

ABSTRACT

Researchers have demonstrated that prolonged, intense exercise is correlated to increased oxidative stress to harmful levels. Despite the common presence in sports and exercise, the effects of dehydration and rehydration on oxidative stress levels has been less researched. Eighty-two athletes were recruited for baseline anthropometric data and blood sampling. Subjects underwent a supervised exercise regimen until they reached a predetermined dehydration-induced mass loss goal: 3 percent body mass for males and 2 percent body mass for females. Post-dehydration, subjects underwent a second series of measurements and blood sampling. Each subject was then provided with an electrolyte-carbohydrate beverage, equal in quantity to the amount of body mass lost during exercise, to drink in 40 minutes. Once body mass was reestablished to baseline values, subjects underwent a final series of measurements and blood sampling 2 hours post rehydration. Plasma samples were snap frozen in liquid nitrogen and stored at -80°C until analysis. Oxidative stress was determined by measuring lipid hydroperoxide levels within the blood plasma of 13 randomly selected subjects using the ferrous oxidation-xylene orange (FOX) assay. Statistical analysis was performed through a 1 way ANOVA with post-hoc testing. All values are reported as mean \pm standard deviation. An increase was seen in mean lipid hydroperoxide levels post-dehydration followed by a return near baseline at the 2 hour post rehydration level, however the values were not statistically significant ($p = 0.3342$). While this study suggests that dehydration and rehydration are coupled with an increase and decrease in oxidative stress, respectively, more samples would need to be analyzed to make a statistically significant conclusion.

INTRODUCTION

Regular physical exercise has been proven to have many health benefits and acts as a primary and secondary prevention mechanism against several chronic diseases (e.g., cardiovascular disease, diabetes, cancer, hypertension, depression, and obesity) and premature death (Warburton et al., 2006). However, research over the past four decades has demonstrated a relationship between prolonged, intense exercise and oxidative stress (Powers and Jackson, 2008). High levels of oxidative stress are associated with an increased risk for several human diseases including Alzheimer's disease, rheumatoid arthritis, diabetes, sepsis, chronic renal failure, cancers, and respiratory distress syndrome (Uttara et al., 2009). Since 1978, extensive research has been put into exercise-induced oxidative stress. A summary of these findings and a possible link between dehydration and oxidative stress are described below.

Free radicals are defined as an atom or group of atoms with one or more unpaired electrons (Powers and Jackson, 2010). Despite their unstable state, free radicals are formed as necessary intermediates in many natural reactions. In biological systems, oxygen-derived radicals, also known as reactive oxygen species (ROS), represent the most important class of free radicals in living systems (Fisher-Wellman and Bloomer, 2009). ROS are constantly generated as a part of normal aerobic life; major reactive oxygen species include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Powers and Jackson, 2010). The most supported hypothesis of ROS generation, is the idea that free radicals from the mitochondria. The mitochondria creates ATP through oxidative phosphorylation, through which electrons are transported across the inner membrane via the electron transport chain. Electrons are reduced as they are passed through a series of proteins via oxidative-reduction

reactins. Normally, oxygen is reduced to produce water, but sometimes (0.1-2% of the time) oxygen is prematurely and incompletely reduced. This misstep most commonly occurs at Complex I and Complex III and generates a superoxide radical (Turrens, 2003).

Additionally, ROS are generated as necessary intermediates in many enzyme reactions.

Oxygen radicals play an important and beneficial part of several regulatory roles in cells: control in gene expression, regulation of cell signaling pathways, and modulation of skeletal muscle force production (Powers and Jackson, 2008). Despite the constructive role ROS play in the body, their high chemical reactivity makes them dangerous at excessive levels.

The body's antioxidant defense system serves to protect cells from excessive ROS levels. These interactions occur both in the cell itself and through the body as a whole to reduce, breakdown, dismutate, or convert ROS into less reactive molecules (Powers and Jackson, 2008). The defense system is composed of both enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, and catalase) and nonenzymatic antioxidants (Glutathione, uric acid, bilirubin, carotenoids, Vitamin E, Vitamin C, etc.) (Givertz, 2016). Of these nonenzymatic antioxidants, Vitamin E, Vitamin C, carotenoids, and several others are exogenous compounds and acquired through diet (Fisher-Wellman and Bloomer, 2009). However, only a few of these antioxidants demonstrate the ability to protect the body during excessive exercise. Although moderate exercise produces manageable and beneficial levels of ROS, exercise of a long duration or high in intensity may produce ROS levels too high for the system to handle.

Oxidative stress occurs when the production of reactive oxygen species is larger than the body's ability to detoxify the reactives. This imbalance leads to oxidative damage to all types of macromolecules including lipids, proteins, and nucleic acids. Several activities lead

to an increase of oxidative stress including: excessive ultraviolet ray exposure, pollution, smoking, unhealthy diet, and most importantly excessive exercise. As mentioned earlier, during normal respiration, mitochondria produce small levels of ROS when oxygen escapes during the electron transport chain. During exercise, it is theorized that the elevated oxygen consumption during increased mitochondrial activity is the cause for the increased ROS generation. When exercise becomes intense and prolonged, the body's antioxidant defense systems can no longer handle the levels of ROS produced (Lenaz, 1998). While this is the most supported theory for increased oxidative stress during exercise, more research needs to be done to draw direct conclusions.

While low-to-moderate levels of oxidants have been proven to be beneficial, high levels can cause very adverse effects. One of the most toxic effects of oxygen radicals is lipid peroxidation in which radicals target lipids in cellular membranes resulting in the formation of highly reactive and unstable lipid hydroperoxides. Peroxidation of membranes has several adverse effects including an increased rigidity of the membrane, altered permeability, decreased activity of membrane-bound enzymes, and altered activity of membrane receptors (Abuja and Albertini, 2000). The quantification of lipid peroxidation through the measurement of the biochemical marker lipid hydroperoxide, allows for a direct calculation of oxidative stress within the human body. Reliable markers for oxidative stress must be chemically unique and detectable, increased or decreased during periods of oxidative stress, possess a relatively long half-life, and be unaffected by other cellular processes (Powers and Jackson, 2008). Lipid hydroperoxides possess each of these qualities making it a functional indicator of oxidative stress.

While there has been a significant amount of research published on exercise and its association with oxidative stress, there are questions on the possible connection between dehydration and oxidative stress. While *in vivo* research linking dehydration and oxidative stress is limited, several animal and *in vitro* human studies have begun to look at the connection.

Schliess and Häussinger have stated through their research that as cellular hydration changes, cells become more or less immune to handling stress. During adequate hydration, cells swell promoting anabolism and protecting the cell from heat and oxidative challenges. Conversely, when dehydrated, cells promote anabolism and become more susceptible to stress-induced damages (Schliess and Häussinger, 2006). These cellular hydration alterations demonstrate a possible relationship between osmotic and oxidative stress.

Testing these stressors through an experimental design, Hillman et al. looked at the effects of exercise-induced dehydration and hyperthermia on oxidative stress through the testing of seven healthy male cyclists. The cyclists performed 90 minutes of cycling and then a 5 km time trial in four different settings: euhydration in a warm environment, dehydration in a warm environment, euhydration in a thermoneutral environment, and dehydration in a thermoneutral environment. The results demonstrated an increase in the oxidative stress marker, GSSG, during only the dehydration trials (Hillman et al., 2010). These results help demonstrate the importance of fluid consumption during exercise to help attenuate thermal and oxidative stress levels.

Penkman et al. examined the effect of dehydration and rehydration on performance, immune cell response, and tympanic temperature after high intensity rowing exercise. Seven

rowers completed two simulated 2000m rowing race trials, one performed in a euhydrated condition and another using a dehydration protocol, separated by 72 hours in a random, cross-over design. While there was no difference in performance between the dehydration and rehydration trials, the dehydration trial produced a higher tympanic temperature and had a significant immune response as shown by the increase in leukocyte, lymphocyte, and lymphocyte subset concentration (Penkman et al., 2008). This suggests that dehydration produces enough stress to activate the immune system – a possible expression of oxidative stress.

In another study, McAnulty et al. examined the effect of exercising at a fixed intensity in a hyperthermic environment (35°C at 70% relative humidity) versus a neutral environment (25°C at 40% relative humidity) on oxidative stress markers. Six male subjects performed low intensity exercise (50% of maximal oxygen consumption) on a treadmill in both environments and were tested for a series of oxidative stress markers (F2 isoprostanes, lipid hydroperoxides, and lactate) (McAnulty et al., 2005). The increase in oxidative stress markers as well as the significantly increased ending core temperatures and heart rates demonstrates how hyperthermia increases oxidative stress. Interestingly, dehydration was also greater in the hot trial in comparison to the neutral trial.

The American College of Sports Medicine defines excessive dehydration as greater than 2 percent weight loss from water deficit (Sawka et al., 2007). Dehydration magnifies stressors on the body, such as fatigue, heart rate and possibly oxidative stress (Cheuvront et al., 2010). Despite the results of these studies, not enough research has been done to draw conclusions, demonstrating the need for a more comprehensive examination of the specific impact of dehydration on oxidative stress in exercising humans. By testing lipid

hydroperoxides in the blood plasma of dehydrated and then rehydrated athletes, this study will investigate how oxidative stress levels change through these processes. It is hypothesized that these results will show an increase of lipid hydroperoxide levels after dehydration and return in levels back to baseline after rehydration.

METHODS

The purpose of this study is to determine if dehydration and rehydration have an effect on oxidative stress, as measured by the presence of the biochemical marker lipid hydroperoxides in blood plasma.

Subjects

A total of eighty-two athletes, fifty-six NCAA Division I male wrestlers and twenty-six female soccer athletes were recruited for this study. Informed written consent was obtained from each subject. 13 of these subjects were analyzed using the ferrous oxidation-xylene orange (FOX) assay.

Research Design

The experiment followed a repeated measures design with all subjects serving as their controls. Subjects were instructed to report to Appalachian State University Human Performance Laboratory at 8:00am in a hydrated state. Upon arrival, subjects provided a series of samples in the following order: urine sample, body mass, skinfold test, ultrasound, and blood samples. These measurements were taken, in the same order at the following four time points: baseline, post dehydration, one hour rehydration, and two hours rehydration. Blood samples were obtained by phlebotomy from both the left and right median cubital veins of each subject.

After baseline measurements, each subject was given a mass loss (hydration loss) goal: 3% of body mass for males and 2% body mass for females. The subjects then underwent a supervised exercise regimen to induce the prescribed weight loss. Scales were

available at all times for the participants to check if they had completed their mass loss goal. Participants were allowed to shower once completing their session and return to the laboratory. Subjects then followed the same measurement order as baseline. After all post dehydration measurements were taken, subjects were given an electrolyte-carbohydrate beverage (6% or 60 g•L⁻¹; Gatorade®, Barrington, IL, USA). The electrolyte-carbohydrate beverage contained 20 mmol•L⁻¹ sodium and 3.2 mmol•L⁻¹ potassium. Each participant was given an amount of the beverage equal to the amount of mass they had lost during exercise. The first half of the beverage, equivalent to half the mass the subject had lost, was consumed within the first 20 minutes of the rehydration protocol. The second half of the beverage was consumed in the second and final 20 minutes of the rehydration protocol. Once pre-exercise body mass was attained, the original series of measurements were completed at 120 minutes after fluid consumption commenced.

Blood Sampling Conditions and Disposition

Blood samples were drawn from the left and right median cubital veins of each subject, from a seated position. Samples were obtained in sodium heparin tubes and centrifuged at 3,500 RPM at 4°C for 10 minutes. Plasma was divided, snap frozen in liquid nitrogen, and then stored at -80°C until further analysis. Specific analysis for this experiment was performed through the Ferrous Oxidation-Xylenol Orange (FOX) assay.

Ferrous Oxidation-Xylenol Orange (FOX)

Lipid Hydroperoxides were measured using the Ferrous Oxidation-Xylenol Orange (FOX) assay according to the modified methodology of John Quindry in 2002. The basis of this assay is that the oxidation of reagent Fe²⁺ to Fe³⁺ binds to xylenol orange to produce a

color complex. The color complex allows for an absorbance reading of the lipid hydroperoxide levels.

In total, 13 of the 82 subjects were used for the FOX assay. Three of the four time points within the dehydration-rehydration study were analyzed: baseline, post-dehydration, and two hours rehydration. The one hour rehydration was excluded to ensure full rehydration of the blood plasma tested and as a time saving measure. Untreated, frozen plasma samples stored at -80°C were used for this study. Each plasma sample served as its own control; $90\mu\text{L}$ of each sample was mixed with $10\mu\text{L}$ of the control substance and reducing agent, tris(2-carboxyethyl)phosphine (TCEP), and then $10\mu\text{L}$ of the testing substance, Methanol (HPLC grade). After 30 minutes of incubation at room temperature, $900\mu\text{L}$ of freshly made work solution (9.8mg ferrous ammonium sulfate combined with 10mL H_2SO_4 ; 79.2mg butylated hydroxytoluene mixed with 90mL Methanol; and 7.6 mg xylene orange) was added to both the control and testing vials. Each sample was incubated again at room temperature for 45 minutes and then centrifuged at $12\text{ K}\cdot\text{g}$ for 15 minutes. After being centrifuged, $250\mu\text{L}$ of each standard and sample were removed and placed in a 96-well glass plate and read at 560 nm. All samples were tested in triplicate. Lipid hydroperoxide concentration was determined from a linear regression line generated from the standard curve of cumene hydroperoxide.

Statistical Analysis

Data was analyzed using a one-way repeated-measures analysis of variance (ANOVA) with post hoc testing through the InStat statistical software (Graphpad StatMate

version 2.00 for Windows, GraphPad Software, San Diego California, USA). All values are reported as mean \pm standard deviation.

RESULTS

The mean values of the 13 subjects' lipid hydroperoxide absorbance levels are presented together in Figure 1 below. In total, hydroperoxide levels did demonstrate a general increase post-dehydration followed by a return near baseline at the 2 hour post rehydration time frame. Baseline values were recorded at $26.3 \pm 41.7 \mu\text{M}$, with a general increase upwards post- dehydration ($70.2 \pm 109.6 \mu\text{M}$) and a notable trend downwards at the 2 hour rehydration time point ($39.7 \pm 60.9 \mu\text{M}$). However, the values were not statistically significant ($p = 0.3342$).

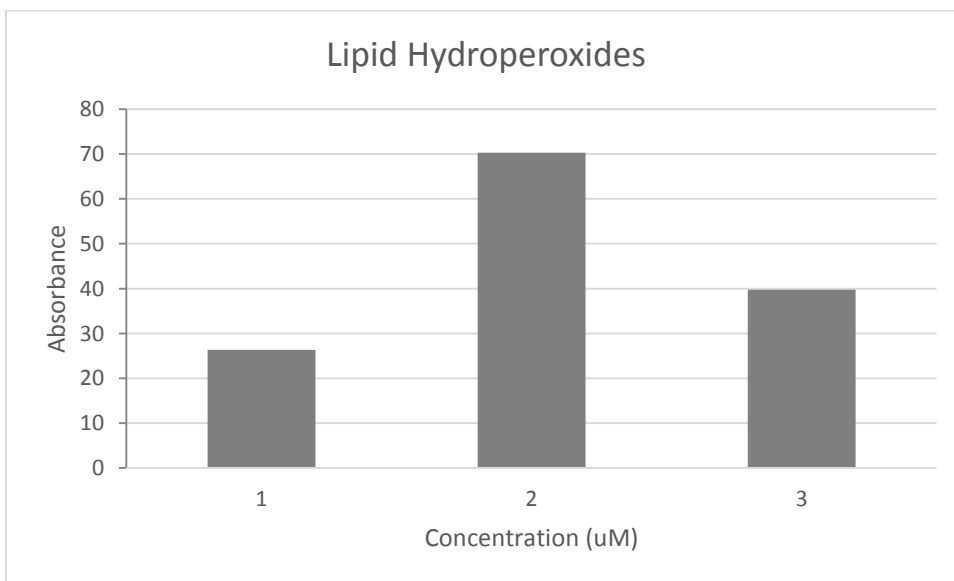


Figure 1. The mean change in lipid hydroperoxide absorbency throughout the three measurement points: 1) baseline, 2) post-dehydration, 3) 2 hours post rehydration.

DISCUSSION/ CONCLUSION

While not statistically significant, this study supports that dehydration in combination with exercise has a potential effect on the increase of oxidative stress. Additionally, the results suggest that post-exercise rehydration returns the oxidative stress to close to normal levels.

A possible limitation of this study is that we did not include a control group that performed exercise without dehydration. However Hillman et al. demonstrated that dehydration alone is a factor in the disruption of the redox state. By setting the conditions to test for exercise-induced dehydration with and without hyperthermia, it allows us to see the specific effect of dehydration on oxidative stressed. The results showed a significant increase in oxidized glutathione post-exercise in the dehydration trials only, demonstrating that dehydration alone can interrupt antioxidant defenses (Hillman et al., 2011).

Additionally, a contributing factor for the change in oxidative stress levels could have been due to an elevated core body temperature. As demonstrated by McAnulty et al., ROS production levels are exacerbated by elevated body temperatures and then return to normal

once temperature levels decrease (McAnulty et al., 2005). The reason for the increased reactive oxygen species production during exercise with elevated body temperature is still unknown, but it may be due to increased oxygen consumption due to the uncoupling of the respiratory chain.

Increased body temperature could also be a byproduct of immune system activation. Jozefowicz and Nowak, examined the effect of elevated body temperature on priming of the respiratory burst of human neutrophils. Specifically, their study looked at how temperature affected reactive oxygen species generation by human peripheral blood neutrophils. The study found a priming effect at 38°C toward human peripheral blood neutrophil ROS production. However, the priming effect was specific to 38°C; higher temperatures produced no effect or even caused an inhibitory response (Jozefowicz and Nowak, 2004).

Paik et al., investigated the effects of hydration status on oxidative stress and exercise performance. 10 subjects ran at 80% of their VO_{2max} until exhaustion in four different conditions: control, 3% dehydration, 3% dehydration with water, and 3% dehydration with a sports drink. The results demonstrated that dehydration significantly decreased performance and increased oxidative stress during exercise, however fluid replacement with both water and a sports drink equally relieved the damage (Paik et al., 2009). Through this study, fluid replacement is shown to prolong exercise endurance and lessen oxidative damage.

Although the study supports that dehydration and rehydration are coupled with an increase and decrease in oxidative stress levels, more samples would need to be analyzed to make a statistically significant conclusion. Therefore, one of the largest limitations of the lipid hydroperoxide analysis was sample size. In conclusion, although this study indicates

that dehydration coupled with exercise may potentially increase oxidative stress levels, respectively, more samples would need to be analyzed to make a statistically significant conclusion.

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