-MS, and liquid chromatograph-tandem MS (99).

RONS can damage DNA through various mechanisms, including modification of all bases, production of base-free sites, deletions, frame shifts, strand breaks, DNA-protein cross-links, and chromosomal rearrangements (100). $OH^{\bullet}$ is the main RONS associated with oxidative damage. This radical species has been shown to react with every component of DNA (the purine and pyrimidine bases as well as the deoxyribose backbone) (100) and produce a variety of base and sugar modification products (101). Such modification products transform into stable end products, which can serve as biomarkers of DNA oxidation. Unfortunately, the relative amounts of these end products depend not simply on the magnitude of DNA damage, but also on reaction conditions (101). As a consequence, no single end product should be considered the sole piece of evidence pertaining to the magnitude of DNA oxidation that occurred. Nonetheless, many investigations have only measured 8-hydroxy-2'-deoxyguanosine (8OHdG) to indicate oxidative damage to DNA (101). This biomarker is most commonly collected via urine sampling. One advantage of this form of sampling is that urinary 8OHdG does not appear to be affected by acute changes in diet (101). However, urinary 8OHdG may not accurately reflect levels of DNA oxidation, because this biomarker can also arise from the degradation of oxidized deoxyguanosine triphosphate in the DNA precursor pool (101). Common methods for assaying urinary 8OHdG include high-performance liquid chromatography, MS, and the enzyme-linked immunosorbent assay (101).

Total Antioxidant Capacity

An organism's antioxidant defense system may be temporarily abated as its components are employed to scavenge RONS. Thus, measuring TAC serves as another method of estimating

oxidative stress. This measurement is performed by adding a known quantity of a given RONS to a sample and measuring the amount of RONS that is scavenged (102). Measuring TAC serves as a better estimation of oxidative stress *in vivo* than measuring individual antioxidants separately, because TAC reflects the fact that antioxidants act synergistically to scavenge RONS (102). Also, TAC is easier to perform and less costly than disentangling the antioxidants and examining each one separately (103). Blood is the preferred biological fluid for TAC assays, because it is influenced by RONS overload and dietary antioxidant consumption, thus providing a more accurate representation of oxidative stress *in vivo* (102). Several methods of assaying TAC are currently practiced, including Total Radical-Trapping Antioxidant Parameter, Ferric Reducing Ability of Plasma, Trolox Equivalent Antioxidant Capacity, and Oxygen Radical Absorbance Capacity. Currently, none of these methods is considered to be the "gold standard" (103). Moreover, "total antioxidant capacity" has been criticized as being a misnomer, because different methods of assaying TAC will yield different results due to the different reaction conditions (most notably, the species of RONS that is used) associated with each method (103). Thus, the emergence of a single method that can successfully determine *total* antioxidant capacity will likely never occur.

Attenuating Oxidative Stress

Three main interventions can attenuate oxidative stress. First, pharmacological interventions can reduce RONS formation. Second, exercise can upregulate the endogenous antioxidant defense system. Finally, antioxidant intake (either through diet or supplementation) and a reduction of dietary energy/dietary lipid can increase tissue antioxidant levels and lead to a decrease in RONS formation, respectively.

Pharmacological Interventions

Several pharmaceuticals have been demonstrated to decrease RONS generation, including thiazolidinediones, statins, angiotensin type I (AT_1) inhibitors, and angiotensin converting enzyme inhibitors. Thiazolidinediones inhibit inducible nitric oxide synthase, an enzyme that diverts electrons from L-arginine to molecular oxygen to form $O_2^{\bullet-}$ (104). Statins, AT_1 inhibitors, and angiotensin converting enzyme inhibitors reduce the activity of nicotinamide adenine dinucleotide phosphate oxidase, an enzyme responsible for increasing $O_2^{\bullet-}$ production (105). Nicotinamide adenine dinucleotide phosphate oxidase activity is stimulated when angiotensin II activates AT_1 receptors. Statins reduce AT_1 receptor expression (106), AT_1 inhibitors prevent angiotensin II from activating AT_1 receptors (107), and angiotensin converting enzyme inhibitors block the conversion of angiotensin I to angiotensin II (105).

Exercise

An exercise bout, whether aerobic or anaerobic, causes an acute elevation in RONS production, which can occur via multiple pathways: 1) increased oxygen consumption leads to increased cellular respiration, prostanoid metabolism, and/or autoxidation of catecholamines; and 2) increased ischemia/reperfusion stimulates oxidase enzymatic activity (108). Moreover, this initial elevation in RONS production can lead to subsequent elevations via proteolysis, inflammation, and/or a loss of balance in calcium homeostasis (108). The amount of RONS elevation depends on a variety of factors, including: age, health, intensity and duration of exercise, training status, as well as the amount and duration of antioxidant intake/supplementation (108).

Exercise-induced elevations in RONS formation cause an upregulation of the endogenous antioxidant defense system in an effort to protect the body from elevated RONS during

subsequent training sessions (109). Specifically, exercise increases the activity of both antioxidant enzymes and oxidative damage repair systems (110). Such an adaptation is consistent with the hormesis hypothesis, which proposes that repeated exposure to low-intensity stressors protects an organism from subsequent exposure to higher-intensity stressors (111). Two considerations are worthy of mention. First, antioxidant supplementation may blunt the acute elevation in RONS that occurs during an exercise bout, thus removing the stimulus necessary for the upregulation of endogenous antioxidant defenses (112, 113). Second, exercise of too high an intensity and/or too long a duration may lead to an exacerbated RONS elevation that inundates antioxidant defenses and contributes to poor health and disease (108). Therefore, more research is needed to establish an "optimal" level of acute RONS elevation.

Antioxidant Intake and Diet Modification

Exogenous antioxidants can be consumed via either diet or supplementation. The exogenous antioxidants that have been most commonly researched are ascorbate (vitamin C), tocopherols (vitamin E), carotenoids, and flavonoids. Once consumed, these antioxidants act synergistically to delay or inhibit oxidative damage (114, 115).

Vitamin C is regarded as one of the most potent and important antioxidants (114, 115). Indeed, vitamin C is particularly effective at quenching $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, and singlet oxygen (114). Also, vitamin C has been demonstrated *in vitro* to regenerate vitamin E from the tocopheroxyl radical that is formed during the inhibition of lipid peroxidation (114). It is recommended that 145 mg of vitamin C be consumed daily to reduce the risk of developing disease (114). The main dietary sources of vitamin C are citrus fruits, kiwis, cherries, melons, tomatoes, leafy greens, broccoli, cauliflower, Brussel sprouts, and cabbage (114).

"Vitamin E" is a generic term for tocopherols and tocotrienols (115), which represent 4 specific forms of tocopherols (alpha, beta, gamma, delta) and 4 specific forms of tocotrienols (alpha, beta, gamma, delta). As an antioxidant, vitamin E mainly quenches lipid peroxyl radicals to produce lipid hydroperoxides and tocopheroxyl radicals (114). The majority of tocopheroxyl radicals are then converted back to vitamin E via either vitamin C or glutathione (114). Also, vitamin E has been shown to scavenge singlet oxygen and peroxynitrite (114). It is recommended that 23 mg of vitamin E be consumed daily to reduce the risk of developing disease (114). The main dietary sources of vitamin E are wheat germ, nuts, several green leafy vegetables, vegetable oils, and vegetable oil products (114). Vitamin E and Vitamin C are currently the only two dietary antioxidants considered to meaningfully reduce oxidative damage in humans (116).

Carotenoids are tetraterpenoid organic pigments that have the ability to dissolve fats, oils, and lipids. These antioxidants are effective scavengers of singlet oxygen and peroxyl radicals (114). Also, β-carotene, the most well-known carotenoid, is particularly effective at quenching trichloromethylperoxyl radicals (115). It is recommended that 5.7 mg of carotenoids be consumed daily to reduce the risk of developing disease (114). The main dietary sources of carotenoids are carrots (α-carotene and β-carotene) tomatoes (lycopene), citrus fruits (β-cryptoxanthin), spinach (lutein), and corn (zeaxanthin) (114).

Flavonoids are polyphenolic compounds that exist in all plant families. The main antioxidant subforms of flavonoids are flavones, flavanones, isoflavones, flavonols, flavononols, flavan-3-ols, and anthocyanidins (117). Flavonoids inhibit xanthine oxidase and protein kinase C, which consequently lowers $O_2^{\bullet-}$ production (117). In addition, flavonoids chelate certain trace metals that could otherwise serve as reactants in the production on RONS (117). Flavonoids

have a very poor bioavailability; in fact, only a few percent of ingested flavonoids are absorbed into the bloodstream (117). Currently, little is known about the antioxidant effects of flavonoids *in vivo*, but it appears that these compounds may have a sparing effect on α-tocopherol and β-carotene (117). The main dietary sources of flavonoids are fruits, vegetables, seeds, nuts, grains, spices, tea, wine, and beer (117).

Aside from the above, many additional antioxidants are available within both whole foods and nutritional supplements. Many nutritionists argue that the best method to ensure intake of a full spectrum of antioxidants is to consume a wide variety of fresh fruits, vegetables, and whole grains. Such a dietary pattern is very low in saturated fat and cholesterol, is relatively low in total dietary energy, and likely results in a low and "healthy" production of RONS. This plan is characteristic of the Daniel Fast.

THE DANIEL FAST

The Daniel Fast derives from two passages in the Book of Daniel. In Daniel 1:8-14 (NIV), Daniel asked the chief official for permission to consume nothing but vegetables and water for ten days. This request was made in an effort to avoid the consumption of Babylonian food that was not prepared in accordance with the Mosaic Law. Later in the same book (Daniel 10:2-3 NIV), Daniel refrained from consuming choice food (meat or wine) for twenty-one days. Based on these two passages, a modern day Daniel Fast allows for *ad libitum* consumption of fruits, vegetables, whole grains, nuts, seeds, and oil. Animal products, refined foods, white flour, preservatives, additives, sweeteners, flavorings, caffeine, and alcohol are each disallowed during this fast. Thus, the Daniel Fast can be described as a variant of veganism and a form of DR. This fast is commonly partaken for twenty-one days, although longer (forty-day) and shorter

(ten-day) fasts have been observed. Although no Biblical passage enjoins the faithful to observe the Daniel Fast at any particular time of the year, this fast is often partaken during the month of January to commemorate the New Year with fasting and prayer.

The only study to date that has examined the Daniel Fast reported favorable changes in cardiovascular health, glucoregulatory health, blood lipids, and oxidative stress (118, 119). Systolic BP, diastolic BP, TC, LDL-C, MDA, and H_2O_2 decreased during the fast. Trolox Equivalent Antioxidant Capacity and nitrate/nitrite both increased from pre to post fast. C-reactive protein (3.15 ± 0.91 vs. 1.60 ± 0.42 $mg \cdot L^{-1}$; $p = 0.13$), plasma insulin (4.42 ± 0.52 vs. 3.37 ± 0.35 $\mu U \cdot mL^{-1}$; $p = 0.10$), and the homeostatic model of insulin resistance (0.97 ± 0.13 vs. 0.72 ± 0.08; $p = 0.10$) were each lowered to a clinically meaningful, albeit statistically insignificant, extent. Plasma VLDL-C and TAG did not change significantly during the fast, nor did the TC:HDL-C ratio.

Some advantages may make the Daniel Fast a superior option to CR and ADF regimens for optimizing health. The Daniel Fast's allowance of *ad libitum* energy intake likely promotes greater satiety and compliance compared to dietary regimens that restrict energy consumption. Also, partaking in a Daniel Fast serves as an education in food choices, as nutrition labels must be checked to see if food selections comply with the fast's restrictions (118).

Unfortunately, a notable drawback of the Daniel Fast appears to be its lowering of HDL-C. Indeed, this lipoprotein decreased by 14% in the lone investigation that examined the Daniel Fast (118). This lowering of HDL-C was outpaced by a 19% reduction in TC, thus resulting in a reduced TC:HDL-C ratio (118). However, given the important role of HDL-C in reverse cholesterol transport, any reduction of this lipoprotein should be avoided if at all possible.

Theoretically, including an intervention known to raise HDL-C could counterbalance the lowering of this lipoprotein that occurs during the Daniel Fast. Even though aerobic exercise is an excellent lifestyle intervention that should be performed by all able-bodied individuals, its minimal effect on HDL-C is likely insufficient to offset a fast-induced 14% reduction of this cholesterol. Moreover, although several pharmaceuticals (statins, niacin, fibrates, thiazolidnediones, glitazars, CETP inhibitors, and HDL infusions) have been shown to substantially raise HDL-C, use of these drugs without a medical need departs from the abstemious philosophy of a Daniel Fast. All-natural nutritional supplements, on the other hand, appear to be ideal intervention candidates. Krill oil is particularly interesting in this regard, as one study reported a 43-60% increase in HDL-C following supplementation (67), while two recent studies (70, 71) reported no change in this cholesterol.

Summary

Favorable changes in a multitude of health-related biomarkers were noted during the initial investigation of the Daniel Fast. Such findings warrant a subsequent investigation designed to replicate (or improve on) the results of the initial study. However, not every change noted in the initial investigation was desirable (e.g., a 14% reduction in HDL-C). Therefore, a subsequent investigation should seek to maintain or improve HDL-C via an intervention that is harmonious with the abstemious philosophy of the Daniel Fast.

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INSTITUTIONAL REVIEW BOARD APPROVAL

THE UNIVERSITY OF MEMPHIS

Institutional Review Board

To: Richard Bloomer
Health & Sport Sciences

From: Chair, Institutional Review Board
for the Protection of Human Subjects

Subject: **Biochemical and Health Effects of the "Daniel Fast" 2 (H11-14)**

Approval Date: **August 13, 2010**

This is to notify you of the board approval of the above referenced protocol. This project was reviewed in accordance with all applicable statutes and regulations as well as ethical principles.

Approval of this project is given with the following obligations:

1. At the end of one year from the approval date an approved renewal must be in effect to continue the project. If approval is not obtained, the human consent form is no longer valid and accrual of new subjects must stop.
2. When the project is finished or terminated, the attached form must be completed and sent to the board.
3. No change may be made in the approved protocol without board approval, except where necessary to eliminate apparent immediate hazards or threats to subjects. Such changes must be reported promptly to the board to obtain approval.
4. The stamped, approved human subjects consent form must be used. Photocopies of the form may be made.

This approval expires one year from the date above, and must be renewed prior to that date if the study is ongoing.