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## Strenuous Exercise Increases the Risk of Oxidative Stress in Ironman Triathlon Participants

Noelle L. Cutter  
ncutter@molloy.edu

Emily Cruz  
ecruz@lions.molloy.edu

Frank Cristall III  
fcristallo@lions.molloy.edu

Kristen Lacey  
klacey@lions.molloy.edu

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# Strenuous Exercise Increases the Risk of Oxidative Stress in Ironman Triathlon Participants

## Peer Review

This work has undergone a double-blind review by a minimum of two faculty members from institutions of higher learning from around the world. The faculty reviewers have expertise in disciplines closely related to those represented by this work. If possible, the work was also reviewed by undergraduates in collaboration with the faculty reviewers.

## Abstract

Regular physical activity has been linked to greater overall health. Literature review and studies have also defined regular physical activity as a reducer of life-threatening illnesses such as cardiovascular disease, diabetes and obesity. However, long increments of strenuous exercise can produce oxidative stress and muscle fatigue in the human body. The increase in oxygen consumption during strenuous exercise leads to elevated reactive oxygen species (ROS). Cells continuously produce free radicals and reactive oxygen species as part of metabolic processes in the body. These free radicals are neutralized by an antioxidant defense system in the body consisting of enzymes, such as catalase, and non-enzymatic antioxidants. An Ironman Triathlon consists of a 2.4-mile (3.86 km) swim, a 112-mile (180.25 km) bicycle ride and a marathon 26.2-mile (42.2 km) run, raced in that order and without a break. It is widely considered by athletes to be one of the most demanding sporting events in the world. It is hypothesized that a physically challenging event such as the Ironman Triathlon can be linked to elevated cortisol levels, increased occurrence of DNA damage, elevated concentrations of ROS, and consequently increased oxidative stress in humans. In order to derive conclusive results regarding the hypothesis, groups containing athletes who completed the full Ironman race, the half Ironman race, and a control group of moderately active individuals were established and individuals were required to report Garmin Smartwatch health and wellness data. The half Ironman consists of a 1.2-mile (1.93 km) swim, a 56-mile (90.12 km) bicycle ride and a marathon 13.1-mile (21.1 km) run, raced in that order and without a break. Several protocols were then applied to derive data necessary to complete the research. After the participants were selected, their saliva was collected in a non-invasive fashion and was used in the Elisa Saliva Kit to determine cortisol concentration. The saliva samples were also utilized to perform DNA and RNA extraction; and the resulting products were analyzed for quantity and quality of the DNA and RNA. Real time PCR allows scientists to monitor PCR while it is occurring. In this technique, luminescence is produced by reporter molecules as the PCR products increase with every cycle. To determine ROS concentration, the ROS-Glo assay, which provides a light signal that is proportional to the ROS in a given sample, was utilized. An additional marker of oxidative stress is 8-oxo-2-deoxyguanosine(8-oxo-dG). The OxiSelect™ Oxidative DNA Damage ELISA uses antibody and antigen interactions to report the concentration of 8-oxo-dG in a sample. Furthermore, the results indicate an increase in enzymatic indicators of elevated ROS, elevated cortisol levels, and disruption of sleep in the participating athletes after the race. In conclusion, the athletes who completed the full Ironman triathlon experienced increased amounts of oxidative stress than their less active counterparts in the control group, as was denoted by the elevated cortisol levels, increased 8-oxo-dG concentrations, and increased ROS concentrations. Such a rigorous event negatively impacted participants and caused oxidative stress.

## Keywords

oxidative stress, athletic endurance, reactive oxygen species, cortisol

**Acknowledgements**

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## **Strenuous Exercise Increases the Risk of Oxidative Stress in Ironman Triathlon**

### **Participants**

**Emily Cruz, Kristen Lacey, Rachel Julian, and Frank Cristallo III**

**Dr. Noelle Cutter**

### **ABSTRACT**

Regular physical activity has been linked to greater overall health. Literature review and studies have also defined regular physical activity as a reducer of life-threatening illnesses such as cardiovascular disease, diabetes and obesity. However, long increments of strenuous exercise can produce oxidative stress and muscle fatigue in the human body. The increase in oxygen consumption during strenuous exercise leads to elevated reactive oxygen species (ROS). Cells continuously produce free radicals and reactive oxygen species as part of metabolic processes in the body. These free radicals are neutralized by an antioxidant defense system in the body consisting of enzymes, such as catalase, and non-enzymatic antioxidants. An Ironman Triathlon consists of a 2.4-mile (3.86 km) swim, a 112-mile (180.25 km) bicycle ride and a marathon 26.2-mile (42.2 km) run, raced in that order and without a break. It is widely considered by athletes to be one of the most demanding sporting events in the world. It is hypothesized that a physically challenging event such as the Ironman Triathlon can be linked to elevated cortisol levels, increased occurrence of DNA damage, elevated concentrations of ROS, and consequently increased oxidative stress in humans. In order to derive conclusive results regarding the hypothesis, groups containing athletes who completed the full Ironman race, the half Ironman race, and a control group of moderately active individuals were established and individuals were required to report Garmin Smartwatch health and wellness data. The half Ironman consists of a 1.2-mile (1.93 km) swim, a 56-mile (90.12 km) bicycle ride and a marathon 13.1-mile (21.1 km) run, raced in that order and without a break. Several protocols were then applied to derive data necessary to complete the research. After the participants were selected, their saliva was collected in a non-invasive fashion and was used in the Elisa Saliva Kit to determine cortisol concentration. The saliva samples were also utilized to perform DNA and RNA extraction; and the resulting products were analyzed for quantity and quality of the DNA and RNA. Real time PCR allows scientists to monitor PCR while it is occurring. In this technique, luminescence is produced by reporter molecules as the PCR products increase with every cycle. To determine ROS concentration, the ROS-Glo assay, which provides a light signal that is proportional to the ROS in a given sample, was utilized. An additional marker of oxidative stress is 8-oxo-2-deoxyguanosine(8-oxo-dG). The OxiSelect™ Oxidative DNA Damage ELISA uses antibody and antigen interactions to report the concentration of 8-oxo-dG in a sample. Furthermore, the results indicate an increase in enzymatic indicators of elevated ROS, elevated cortisol levels, and disruption of sleep in the participating athletes after the race. In conclusion, the athletes who completed the full Ironman triathlon experienced increased amounts of oxidative stress than their less active counterparts in the control group, as was denoted by the elevated cortisol levels, increased 8-oxo-dG concentrations, and increased ROS concentrations.

Such a rigorous event negatively impacted participants and caused oxidative stress (Meeusen 2005).

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## **INTRODUCTION**

Regular physical activity has been linked to greater overall health. According to the Mayo clinic, exercising regularly has various benefits such as improvement of cardiovascular function (Mayo Foundation for Medical Education and Research). Regular Physical activity increased high-density lipoproteins, which is commonly known as beneficial cholesterol that absorbs excess cholesterol and transports it back to the liver (Mann 2014). This cholesterol is linked to increase smooth blood flow, allowing a decrease risk for heart disease (Mann 2014). Additionally, regular physical activity is linked to positive change in mental well-being. Physically fit individuals experienced fewer symptoms of depression and anger (Meeusen 2005). Regular physical activity is crucial to optimal health in humans (Meeusen 2005) and the Department of Health and Human Services consequently recommends 30 minutes of exercise, three to five times a week. Physical fitness is defined as a person's ability to carry out tasks without undue fatigue (Caspersen 1985.). Activities can be characterized as rigorous, moderate and light. Vigorous activities include any actions which require larger effort such as running, various sports, and swimming rapidly. Moderate activities, which are less demanding, include physical engagements such as aerobics, walking briskly, and dancing (Lee 2000). Both types of activity count toward meeting the physical activity requirements in humans. Proceeding in this research, emphasis was placed on collecting data concerning the

effects of strenuous exercise on the body. Strenuous exercise surpasses the energy exerted in vigorous or moderate exercise (Lee 1995). What is considered strenuous exercise can vary based on an individual's level of physical fitness and his or her mental perception of how hard the activity is (Caspersen 1985). Therefore, strenuous exercise has a subjective definition that varies from individual to individual. However, aerobic exercise increases the rate of oxygen consumption, which, in turn, increases the production of reactive oxygen species (ROS). The consumption of oxygen for aerobic production of adenosine triphosphate (ATP) may increase from 10 to 20 times during exercise compared with resting levels. Consumption of oxygen is necessary for energy production within muscles (Alessio 2000). Consequently a rise in oxygen consumption, causes an increase in the production of reactive oxygen species (ROS) and oxidative damage to cellular structure because of oxidation of membrane lipids, carbohydrate oxidation, and damage to nucleic acids (Lovrić 2005). In some circumstances, low concentrations of ROS are known to have a stimulating effect and can initiate apoptosis. However, large amounts of ROS, like those identified to occur from ultra-endurance exercise, can damage vital cellular structures, and oxidative damage can result (Radak 2013).

Cellular damage and oxidative stress have been associated with a number of pathophysiological conditions such as atherosclerosis, malignancies, and neurologic diseases such as Alzheimer's and Parkinson's. In the Harvard Alumni Health Study, individuals in the group with the highest energy expenditure had an increased relative risk of death compared with two groups who completed less exercise (Paffenbarger 1986). This finding is consistent with that reported in the British Regional Heart Study, where vigorously active men had higher rates of heart attacks than men performing moderate or moderately vigorous activity (Shaper 1991). A plausible rationale linking high-volume physical activity and increased disease incidence may include oxidative stress in disease etiology (Lee, 2000).

The Ironman triathlon consists of a sports competition including three different sports in one single event (3.8km swim, 180 km bike ride, and 42.2 km run), raced in this order and without a break, during which athletes exercise for a long period of time. These competitions require high resistance and can lead to heat stress and dehydration, muscle injury, oxidative stress, and inflammation (Sahlin 2010). Individuals who undergo intense and prolonged exercise or exhaustive training, or even those with very high training frequency may exceed the capacity of endogenous antioxidant system and, consequently, cause severe muscle injuries, with subsequent inflammation and oxidative stress (Sahlin 2010). Therefore, high rates of oxidative stress may contribute to decreased performance, fatigue, muscle damage, and muscle pain.

The body has cellular and hormonal indicators of when it is exposed to stress or increased oxidative stress. Humans experience a natural rise in cortisol during exercise. Cortisol levels that remain elevated after a workout are seen in athletes who are overtraining, overreaching, and not allowing their bodies significant recovery time (Filaire 1996). We hypothesize that a physically challenging event such as the Ironman Triathlon can be linked to elevated cortisol levels, increased occurrence of DNA damage, elevated concentrations of ROS, and consequently increased oxidative stress in humans.

## **MATERIALS AND METHODS**

Human subject research approval was obtained through the Molloy College Institutional Review Board prior to the study (2017-2018). Subjects gave signed informed consent prior to participating. All subjects were cleared by a physician prior to starting the Ironman training.

### **Selection**

One of the most crucial tasks in the study design phase is identifying appropriate participants. The participants of the study were acquired through the means of convenience sampling. Furthermore, in order to complete the study, participants of a specific criteria had to be selected. Participants for selection included eleven well-trained triathletes who were scheduled to compete in the Ironman Triathlon (3.8-km swim, 180-km cycle, 42.2-kmrun). Mean environmental conditions ranged from 20 to 25°C and from 79% to 85% relative humidity. Males and females between the ages 25 and 45 were selected, all of which were of good health and cleared by a physician to perform in the Ironman Placid. Selected athletes did not use any medication, antioxidant, or related supplements; are non-smokers; and did not have any febrile illness as noted by a physician prior to enrollment in the study. The selected participants must have competed in an Ironman triathlon prior to competing in the one for the study. The chosen few must have signed up to participate in the Full Ironman Placid or Ironman Placid Half for summer 2017 and summer 2018. In addition, the selected participants must give a written consent form that they will be participating and are responsible for any minor casualties that may occur. Before, during and after the race at selected time points, athletes were surveyed based on their general health, training, and diet. The control group consisted of individuals who were not registered or training for the Ironman triathlon or a comparable event. Several corresponding factors were recorded, including age, height (cm), weight (kg), heart rate (bpm), heart rate max (bpm), exercise time (hours per week), sleep average (hours per day), and VO<sub>2</sub> max. Information such as heart rate, heart rate max, exercise time, sleep average, and VO<sub>2</sub> max were derived utilizing Garmin smartwatch data.

### **Saliva Collection**

Cortisol levels that remain elevated after a workout are seen in athletes who are overtraining, overreaching, and not getting significant recovery time (Filaire 1996). The measurement of cortisol levels in general can be determined from a sample of saliva, and it usually indicates adrenal function. To collect saliva from the participants, a sterile swab of 125mm was used and the subject held it firmly under their tongue to generate saliva. The used swabs were collected separately and placed into sterile test tubes. Additionally, subjects were requested to salivate into a collection tube from Salimetrics. Pre and post exercise saliva aliquots of 500 to 1000 µl were collected and put on ice immediately for transportation back to the lab and then frozen at -80.C within the hour for future functional assays. Analysis of samples took place within 21 days of being frozen at -80.C.

### **Cortisol Saliva Assay**

The ENZO Cortisol ELISA kit was used to quantitatively determine cortisol in human saliva by an enzyme immunoassay. The concept of the procedure imitates the standard competitive binding scenario. The competitive binding took place between the enzyme-labeled antigen, the conjugate, and the unlabeled antigen, existing in the standards, controls, and samples. This occurred only for a restricted number of antibody binding sites on the microwell plate. Unbound materials were then removed by washing and decanting procedures. Next, the enzyme substrate p-nitrophenyl phosphate was added. The reaction was allowed to sit for one hour at room temperature and then stopped using a trisodium phosphate stop solution. The absorbance was measured using the Biotek EL800 plate reader at 405 nm. The quantity of cortisol in the saliva sample was inversely proportional to the intensity of the color formed by the plate reader. A standard curve was created with the data collected, depicting the concentration of cortisol found within the saliva samples.

## DNA and RNA Extraction

DNA and RNA extraction is used to quantify, discover, and profile nucleic acids. To isolate DNA and RNA from approximately 1000 $\mu$ l saliva collected by the participants, the QIAGEN mini kit was utilized. The protocol was followed accordingly. In short, cells were lysed and DNA was isolated, cleaned, and extracted following the spin protocol as outlined by Qiagen. Extracted isolates were analyzed for quantity and quality using the Thermo Nanodrop 2000.

## Real-time qPCR

Gene expression analysis is a fast and convenient PCR method that combines standard RT-PCR with the idea of fluorescence resonance energy transfer (FRET) using fluorogenic primers. The recognition of changes in fluorescence intensity during the reaction allowed us to follow the PCR reaction in real time. Total cellular RNA was isolated using RNeasy kit and 1 $\mu$ g of RNA was reverse transcribed to cDNA using the SuperScript One Step RT-PCR system by Invitrogen, according to the manufacturer's instructions. cDNA at a 1:10 dilution was used for all PCR reactions and primers were designed by BioRad. The primer sequences utilized in RT-qPCR amplification is depicted in Table 2. All PCR reactions were performed on CFX-96 Bio-Rad RT System in triplicate and validated by the presence of a single peak in the melting curve analysis. Changes in gene expression were calculated relative to the actin control. PCR products were electrophoresed through 1.0% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. Band intensity was calculated using Image-J software (Bio- Levels of mRNA were expressed as the ratio of band intensity relative to that for control).

## ROS-Glo Assay

The ROS-Glo<sup>TM</sup> H<sub>2</sub>O<sub>2</sub> Assay is a homogeneous bioluminescent assay that measures the level of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a reactive oxygen species (ROS), directly in saliva samples from our participants. A derivatized luciferin substrate is incubated with sample and reacts directly with H<sub>2</sub>O<sub>2</sub> to generate a luciferin precursor. Addition of ROS-Glo<sup>TM</sup> Detection Solution converts the precursor to luciferin and provides Ultra-Glo<sup>TM</sup> Recombinant Luciferase to produce light signal that is proportional to the level of H<sub>2</sub>O<sub>2</sub> present in the sample. Samples were run in triplicate and repeated at least two times for statistical significance. Relative luminescent values were read using Biotek EL800 plate reader.

## OxiSelect<sup>TM</sup> Oxidative DNA Damage ELISA

Among numerous types of oxidative DNA damage, the formation of 8-hydroxydeoxyguanosine (8-oxo-dG) is a ubiquitous marker of oxidative stress. This kit uses an ELISA for the quantitative measurement of 8-oxo-dG. The unknown 8-oxo-dG samples or 8-oxo-dG standards are first added to an 8-oxo-dG/BSA conjugate preabsorbed microplate. Clear saliva samples were diluted in Assay Diluent and used directly in the assay. Samples were incubated for 10 minutes at room temperature with orbital shaking. Next, an anti-8-oxo-dG monoclonal antibody is added and left to sit for one hour at room temperature. The samples are washed and incubation is followed by an HRP conjugated secondary antibody for an additional one hour. The 8-oxo-dG content in unknown samples is determined by comparison with predetermined 8-oxo-dG standard curve using the Biotek EL800 plate reader at 450 nm primary wavelength. Samples were run in triplicate and repeated at least two times for statistical significance.



## Data Analysis

All data represented in our study is shown as the averages with standard deviation bars of three independent trials per experiment. T-tests were conducted in comparison to the untreated group with Microsoft Excel®, with  $p < 0.05$  and  $p < 0.01$  for significance. Normality was evaluated by Kolmogorof–Smirn-off test, and a paired test was used to test for differences between pre-race and post-race. All experiments were repeated three times for accuracy, in replicates of 10, unless otherwise stated. Statistical analyses were performed using SPSS/Windows version 12.5S statistical package (SPSS, Chicago, IL, USA). Statistical significance was accepted at the level of  $p < 0.05$ . The Bonferroni correction calculation was used to compare t-tested paired samples.

## RESULTS

### Athletic Profile

The ages of the male and female athletes, all of whom were of good health and cleared by a physician to participate, varied from 25 to 45 years of age. The body composition varied with different body compositions and different lifestyles choices such as time spent sleeping and exercising (Figure 1). Data on the following parameters, namely heart rate, heart rate max, exercise time per week, average sleep time per day, and VO<sub>2</sub> max were all obtained from various versions of the Garmin Smartwatch. The average heart rate of the full Ironman participants was lower in comparison to the half Ironman participants and those in the control group, while the half Ironman participants had lower average heart rates than those in the control group. The heart rate max data, which is the highest heart rate an individual can have during exercise, showed an opposite trend. The full Ironman participants had the highest heart rate max out of the three groups. The half Ironman participants had a lower heart rate max than the full Ironman participants, but a higher heart rate max than the participants in the control group (Fraga 1990). The control group presented the lowest heart rate max values out of the three groups. The VO<sub>2</sub> max data followed a similar trend, with the full Ironman participants possessing the highest VO<sub>2</sub> max values. The half Ironman participants possessed a lower VO<sub>2</sub> max value than those in the full Ironman group, however the control group participants had the lowest VO<sub>2</sub> max values of each of the three groups. The exercise time per week performed by those in the full Ironman group exceeded both the half Ironman and control group participants; however the participants in the half Ironman group exercised more per week than those in the control group.

### Cortisol Levels

Cortisol plays an essential role in the bodies' response to stress. Our results indicate elevated cortisol levels both before and after the Ironman race when compared to the average cortisol levels of participants in the control group, as hypothesized. Cortisol levels in Ironman and Half-Ironman participants remained elevated 24 hours after the race in comparison to the cortisol levels derived 24 hours pre-race (Figure 5). The sustained elevation in cortisol levels is due to the athletes overtraining, overreaching, and not allowing their bodies significant recovery time (Hassmen 2000). The actual cortisol levels derived from the participants are denoted in Table 2.

### Sleeping Patterns

The sleeping patterns of the participant athletes differed from their documented average sleep. The average of those participants was 7 hours but after the Ironman triathlon, the athletes slept more hours, which may indicate higher need for recovery (Figure 2). This trend is noticed in the participants who partook in the half triathlon. The athletes slept more after the rest (Figure 2) Out of the total athletes, 66% reported that they slept worse than normal at least once prior to an important competition. 80% reported problems falling asleep. 43% reported waking up early in the morning and 32% reported waking up at night. Factors that contributed were nervousness, thoughts on competition, unusual surroundings, noise in the room (Geda 2010).

### **Elevated ROS**

An important mechanism of chemical toxicity is the induction of oxidative stress through the production of excess reactive oxygen species (ROS) such as superoxide, H<sub>2</sub>O<sub>2</sub>, singlet oxygen, and hydroxyl radical. (Schroeder 2001). The alteration of redox status, which is induced by increased generation of ROS, results in increased vulnerability to oxidative stress. Therefore, we examined the capacity of the samples to produce ROS as utilized directly in the saliva cultures derived from participants. This bioluminescent assay measures the total concentration of H<sub>2</sub>O<sub>2</sub> within a culture sample (Filaire 1996). Results from the assay indicate that the IM participants but not the half IM participants experienced a significant ROS accumulation 48 hours after endurance based activity. These results suggest a larger amount of oxidative stress is placed on the participants of the full IM in comparison to the half IM. This test was repeated in triplicate and results presented statistically significant (p<0.05).

### **qPCR/Gene expression**

The function of the cellular indicators of increased ROS are explained in Table 1. The enzymes tested for include MDA, SOD, CAT, GPx, and GST. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) are the primary biomarkers which indicate oxidative stress in the body. The enzymes which combat increased ROS in the body by neutralizing them were elevated in response to the athletes completing the race. SOD, CAT, and GPx are the front line of protection antioxidants, known to combat oxidative stress in the body (Ighodaro 2018.). An elevation in these enzymes indicate elevated levels of oxidative stress as well as ROS in the body. Our findings demonstrate the upregulation of all the genes, confirming our hypothesis that oxidative stress levels and ROS concentrations will be elevated after the triathlon was completed. The greatest change was seen in GST and SOD, indicating an important upregulation of their expression 48-72 hours post event. (Figure 4)

### **DNA Damage**

Both a systemic inflammatory response as well as DNA damage has been observed following exhaustive endurance exercise (König 2001). Cell lysates from participants were collected 48 to 72 hours after completing their respective races. The concentrations of 8-oxo-2-deoxyguanosine, or 8-oxo-dG, were elevated in the participants who completed the Ironman and Half-Ironman race. On average there was a two fold increase in 8-oxo-2-deoxyguanosine. This was a significant change to the half Ironman and control population. The concentrations of 8-oxo-dG in the control group serve as measure of comparison for the Ironman and Half-Ironman groups (Figure 6). The results indicate that IM based endurance exercise does cause DNA damage in well-trained athletes (Fraga 1990).

## DISCUSSION

A significant feature of this study is the use of noninvasive and cost-effective methods to determine whether a physically challenging event, namely the Ironman Triathlon, correlates with increased oxidative stress in participants (Scheffer 2012). The use of saliva samples and Garmin Smartwatch technology were both employed to address cost-effectiveness and utilize a noninvasive method to obtain data on sleep patterns, vital information, cortisol levels, ROS concentrations, DNA damage, and gene expression data. Another cost-effective measure employed in this research that distinguishes the methodology from previous studies is the assay of transcript levels. Previous research on the analysis of the gene expression has often employed the use of techniques such as Western blot to analyze protein expression (Bass 2017) However, the current study is distinguishable from comparable prior research in that transcription levels of the genes in question, namely GST, SOD, MDA, CAT, and GPx, were assayed rather than the protein products (Ighodaro 2018).

Cortisol is the body's main stress hormone, and it is involved in numerous stress responses produced in the body. Among its many functions, cortisol is instrumental in moderating metabolism of carbohydrates, fats, and proteins, regulation of blood pressure, and regulation of blood sugar (McEwen 2008.) Measurement of cortisol is an excellent indicator of the amount of stress that the body is undergoing, as increased cortisol levels point to an increase in stress. In order to determine the amount of stress the athletes underwent, an ELISA based assay was utilized to measure cortisol levels in the participants (Flint M.). When compared with the average cortisol levels of participants in the control group, it was noted that the average cortisol level of the athletes completing the full and half Ironman remained elevated 24 hours prior to the race and that this elevation increased 24 hours after the race (Valavanidis 2009). The pre-race elevation in cortisol levels was attributed to overtraining and overreaching without significant recovery time. These activities would lead to increased stress in the body, hence the rise in cortisol levels. The post-race elevation, which increased from the pre-race cortisol levels, was expected, as completing the Ironman triathlon requires an athlete to exert an enormous amount of energy and undergo strenuous exercise for a prolonged period of time. The elevation in cortisol levels after the race has been attributed to these factors (Jacks 1999.)

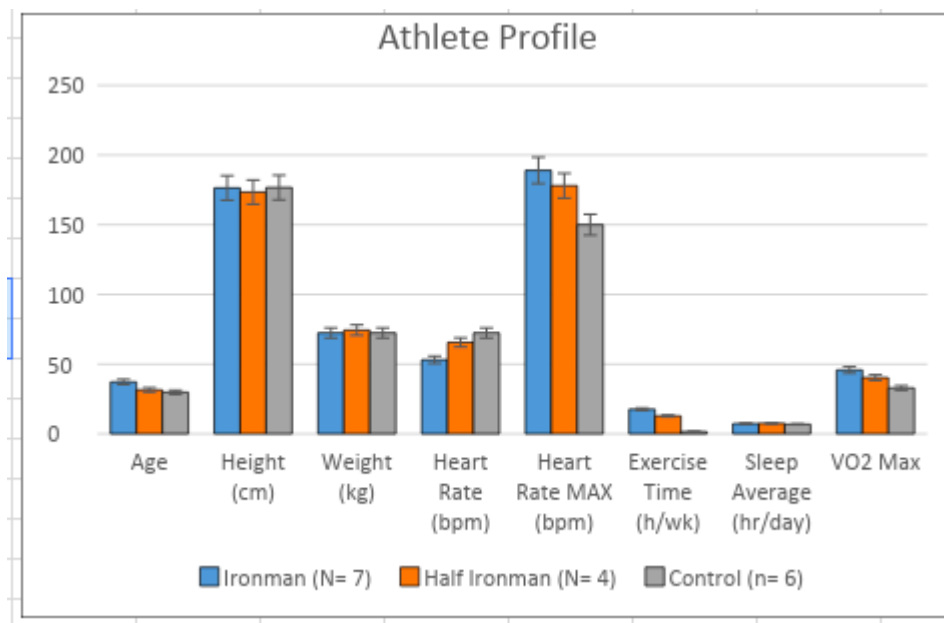
Previous research has found that high intensity exercise can increase the production of ROS, which can damage cellular integrity by modifying DNA. As the Ironman participants were competing in an event that calls for a large amount of strenuous exercise completed over an extended time interval, we hypothesized that our participants would likely accumulate ROS (Alessio 2000) In order to measure this accumulation of H<sub>2</sub>O<sub>2</sub>, a specific ROS, the participants, a ROS Glo H<sub>2</sub>O<sub>2</sub> assay was utilized directly in the cell cultures derived from participants. This bioluminescent assay measures the concentration of H<sub>2</sub>O<sub>2</sub> within a cell culture sample; and after completing the assay, our results indicated that the participants did experience significant ROS accumulation 48 hours after endurance based activity. This significant accumulation of ROS was attributed to the athletes partaking in an event that called for high intensity exercise (Salin 2010.)

Oxidative damage is an important consequence of oxidative stress, which is a state characterized by an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense capacity (Fraga 1990 & Demple 1994). We hypothesize that post-IM athletes endure an increased amount of stress even at the molecular level. To test this, we examined several biochemical markers such as MDA, SOD, CAT, GPx, and GST. In general, antioxidant defense systems consist of low-molecular-weight antioxidants and antioxidant enzymes including SOD, CAT, GPx and GST (Pan 2016). These antioxidant enzymes activities are closely related to their

mRNA levels. The expression of these antioxidant enzymes depends on the quantity and quality of generated free radicals into the biological system (biological membranes, cytosolic compartments, nucleus, tissues or artificial systems), as well as it depends on different cell types and diseases. It will be interesting to follow up with these results in the future to see if elevated mRNA is seen beyond the 72 hour mark (Radak 2013). It would also be beneficial to look at mRNA involved in the inflammatory response as oxidative stress and inflammation often are seen simultaneously (Ghamin 2011).

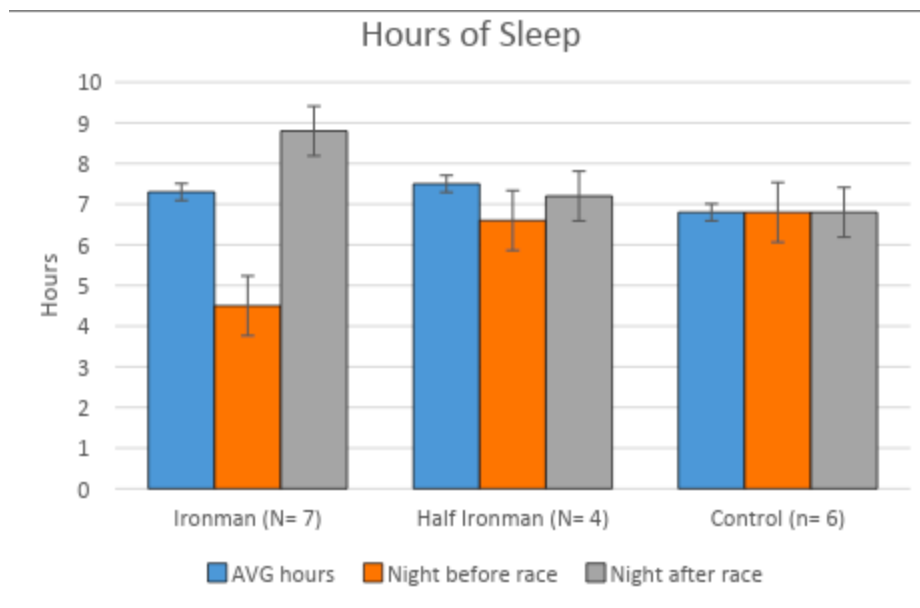
In order to quantify the amount of DNA damage that occurred due to the presence of ROS within the cells of the participants, another ELISA based assay was utilized. This kit allowed us to indirectly measure the amount of DNA damage that occurred in our participants by quantifying a byproduct of DNA damage known as 8-oxo-2-deoxyguanosine, or 8-oxo-dG. 8-oxo-dG is a byproduct of DNA damage that occurs as a result of oxidative stress (Wu 2004). By measuring the concentration of 8-oxo-dG in our cells of study, we were able to determine the relative amount of DNA damage that occurred within the cells. Previous research has noted that those who exercise incur increased DNA damage in comparison to less active individuals, as exercise increases the amount of free radical oxygen and nitrogen species in the body (Stroth 2009). These free radical species can be responsible for DNA modifications in the cells by causing the hydroxylation of guanosine. Our results did indicate that athletes incurred more DNA damage than their less active counterparts in the control group. Those who completed the full Ironman experienced an increase in relative 8-oxo-dG concentrations in comparison with those who completed the half Ironman. The athletes who completed the half Ironman did have higher concentrations of 8-oxo-dG in their cells than those in the control group (Shaper 1991). 8-hydroxy-deoxyguanosine (8-oxo-dG) is a sensitive marker of the DNA damage due to hydroxyl radical attack at the C8 of guanine. This damage, if left unrepaired, has been proposed to contribute to mutagenicity and cancer promotion (Valavanidis 2009).

The role of dietary antioxidants and sleep cycles in participating athletes are subsets of this research that would be interesting to investigate further. Results showcase the sleeping patterns of the athletes were disrupted days before the race as 80% of the athletes reported having trouble falling asleep. Furthermore, dietary antioxidants are linked to shorter recovery and supplemental defense against oxidative stress in the body (Mittler 2002). Both sleep cycles and dietary antioxidants can have an impact in the oxidative stress levels found in athletes and can be further researched. Exercise is a fundamental part of life and one that all should receive the benefits from. At all levels of fitness, it is important to understand both the hazards and rewards of the activity.



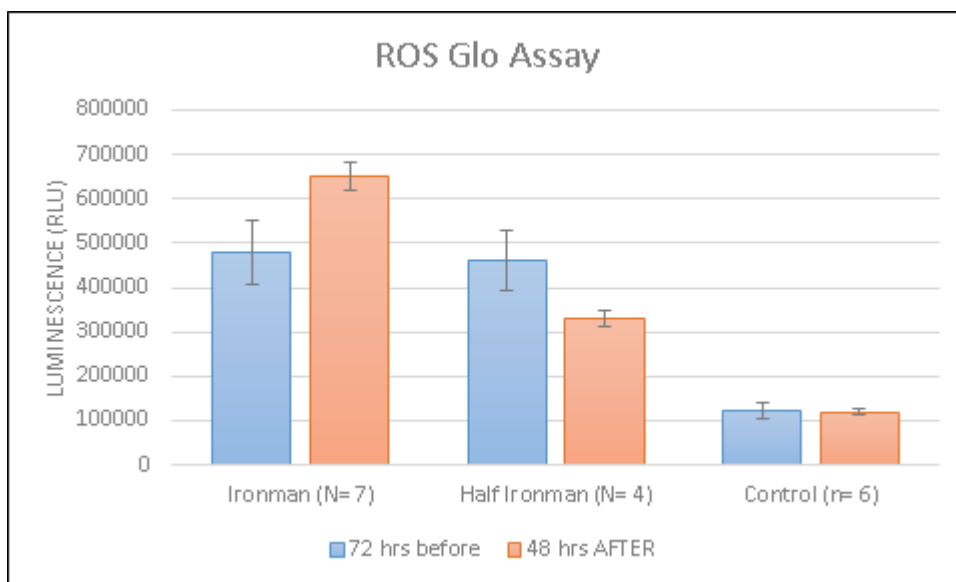
**Figure 1. Athletic Profile**

This table represents that overall athlete profile for participants in the study. Athletes completed questionnaires which requested information on health habits. Additionally, participants recorded exercise, sleep, heart rate, and VO2 max using the Garmin Smartwatch device.



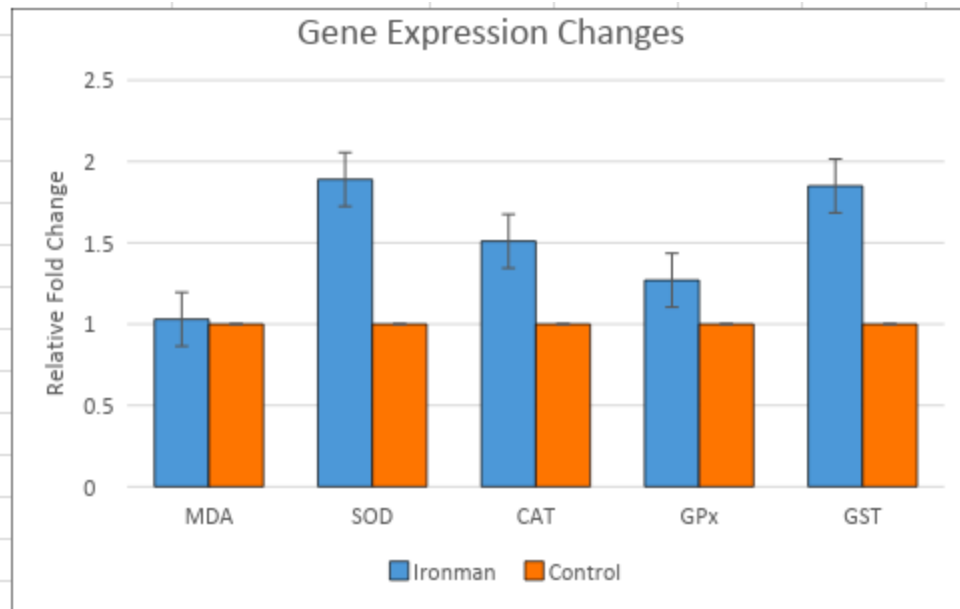
**Figure 2. Hours of Sleep**

This figure represents the amount of hours slept on average, and before and after the IM race by participating athletes. IM and half IM athletes experienced less sleep the night before the race and a slight increase in the amount of sleep immediately after the race. On average, most participants slept approximately 7-8 hours per night, which is the recommended amount.



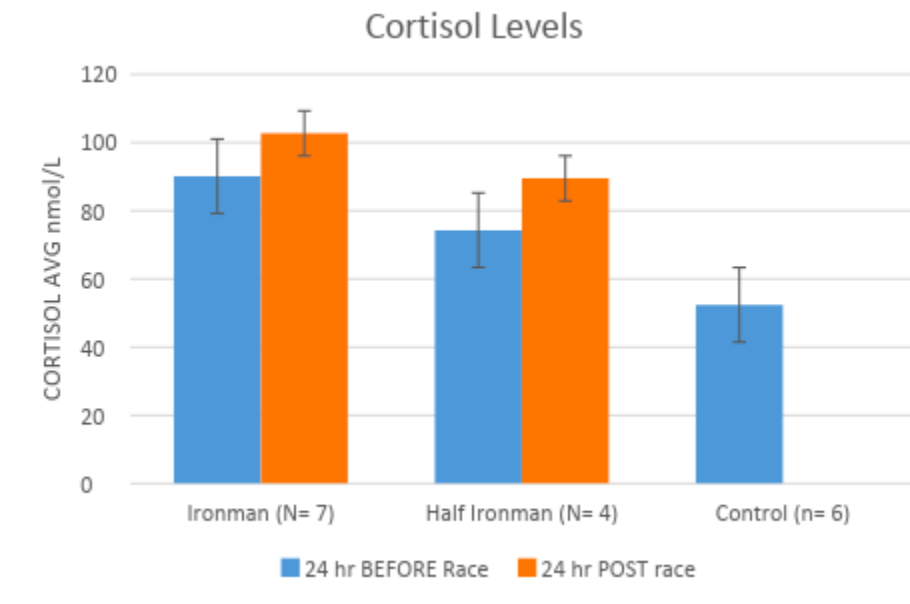
**Figure 3. ROS-Glo Assay for Oxidative Stress.**

This graph represents changes in bioluminescence 72 hours before and 48 hours after the selected participants' race. IM participants experienced a significant increase in total ROS 48 hours after the race.



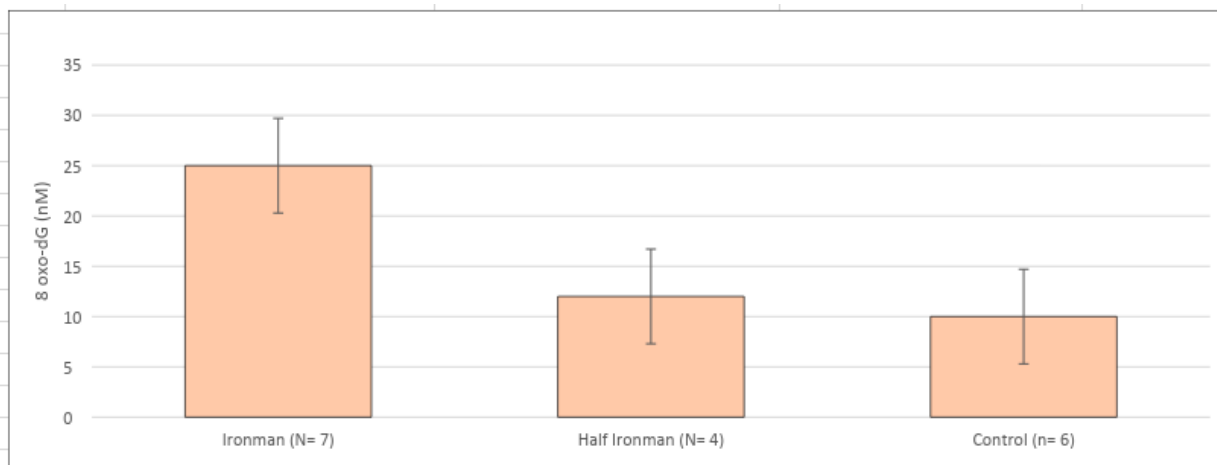
**Figure 4: Gene Expression Changes**

Gene expression changes were analyzed in several markers for physiological stress in the body. Overall, elevated stress response expression at the molecular level was seen in IM participants compared to the control population. Most significant changes were seen in SOD and GST.



**Figure 5. Cortisol Levels of Control and Participating Athletes Before and After the Full and Half-Ironman.**

IM and half IM athletes indicate elevated cortisol levels 24-hours post event. Cortisol levels in the Ironman and Half-Ironman group are elevated 24 hours post-race in comparison to the pre-race levels. In comparison to the control group, both the pre-race and post-race cortisol levels in the Ironman and Half-Ironman participants were elevated.



**Figure 6. 8 oxo-dG Concentration in Participants.**

8 oxo-dG is the most frequent oxidative DNA damage as represented by the 8-oxo-dG assay results. Our results indicate a 2-fold increase in 8-oxo-dG damage to IM athletes compared to half IM and control counterparts.

Gene Symbol	Gene Name	Function
MDA	Malondialdehyde	Member of the paraoxonase family of enzymes and exhibits lactonase and ester hydrolase activity
SOD	Superoxide dismutase	Binds copper and zinc ions and is one of two isozymes responsible for destroying free superoxide radicals in the body
CAT	Catalase	Converts the reactive oxygen species hydrogen peroxide to water and oxygen and thereby mitigates the toxic effects of hydrogen peroxide
GPx	Glutathione peroxidase	Reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.
GST	Glutathione-S-transferase	Endogenous Thiel group-containing antioxidant that reacts with ROS as a co-factor of the antioxidant enzyme glutathione peroxidase

**Table 1:** Gene symbol, name, and functional information.

<u>Primer Name</u>	<u>Sequence FWD</u>	<u>Sequence REV</u>
<b>APOE</b>	CCGTTTCTCCTTCCCCAGAC	GGGGTCAGTTGTTCCCTCCAG
<b>Notch</b>	GGGACCAACTGTGACATCAA	GTAGCCACTGGTCATGTCTTT



<b>CD34</b>	TAGCCTGTCACCT GGAAATG	TGCCTTGATGTCACTTAGGATAG
<b>CDCP1</b>	AAGGACACAGAC ATTCCCTTAC	GTCTCAGTGCCCT GCTTTAT
<b>NANOG</b>	TCCTGAACCTCAG CTACAAAC	GCGTCACACCATT GCTATTC
<b>RPL4</b>	GGCTACAAGAAG ACCAAGGAA	CTCATTCGCTGAG AGGCATAG
<b>CAT</b>	ATCCGTGTAACCCGCTCATC	GAGTCCTCGAGATACTGGCA
<b>FOXM1</b>	GTGGATCTGCTTGCCAGAGT	TGCCTTAGGAATCAGACGCC
<b>GPX1</b>	ACACCCAGATGAACGAGCTG	CGTTCTTGGCGTTTCTCCTGA
<b>MPO</b>	TTTGACAACCTGCACGATGAC	CGGTTGTGCTCCCGAAGTAA
<b>SOD1</b>	CATCAGCCCTAATCCATCTGA	CGCGACTAACAAATCAAAGTGA

**Table 2.** Primer sequences utilized in RT-qPCR amplification.

<b>CORTISOL Measurements</b>	<b>Ironman (N= 7)</b>	<b>Half Ironman (N= 4)</b>	<b>Control (n= 6)</b>
AVG nmol/L			
24 hr BEFORE Race	90.1	74.3	52.4
24 hr POST race	102.7	89.5	n/a

**Table 3.** Cortisol measurements from control, IM, and half IM participants.

<b>DNA Damage Assay</b>	<b>Ironman (N= 7)</b>	<b>Half Ironman (N= 4)</b>	<b>Control (n= 6)</b>
8-oxo-dG (nM)	25	12	10

**Table 4.** Average values of 8-oxo-dG concentration, a byproduct of DNA damage, measured in nM and reported for the Ironman, Half-Ironman, and Control groups.

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