



Effects of Intermittent Fasting and Physical Activity on Salivary Expression of Reduced Glutathione and Interleukin-1 β

COLTON ALLEN*¹, BRANDON SELLERS*¹, MACKINZIE SMITH*¹, ALYSSA EDWARDS*¹, KIRSTEN GATELESS*¹, BAILEY AAB*¹, KALEE SHERRARD*¹, CARL BOLYARD^{‡2}, and SHAWN STOVER^{‡1}

¹Department of Biology and Environmental Science, Davis & Elkins College, Elkins, WV, USA;

²Emergency Department, Davis Medical Center, Elkins, WV, USA

*Denotes undergraduate student author, †Denotes professional author

ABSTRACT

International Journal of Exercise Science 13(7): 1063-1071, 2020. Previous research has consistently demonstrated that regular exercise promotes antioxidant production and decreases the expression of inflammation markers. However, there is very little research examining the effects of intermittent fasting (IF) on oxidative stress and inflammation. The present study investigated the hypothesis that a combination of IF and physical activity will reduce the need for glutathione (GSH) production by decreasing oxidative stress. In addition, it was hypothesized that a combination of IF and physical activity will significantly reduce inflammation, as indicated by a decrease in interleukin-1 β (IL-1 β) concentration. For three months, subjects practicing IF (n=7) ate only during an eight-hour window each day and fasted for the next 16 hours. A standard diet control group (n=18) maintained a normal, balanced diet spread out over the course of 14-18 hours each day. Based on data obtained from fitness-tracking devices, subjects were placed into one of three activity level groups: minimum, moderate, and maximum physical activity. Subjects provided fasting saliva samples monthly. The samples were subjected to a glutathione microplate assay and an interleukin ELISA test to determine salivary concentrations of GSH and IL-1 β , respectively. For GSH concentration, there were no significant differences between the diets at any physical activity level. However, moderate to maximum physical activity, in conjunction with fasting, led to significant decreases in IL-1 β concentration. In summary, results suggest that a combination of moderate physical activity and intermittent fasting promotes the maintenance of antioxidant function while inhibiting the inflammatory process.

KEY WORDS: Cytokine, reactive oxygen species, time-restricted feeding

INTRODUCTION

The generation of reactive oxygen species (ROS) occurs as a consequence of normal cellular metabolism (25). These “free radical” oxygens have unpaired electrons, making them highly reactive and potentially destructive. Prolonged ROS exposure can result in oxidative damage to cellular proteins, nuclear DNA, and membrane lipids (7). Neutralization of excessive ROS is undertaken by the body’s endogenous antioxidant defense system, which includes the enzymes

superoxide dismutase and glutathione peroxidase, in conjunction with exogenous dietary antioxidants (25). Oxidative stress results from an imbalance between ROS production and antioxidant defenses. Reduced glutathione (GSH) plays a major role in the cellular defense against oxidative stress by employing a thiol group as a reducing agent (13).

Inflammation is an important part of the body's immune response to infection, injury, or allergy. The immune system releases pro-inflammatory signaling molecules called cytokines to promote blood vessel dilation, allowing white blood cells to rapidly reach the site of infection or injury. Inflammation is normally a protective response, but it can potentially become a chronic condition, even in the absence of infection or injury. Chronic inflammation is associated with heart disease, diabetes, and cancer (27). Interleukin-1 β (IL-1 β) is a cytokine released early in the immune response by monocytes and macrophages (28). Since it mediates the expression of other cytokines, overproduction of IL-1 β can lead to chronic inflammation (24).

Previous research has demonstrated that regular physical exercise promotes an upregulation of GSH synthesis to provide protection against exercise-induced oxidative stress (5, 8). Furthermore, the muscle damage associated with strenuous exercise is a catalyst for local inflammation (3), and a crucial cytokine in exercise-associated inflammation is IL-1 β (19). Research by Petersen and Pedersen (20) indicates that long-term exercise training actually has anti-inflammatory effects, including a decrease in IL-1 β levels. While multiple studies have examined the complex relationship between exercise, oxidative stress, and inflammation (22, 23), and there has been considerable research into the effects of food and supplements on exercise-associated oxidative stress and inflammation (6), the impact of fasting on those variables has received far less attention.

Intermittent fasting (IF) involves the regular cycling of periods of fasting and non-fasting (14). Previous work indicates that IF can reduce markers of oxidative stress in overweight adults over the course of eight weeks (10). A 2016 study (16) concluded that time-restricted feeding significantly decreases circulating markers of inflammation, including IL-1 β . Furthermore, a study of volunteers participating in the month-long intermittent religious fast associated with Ramadan demonstrated decreased blood levels of multiple inflammation markers, compared to non-fasting control subjects (1).

Changes in salivary biomarkers have proven to be effective indicators of systemic changes associated with various medical conditions, including cardiovascular disease (15) and type II diabetes mellitus (2). The present study investigated the hypothesis that a combination of IF and physical activity will reduce the need for GSH upregulation by decreasing oxidative stress. The oxidative status of subjects was assessed by measuring salivary concentrations of GSH. In addition, it was hypothesized that a combination of IF and physical activity will significantly reduce inflammation, as indicated by a decrease in the salivary concentration of IL-1 β .

METHODS

Participants

This research was carried out fully in accordance to the ethical standards of the International Journal of Exercise Science (17) and was approved by the Institutional Review Board (IRB) of Davis & Elkins College (D&E). Participants were recruited from a population of students and staff at D&E and a population of healthcare workers at a regional medical center. Data pertaining to individuals who did not complete the entire three-month study were excluded from the final analysis. A total of 12 males (age 21-57) and 13 females (age 21-58) completed the study. For three months, subjects practicing IF (n=7) ate only during an eight -hour window each day (from 10:00 a.m. to 6:00 p.m., for example) and fasted for the next 16 hours. A standard diet control group (n=18) maintained a normal, balanced diet spread out over the course of 14-18 hours each day. IRB guidelines prevented the prescription of specific diets for research subjects. The small number of subjects in the IF group initiated the diet on their own and had been fasting regularly for at least one year prior to the study.

Based on three months of data obtained from wristband-embedded fitness tracking devices (Fitbit Flex, Fitbit Inc., San Francisco, CA), individuals were placed into one of three activity level groups: minimum (MIN; fewer than 8,000 steps per day), moderate (MOD; between 8,000 and 12,000 steps per day), and maximum physical activity (MAX; more than 12,000 steps per day). Because the American Heart Association recommends 10,000 steps per day for a heart-healthy lifestyle (21), 10,000 steps per day was designated as the midpoint of the moderate physical activity range.

Protocol

At the onset of the study, each subject signed a consent form and agreed to fast and drink only water for at least eight hours prior to each sample collection. Each subject was given a wristband-embedded fitness tracker and a brief tutorial on how to use it. Approximately one month later, subjects reported for the first sample collection. Using a saliva collection aid (Salimetrics LLC, State College, PA) to maximize the acquisition of whole saliva (serous and mucus secretions), each subject provided a 0.5-1.0 ml sample, which was stored in a cryovial at -20°C until analyzed. Subjects' body weight and body fat percentage data were obtained using a digital scale capable of measuring electrical impedance (Fitbit Aria, Fitbit Inc., San Francisco, CA). Although it is common practice to restrict water intake for several hours prior to measuring electrical impedance, subjects were encouraged to consume approximately eight ounces of water just before sample collection in order to facilitate saliva production. Resting heart rate and blood pressure were determined via fingertip pulse oximeter and sphygmomanometer, respectively. Fasting saliva samples were collected twice more, at approximately one-month intervals. All sample collections took place between 7:30 a.m. and 8:30 a.m. Body weight, body fat percentage, resting heart rate, and blood pressure were measured, and fitness tracker data were downloaded, at the time of each sample collection. To ensure that acute immune responses did not skew results of the study, subjects confirmed, to the best of their knowledge, that they were infection- and injury-free at each sample collection session.

Fifty (50) μl of each saliva sample was diluted with 150 μl of 5% metaphosphoric acid and centrifuged at 13,000 $\times g$ for two minutes. Resulting supernatants were reacted with 5,5'-dithiobis-2-nitrobenzoic acid in a 96-well microtiter plate to generate a stable GSH chromophore with maximal absorbance at 405 nm (Total Glutathione Microplate Assay, Oxford Biomedical Research, Oxford, MI). Total GSH concentrations were calculated via plate reader (SpectraMax M2 Microplate Reader, Molecular Devices LLC, San Jose, CA).

Two hundred (200) μl of each saliva sample was centrifuged at 1,500 $\times g$ for 15 minutes. Resulting supernatants were subjected to an enzyme-linked immunosorbent assay (ELISA) in a 96-well microtiter plate to generate a stable IL-1 β antigen-antibody-conjugated enzyme complex with maximal absorbance at 450 nm (Salivary IL-1 β ELISA Kit, Salimetrics LLC, State College, PA). IL-1 β concentrations were calculated via plate reader.

Statistical Analysis

ProStat version 5.5 (Poly Software International, Pearl River, NY) was used for statistical analysis. GSH and IL-1 β data were subjected to a multiple comparison analysis of variance (ANOVA). Fisher's least significant difference test was employed to compare specific groups in the ANOVA. An alpha level of $P < 0.05$ was regarded as statistically significant. Data are expressed as mean \pm standard error.

RESULTS

In terms of salivary GSH concentration, there was no significant difference between the standard diet and IF. However, IF produced significantly less inflammation than the standard diet, as indicated by salivary IL-1 β concentration (Table 1). There were no significant age- or gender-related differences associated with GSH or IL-1 β concentrations.

Table 1. Effects of intermittent fasting on GSH and IL-1 β concentrations.

	Standard Diet (n=18)	Intermittent Fasting (n=7)
GSH (μM)	8.18 \pm 0.85	7.04 \pm 0.57
IL-1 β (pg/ml)	133.44 \pm 27.28	48.88 \pm 14.16*

*Significantly different from standard diet.

For GSH concentration, there were no significant differences between the diets at any physical activity level (Figure 1). However, moderate to maximum physical activity, in conjunction with fasting, led to significant decreases in IL-1 β concentration (Figure 2).

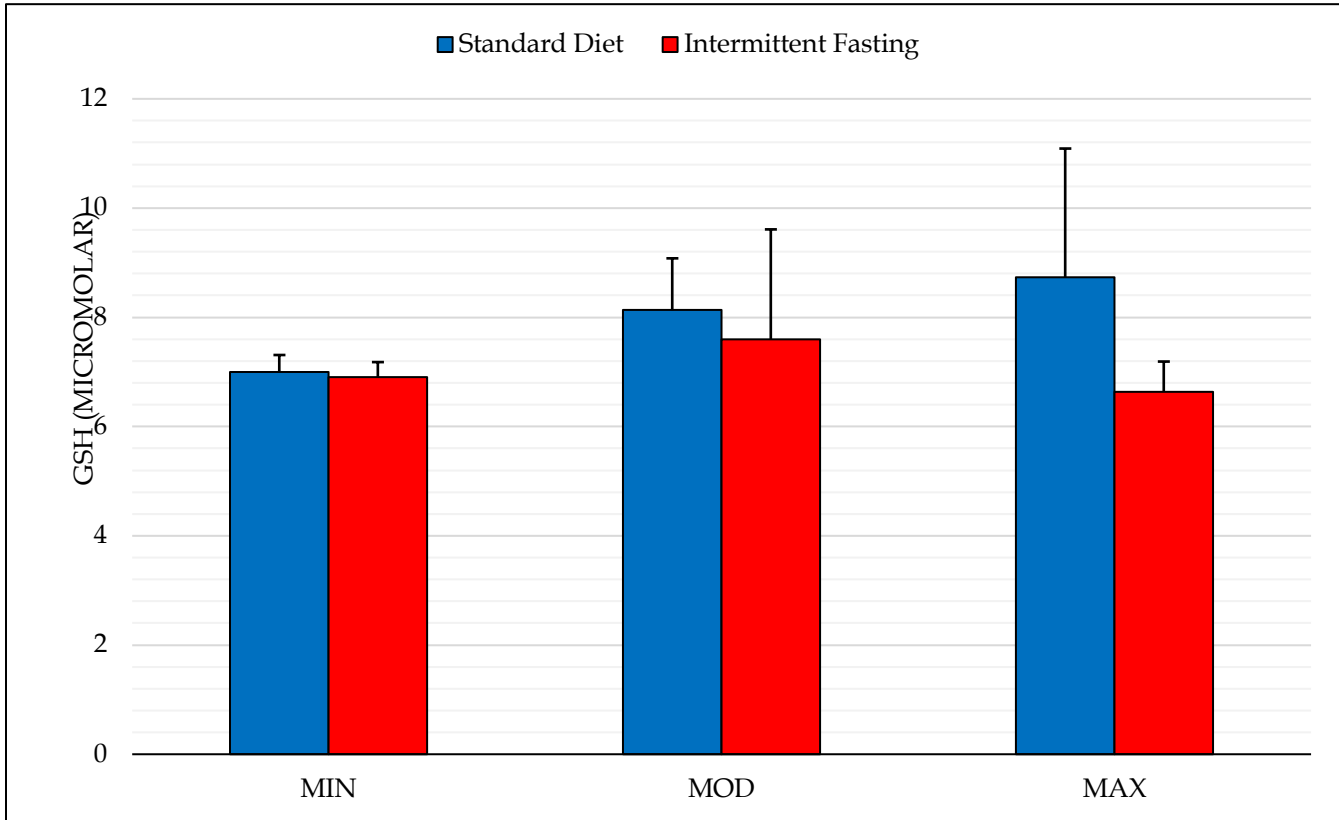


Figure 1. Physical activity effect on GSH. Std. Diet, MIN (n=3); MOD (n=10); MAX (n=5). IF, MIN (n=2); MOD (n=3); MAX (n=2).

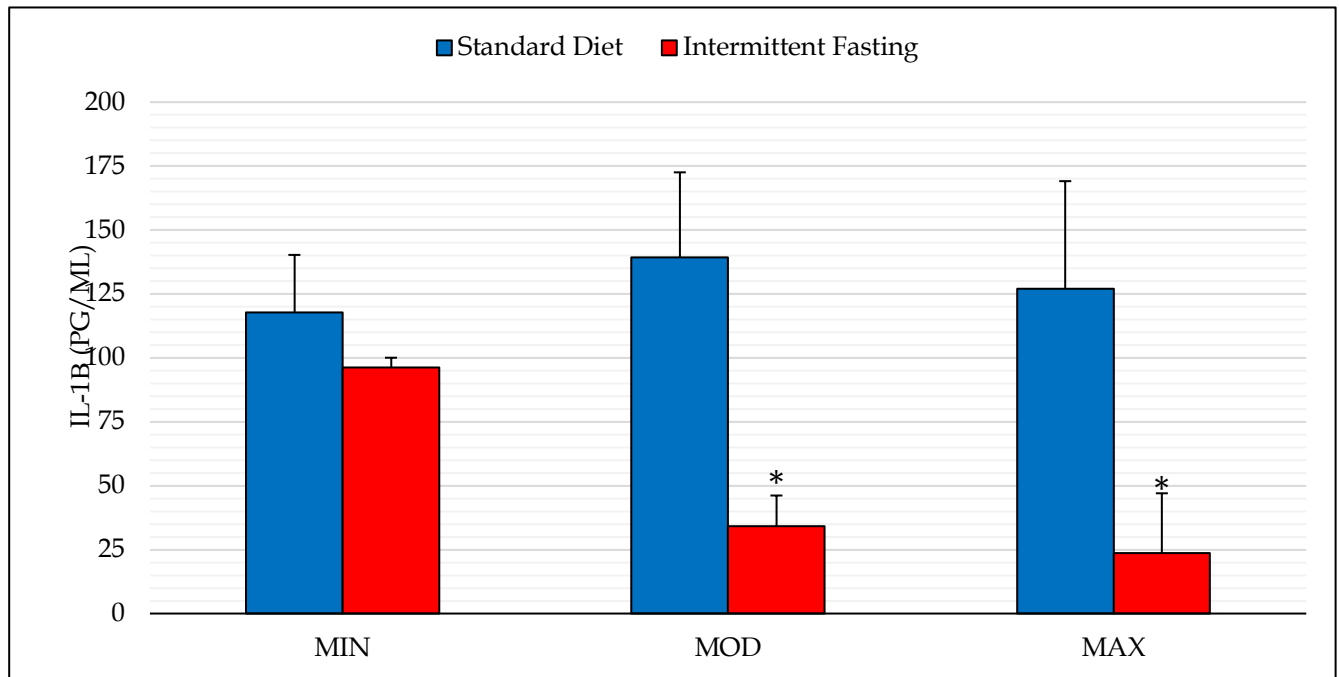


Figure 2. Physical activity effect on IL-1β. *Significantly different from minimum exercise. Std. Diet, MIN (n=3); MOD (n=10); MAX (n=5). IF, MIN (n=2); MOD (n=3); MAX (n=2).

Although the IF group was significantly older than the standard diet group, on average, anthropometric and cardiovascular variables were comparable (Table 2). There were no significant age-related differences associated with any demographic variable. The only gender-related difference was associated with body fat. Females, on average, exhibited a significantly higher body fat percentage (28.6 ± 1.8) than males (21.4 ± 1.8).

Table 2. Group demographics.

	Standard Diet (n=18)	Intermittent Fasting (n=7)
Age	30.9 \pm 2.9	43.0 \pm 4.6*
Steps/Day	10,374.8 \pm 965.1	10,427.5 \pm 1,581.4
Body Mass Index	25.1 \pm 0.7	25.9 \pm 1.7
Body Fat Percentage	24.0 \pm 1.7	26.5 \pm 2.9
Resting Heart Rate (beats/min)	59.8 \pm 2.1	61.9 \pm 3.3
Systolic Blood Pressure	115.5 \pm 0.8	116.4 \pm 1.8
Diastolic Blood Pressure	79.1 \pm 0.7	79.1 \pm 1.5

*Significantly different from standard diet.

DISCUSSION

In the current study, no difference was found between IF and the standard diet in terms of salivary GSH concentration (Table 1). Furthermore, as physical activity level increased, GSH concentration remained relatively consistent (Figure 1). Because GSH is oxidized during exercise (9, 11), its production may have been upregulated to maintain a stable concentration. Previous research has demonstrated GSH upregulation in liver (5) and skeletal muscle (8) in response to regular aerobic exercise. Further research will be necessary to determine if expression of the enzymes associated with GSH synthesis, glutamate-cysteine ligase and glutathione synthetase, is actually elevated in response to increasing physical activity levels. Although not statistically significant, the combination of IF and maximum physical activity in the current study appeared to generate less GSH than maximum physical activity alone. This might indicate a slight decrease in oxidative stress. For future studies, markers of lipid peroxidation and oxidative protein modification could be examined to determine whether a combination of IF and vigorous physical activity is sufficient to significantly decrease oxidative stress.

IF produced significantly less inflammation than the standard diet, as indicated by salivary IL-1 β concentration (Table 1). While the combination of IF and minimum physical activity was not effective at decreasing inflammation, it appears that moderate to maximum physical activity, in conjunction with fasting, significantly reduces the inflammatory activity of IL-1 β (Figure 2). Other markers of inflammation, including interleukin-6 and C-reactive protein, could be assayed in the future to determine the extent of inflammatory control offered by a combination of IF and moderate-to-vigorous physical activity. Furthermore, previous work has demonstrated a decrease in inflammation in response to a combination of IF and resistance training (16). Perhaps a combination of both aerobic training and resistance training, in conjunction with time-restricted feeding, could optimize anti-inflammatory pathways. In

addition, the IF model employed in the current study is not the only model in use. Johnson *et al.* (10) demonstrated a significant decrease in the inflammation marker TNF- α in response to alternate day calorie restriction. It remains to be determined whether one particular IF strategy is superior.

The IF and standard diet groups in the current study were very similar in terms of anthropometric and cardiovascular variables (Table 2), suggesting that the dramatic differences observed in IL-1 β concentrations were influenced significantly by their respective diets. On average, members of the IF group were significantly older than members of the standard diet group. Only one college student was practicing IF. Other members of the group were faculty members or healthcare workers. However, no significant age-related differences were found to be associated with either GSH or IL-1 β concentration. Furthermore, all members of the IF group had been fasting for at least one year prior to the study. In a concurrent study, using many of the same subjects, we investigated the effects of IF on resting metabolic rate (26). We wanted to assess IF over a long period of time to address any impact it might have on the adaptive thermogenesis that can eventually slow metabolic rate and inhibit weight loss. We found that, after more than a year, resting metabolic rate had not decreased for individuals practicing IF.

Saliva collection is easy and non-invasive. It is safer to work with than blood and has been used effectively to quantify enzymes, hormones, and cancer mutations (4). Both GSH (18) and IL-1 β (12) are consistently detectable in saliva at concentrations comparable to, or greater than, that of blood. Salivary concentrations for GSH and IL-1 β obtained in the current study were comparable to those of previous studies.

Finally, it should be noted that sample sizes in the current study were diminished considerably when subjects were placed into separate groups based on activity level. The IF group was small at the onset (n=7) due to IRB restrictions. IF groups designated as MIN and MAX each contained only two subjects. Such small sample sizes decrease the power of statistical analysis. While the results of this preliminary study are encouraging, follow-up studies with larger sample sizes will be necessary to confirm the anti-oxidative and anti-inflammatory potential of intermittent fasting.

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REFERENCES

1. Aksungar FB, Topkaya AE, Akyildiz M. Interleukin-6, C-reactive protein and biochemical parameters during prolonged intermittent fasting. *Ann Nutr Metab* 51(1): 88-95, 2007.
2. Al-Rawi NH. Oxidative stress, antioxidant status and lipid profile in the saliva of type 2 diabetics. *Diab Vasc Dis Res* 8(1): 22-28, 2011.

3. Aoi W, Naito Y, Takanami Y, Kawai Y, Sakuma K, Ichikawa H, Yoshida N, Yoshikawa T. Oxidative stress and delayed-onset muscle damage after exercise. *Free Radic Biol Med* 37(4): 480-487, 2004.
4. Aro K, Wei F, Wong DT, Tu M. Saliva liquid biopsy for point-of-care applications. *Front Public Health* 5: 77, 2017.
5. Chang SP, Chen YH, Chang WC, Chen JT. Increase of anti-oxidation by exercise in the liver of obese Zucker rats. *Clin Exp Pharmacol Physiol* 31(8): 506-511, 2004.
6. Dannecker EA, Liu Y, Rector RS, Thomas TR, Sayers SP, Leeuwenburgh C, Ray BK. The effect of fasting on indicators of muscle damage. *Exp Gerontol* 48(10): 1101-1106, 2013.
7. Ibrahim WH, Habib HM, Jarrar AH, Al Baz SA. Effect of Ramadan fasting on markers of oxidative stress and serum biochemical markers of cellular damage in healthy subjects. *Ann Nutr Metab* 53(3): 175-181, 2009.
8. Jagannathan R, Hussain K, Somani SM. Interaction of pyridostigmine and physical stress on antioxidant defense system in skeletal muscle of mice. *J Appl Tox* 21(4): 341-348, 2001.
9. Ji LL, Fu RG. Responses of glutathione system and antioxidant enzymes to exhaustive exercise and hydroperoxide. *J Appl Physiol* 72: 549-554, 1992.
10. Johnson JB, Summer W, Cutler RG, Martin B, Hyun DH, Dixit VD, Pearson M, Nassar M, Telljohann R, Maudsley S, Carlson O, John S, Laub DR, Mattson M. Alternate day caloric restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma. *Free Radic Biol Med* 42(5): 665-674, 2007.
11. Kretzschmar M, Muller D. Aging, training, and exercise: a review of effects of plasma glutathione and lipid peroxidation. *Sports Med* 15: 196-209, 1993.
12. La Fratta I, Tatangelo R, Campagna G, Rizzuto A, Franceschelli S, Ferrone A, Patruno A, Speranza L, De Lutiis MA, Felaco M, Grilli A, Pesce M. The plasmatic and salivary levels of IL-1 β , IL-18 and IL-6 are associated to emotional difference during stress in young male. *Sci Rep* 8: 3031, 2018.
13. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: impact on human health. *Pharmacogn Rev* 4(8): 118-126, 2010.
14. Longo VD, Mattson MP. Fasting: molecular mechanisms and clinical applications. *Cell Metab* 19(2): 181-192, 2014.
15. Miller CS, Foley JD, Bailey AL, Campell CL, Humphries RL, Christodoulides N, Floriano PN, Simmons G, Bhagwandin B, Jacobson JW, Redding SW, Ebersole JL, McDevitt JT. Current developments in salivary diagnostics. *Biomark Med* 4(1): 171-189, 2010.
16. Moro T, Tinsley G, Bianco A, Marcolin G, Pacelli Q, Battaglia G, Palma A, Gentil P, Neri M, Paoli A. Effects of eight weeks of time-restricted feeding (16/8) on basal metabolism, maximal strength, body composition, inflammation, and cardiovascular risk factors in resistance-trained males. *J Transl Med* 14(1): 290, 2016.
17. Navalta JW, Stone WJ, Lyons TS. Ethical issues relating to scientific discovery in exercise science. *Int J Exerc Sci* 12(1): 1-8, 2019.
18. Ngamchuea K, Batchelor-McAuley C, Compton R. Rapid method for the quantification of reduced and oxidized glutathione in human plasma and saliva. *Anal Chem* 89(5): 2901-2908, 2017.

19. Peake J, Nosaka K, Suzuki K. Characterization of inflammatory responses to eccentric exercise in humans. *Exerc Immunol Rev* 11: 64-85, 2005.
20. Petersen AM, Pedersen BK. The anti-inflammatory effect of exercise. *J Appl Physiol* 98(4): 1154-1162, 2005.
21. "Physical Activity." American Heart Association. Web. 12 June 2015.
22. Pillon Barcelos R, Freire Royes LF, Gonzalez-Gallego J, Bresciani G. Oxidative stress and inflammation: liver responses and adaptations to acute and regular exercise. *Free Radic Res* 51(2): 222-236, 2017.
23. Sallam N, Laher I. Exercise modulates oxidative stress and inflammation in aging and cardiovascular disease. *Oxid Med Cell Longev* 2016: 7239639, 2016.
24. Schett G, Dayer J, Manger B. Interleukin-1 function and role in rheumatic disease. *Nat Rev Rheumatol* 12: 14-24, 2016.
25. Sen CK, Marin E, Kretzschmar M, Hanninen O. Skeletal muscle and liver glutathione homeostasis in response to training, exercise and immobilization. *J Appl Physiol* 73: 1265-1272, 1992.
26. Smith M, Edwards A, Gateless K, Aab B, Sherrard K, Bolyard C, Stover S. The impact of intermittent fasting and exercise on resting metabolic rate and respiratory quotient. *J Exerc Physiol Online* 22(7): 22-29, 2019.
27. "What is an inflammation?" Institute for Quality and Efficiency in Healthcare. Web. 23 Nov. 2010.
28. Zhang L, Liu Y, Wang B, Xu G, Yang Z, Tang M, Ma A, Jing T, Xu X, Zhang X, Liu Y. POH1 deubiquitinates pro-interleukin-1 β and restricts inflammasome activity. *Nat Commun* 9(1): 4225, 2018.

